THROMBOTIC MICROANGIOPATHIES, DIAGNOSTIC AND THERAPEUTIC ADVANCES

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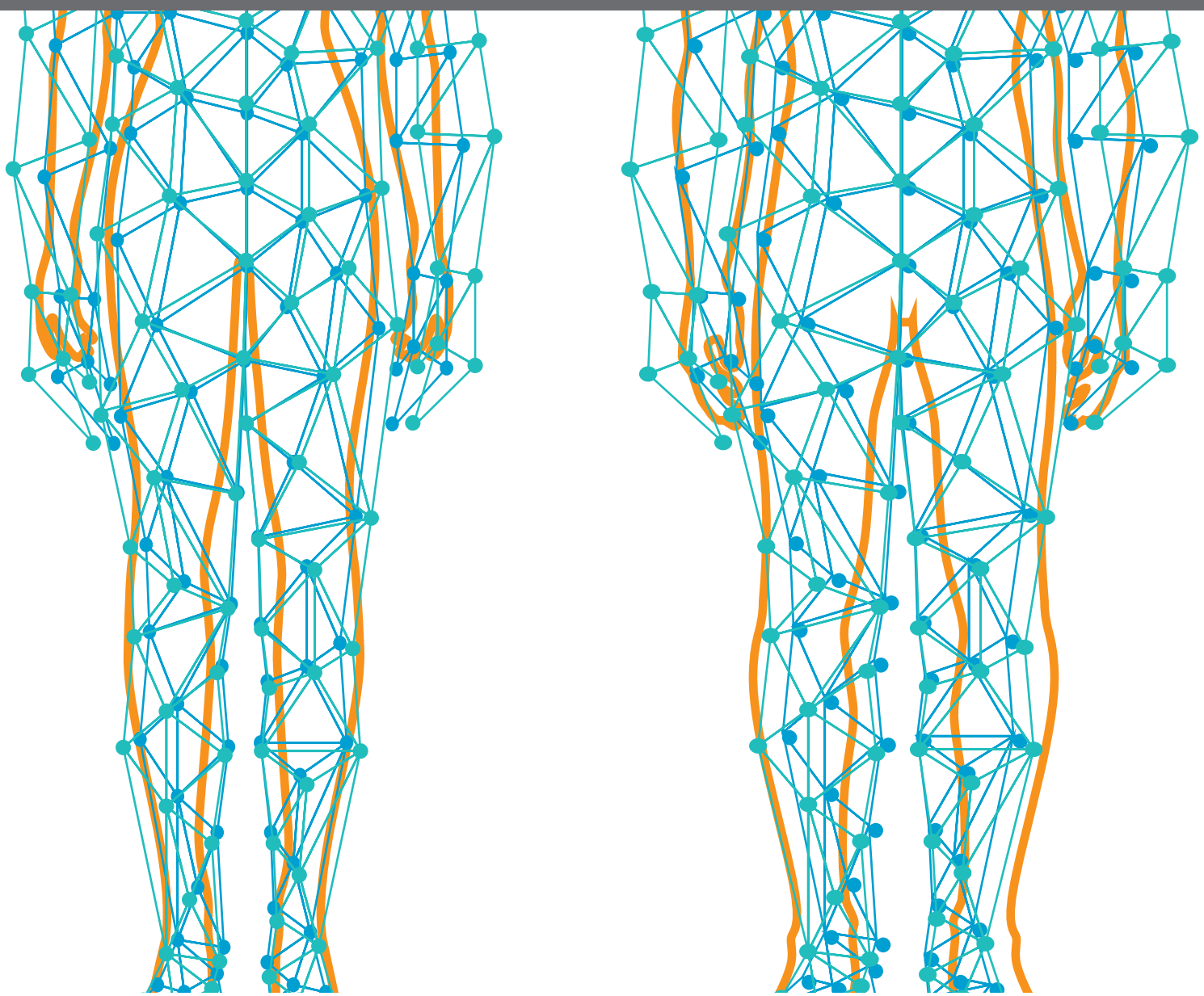

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THROMBOTIC MICROANGIOPATHIES, DIAGNOSTIC AND THERAPEUTIC ADVANCES

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EDITORIAL
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Editorial: Thrombotic Microangiopathies, Diagnostic and Therapeutic Advances

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Editorial on the Research Topic

Thrombotic Microangiopathies, Diagnostic and Therapeutic Advances

Thrombotic microangiopathies (TMAs) as a disease group represent a diagnostic and clinical challenge to practitioners. Presentations, which at times have overlapping symptomatology or have undetermined etiologies, emphasize the need for a constant reassessment of what is known about this disease group in order to determine not only how to best treat patients but also to develop targeted approaches to test for a given etiology. Likewise, in those instances when excluding diagnoses is the only available option, timely adjustment of therapy due to lack of therapeutic response needs to be achieved in the most efficient way. It is with this in mind that this topic-specific collection of articles was conceptualized to provide a forum to review not only what is currently known about these disease entities but also to present new findings in order to keep readers abreast of ways in which these diseases can be discerned. This is important since each of these etiologies responds to different therapies with a distinct road to recovery.

Sometimes diseases at presentation may have a preponderance in complement pathway activation characterized by C3 deposition similar to cases of immune-complex membranous glomerulonephritis (Hanna et al.). In these patients, thrombocytopenia may not be as significant as their renal impairment, and complement abnormalities are at the center of their presentation. This is discussed in more detail in this issue whereby using a case-driven approach, contributors suggest that genetic testing by itself may not always lead to the discovery of complement-related mutations. Instead, C3 staining patterns or presence of anti-factor H antibodies can ultimately lead to rapid recognition and initiation of appropriate therapeutic regimens while patients await renal transplantation. Along the same lines, complement mediators can not only mediate disease pathology but also serve as biomarkers in cases when thrombocytopenia occurs post-hematopoietic stem cell transplantation. In such patients, a report in this issue indicated that increases in soluble-C5b-9 can be seen and may be a diagnostic marker of patients who develop transplant-associated TMA (Mezö et al.).

Thrombocytopenia can also be an unintended adverse event from an increasing list of medications that include chemotherapeutic agents, monoclonal antibodies, and antimicrobials, to name a few. Unfortunately, this can go unrecognized in clinical practice and remain lower in the differential due to a lack of clinical suspicion. Adding complexity to this problem, mechanisms leading to drug-induced TMA are likely distinct from one another due to the different drug agents involved and intended corresponding cellular targets; however, this TMA may involve either antibody formation or dose-dependent and cumulative toxicity, and recovery occurs upon

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discontinuation of the offending drug (Chatzikonstantinou et al.). This is discussed in this issue where an update of drugs leading to this type of TMA and potential mechanisms are reported.

It is understandable that when clinicians see a patient with acute thrombocytopenia in the presence of schistocytes in the peripheral smear, a very non-specific test without standardization, the *gravitas* of the clinical picture may make them think of the most serious presentation. This is the case with thrombotic thrombocytopenic purpura (TTP), which is associated with high morbidity and mortality, requiring prompt initiation of therapy in the form of therapeutic plasma exchange (TPE) and concurrent immunosuppression. The latter specifically can be a matter of concern to practitioners because current literature is still not clear on which long-term immunosuppression regimen best leads to platelet count recovery and reduces relapses. It is thus encouraging that a report in this issue found similar efficacy in treating TTP patients with either rituximab or cyclophosphamide (Abou-Ismail et al.). Nowadays, it is well-accepted that the test to confirm the clinical diagnosis of TTP is ADAMTS13 activity. However, this test is often performed at reference laboratories and results may not be available at the time of therapy initiation. Notably, it should be of interest to readers that a broad platelet differential that focuses on immature platelets produced at the bone marrow can aid in differentiating TTP from other thrombocytopenias (Reeves and Maitta). On the other hand, in those thrombocytopenic cases in which ADAMTS13 activity is normal, a comprehensive body survey may be required to identify possible metastatic malignancies that have gone unnoticed (Osti et al.). With the advent of caplacizumab, the first TTP-specific approved therapy, it should be of interest that cases refractory to TPE and steroids respond to the agent even when initiated during the second week of hospitalization leading to recovery from thrombocytopenia and thrombi-related complications such as neurological impairments (Mellaza et al.). Finally, this issue will discuss congenital TTP unveiled by an infectious agent that led to a thorough workup that resulted in proper diagnosis and successful therapy (Wendt et al.).

In clinical practice, TMA patients can have a broad range of symptomatology and a report looking at almost 300 patients diagnosed with TMA serves as a good source to exemplify the challenges when encountering such patients (Henry et al.). Without a doubt, the vast majority of patients will have a TMA secondary to a primary insult rather than a primary TMA such as TTP or atypical hemolytic uremic syndrome (aHUS). This study stresses the need of proper testing that does not neglect ADAMTS13 activity and/or complement mutation studies, when applicable, to avoid misdiagnosing patients and yielding an accurate and timely diagnosis (Henry et al.). Notably, another contribution to this collection brings attention to and argues in favor of potentially new complement circulating factors mediating familial cases of aHUS when mutations in known complement genes are not identified (Piras et al.).

To conclude, as new discoveries in the field of TMA research that include reports of new highly-specific therapies in the form of monoclonals, among others, come to fruition, the future looks promising that some of these diseases will be treated in a targeted way earlier in the clinical course resulting in improved patient outcomes. Thus, it is the hope of the topic editors and contributors of this special collection that it will serve as a conduit to continue the discussion of TMA presentations while providing guidance on how to best treat patients under our care.

AUTHOR CONTRIBUTIONS

All authors contributed to writing and editing the manuscript.

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OPINION
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An Update in Drug-Induced Thrombotic Microangiopathy

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INTRODUCTION

Drug-induced thrombotic microangiopathy (DITMA) is a life-threatening complication that is often under-recognized and under-reported (1). Despite recent systematic reviews published in 2015 (2) and 2018 (3), the list of drugs implicated in TMA continues to expand (4–9). In addition, novel reports have unraveled potential new mechanisms that might contribute to a targeted therapy of this syndrome. In this opinion article, we aimed to summarize recent data on DITMA, categorizing drugs based on mechanisms of actions and specialties.

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MECHANISMS OF ACTION

Two decades ago, George et al. introduced the term "drug-induced thrombocytopenia." Clinically based criteria were proposed and levels of evidence were stratified in order to solidify a definite, propable, possible, or an unlikely causal relationship between a drug and thrombocytopenia (10). Although the mechanisms of endothelial injury during DITMA still remain unknown; immune-mediated mechanisms or dose-dependent and cumulative toxicity are implied (11). The hypothesis is based on the observation of the timing of TMA occurrence, the pattern of disease, the exclusion of a better explanation thorough investigation. DITMA suspicion is ampilified by TMA resolution when the drug is withdrawn or recurrent endothelial injury during re-exposure to the drug.

During the last decade, laboratory criteria have been added to support the causal relationship between a drug and TMA (2). Some examples of drugs in which antibody mediated DITMA has been confirmed with identification of drug-dependent antibodies to platelets or other cells as the pathophysiologic mechanism of TMA are quinine, oxaliplatin, and vancomycin (12). On the other hand, the dose-dependent and cumulative toxicity model seems to fit for opana's abuse, bevacizumab, levofloxacin, alemtuzumab, and interferon's cases of DITMA (9, 13–15). It is important to exclude any other diagnosis before attributing TMA to a drug. For example, in some cases such as these of ipilimumab, pazopanib, ustekinumab, and golimumab severe ADAMTS13 deficiency was found, plasma exchange was effective and no drug-dependent antibody inhibition of ADAMTS13 activity was reported, making drug-indused causal relationship unlikely (7, 16–18). **Figure 1** summarizes postulated mechanisms in DITMA.

HEMATOLOGY/ONCOLOGY

DITMA is caused by various drugs used in Hematology and Oncology.

Chemotherapy

Chemotherapeutic agents were the first to be implicated in causing DITMA. Mitomycin and gemcitabine have numerous reports of dose-related DITMA, while one report describes an

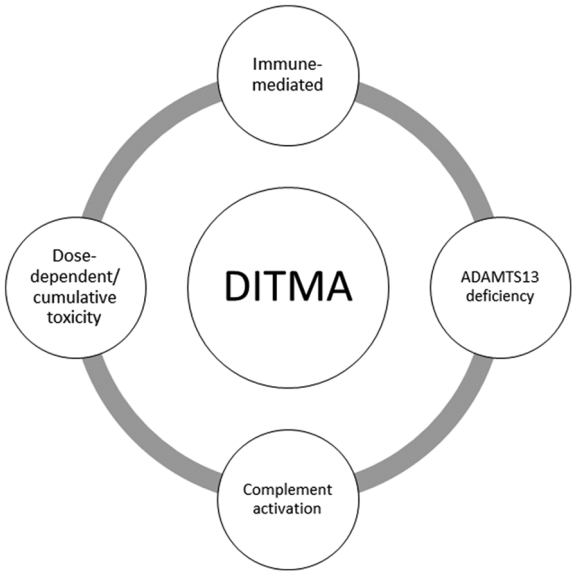

FIGURE 1 | Summary of postulated mechanisms in drug-induced thrombotic microangiopathy (DITMA). ADAMTS13: a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13.

immune-DITMA as a result of gemcitabine administration (19–26). Despite their use as a combination with other drugs, which makes the direct causal relationship difficult in some cases, many well-described cases support a clear-cut association (21, 27, 28).

Three reports have described DITMA caused by pentostatin, a purine analog used in lymphoproliferative diseases (29). Docetaxel and vincristine have also been reported to induce TMA (30, 31). Oxaliplatin has been implicated as a cause of DITMA in a review by Al-Nouri et al. (2), although the authors of the original report described gemcitabine as the causative factor (32). Renal-limited TMA has been reported in three patients treated with pegylated liposomal doxorubicin (33) and in one patient receiving treatment with a short interfering RNA targeted against Myc (DCR-MYC) (5).

Multiagent Chemotherapy

Drug-induced TMA has been reported in children with acute lymphoblastic leukemia (34, 35) and adults with solid tumors (36, 37), receiving multiagent chemotherapy. Jodele et al. described 13 patients developing TMA after high-dose chemotherapy and autologous stem cell transplantation for neuroblastoma (12 patients receiving carboplatin/etoposide/melphalan and one cyclophosphamide/thiotepa) (38). Finally, a high incidence of TMA was observed in melanoma patients receiving a lymphodepleting preparative chemotherapy regimen with total body irradiation (TBI) prior to autologous T cell therapy (39). In all cases, the co-administration of multiple drugs hinders the identification of the causative agent.

Proteasome Inhibitors

Proteasome inhibitors are mainly used in multiple myeloma treatment and have been associated with DITMA (bortezomib, carfilzomib, ixazomib). The majority of reports have implicated bortezomib and cafilzomib (3, 40). Recent reports also support a causal association of ixazomib with DITMA (41–43). Some authors report successful treatment of carfilzomib-induced TMA with eculizumab (44, 45).

VEGF, Kinase and Immune Checkpoint Inhibitors

Vascular endothelial growth factor (VEGF) inhibitors are used for the treatment of various malignancies. Many cases reported DITMA as a result of bevacizumab use, a humanized monoclonal antibody directed against VEGF (46–49). In some, treatment with eculizumab was successful (50). Ramucirumab, anti-VEGF receptor 2 monoclonal antibody, and cetuximab, a monoclonal antibody against epidermal growth factor receptor (EGFR), are also implicated in causing renal-limited TMA (51, 52).

Tyrosine kinase inhibitors (TKIs) are effective in the treatment of hematologic malignancies and solid tumors. Sunitinib is a small-molecule TKI that targets VEGFR-2 and PDGFR-b. Imatinib and ponatinib are small-molecule BCR-ABL TKIs, used mainly in the treatment of chronic myeloid leukemia. Palbociclib inhibits the cyclin-dependent kinases CDK4 and CDK6. Cases reporting a link between the aforementioned TKIs and drug-induced TMA have been described in literature (6, 53–56).

Two cases of TMA, one in a patient receiving the immune checkpoint inhibitor ipilimumab, and one in a patient treated with multi-targeted receptor tyrosine kinase inhibitor pazopanib have also been reported. However, these reports differ from other DITMAs, due to the severe ADAMTS13 deficiency (7, 16).

Calcineurin and mTOR Inhibitors

Numerous reports implicate cyclosporine and tacrolimus in causing dose-dependent TMA (2). Most of these reports described calcineurin inhibitor-induced TMA in patients that have undergone hematopoeitic stem cell or solid organ transplantation (57). Calcineurin inhibitor-induced TMA mostly affects the kidneys (58, 59). The inhibitors of the mechanistic target of rapamycin (mTOR), can also cause DITMA, most frequently associated with sirolimus or tacrolimus administration, than everolimus (60–62). Successful treatment with complement inhibition has been described in several patients, since this condition along with TA-TMA has been considered to resemble atypical hemolytic uremic syndrome (aHUS) (63, 64).

MONOCLONAL ANTIBODIES

The first reported case of monoclonal antibody-induced TMA described a patient treated with anti-T cell monoclonal antibody muromonab-CD3 (OKT3) (65). Emicizumab, a monoclonal antibody used in Hemophilia A, co-administered with high doses of activated prothrombin complex concentrate (aPCC) has been linked with TMA in three patients (66). Discontinuation of

aPCC resulted in resolution of TMA, highlighting the fact that emicizumab monotherapy may not be sufficient to cause DITMA.

Various monoclonal antibodies against tumor necrosis factor alpha (TNF-a), such as adalimumab, golimumab, and certolizumab pegol have been reported to cause TMA in a few cases (17, 18, 67). Another report describes a patient with psoriasis developing TMA after treatment with methotrexate and ustekinumab, a monoclonal antibody that blocks interleukin (IL)-12 and IL-23 (68). However, in the cases where ustekinumab and golimumab were suspected to be the causative factor of DITMA, the authors reported low levels of ADAMS13 (<5%) and an initial response to plasma exchange, making DITMA diagnosis unlikely (17, 18).

In phase 1 study, moxetumomab pasudotox, an anti-CD22 immunotoxin used in the treatment of childhood acute lymphoblastic leukemia, caused TMA in 13% of patients. In the majority of cases, TMA resolved with drug discontinuation (69).

OPIOIDS AND OTHER DRUGS OF ABUSE

Intravenous use of the extended-release opioid oxymorphone and oxycodone tablets reformulated with polyethylene oxide (PEO) have been reported to cause DITMA in many patients (3). Subsequently, intravenously administrated high molecular PEO was determined as the causative factor (15). Cocaine and ecstasy have caused DITMA in recreational users (70–72).

NEUROLOGY

One of the biggest challenges in neurology is the lack of disease-specific drugs that contributes to the increasing global burden of neurological disorders (73). Traditionally, epilepsy benefited from a wide variety of available medicines but during the last decade numerus drugs were introduced at multiple sclerosis (MS) treatment raising long-term safety considerations (74, 75). Until 2018, interferon beta 1-a and 1-b, disease modifying treatments (DMTs) of MS and anticonvulsive valproic acid were the only neurologic drugs associated with thrombotic microangiopathy (3).

Recently, alemtuzumab; which was approved by US Food and Drug Administration (FDA) for treatment of relapsing-remitting MS (RRMS) at 2014; was associated for the first time with DITMA (8, 9). Administration of alemtuzumab was known to rarely cause severe renal adverse effects (76). Nevertheless, in that case report the causal relationship of alemtuzumab with TMA is supported by the fact that (a) symptoms started immediately after the first infusion and (b) the patient did not respond to plasma exchange (9). Another DMT, fingolimod was linked with TMA in an induced-malignant hypertension animal model; in contrast with control group in which fingolimod was not administrated (77). Interferon (IFN) has also been correlated with TMA with a dose-dependent manner (14). Further studies confirm that TMA is a severe complication of IFN-beta RRMS treatment. Lately, five patients were reported to have IFN-induced TMA following long-term treatment (78–80). Interestingly, renal function of three patients improved only after administration of eculizumab, not after withdrawal of IFN (80). Clinical translation of those studies raises awareness of neurologists for early recognition and management of TMA when prescribing DMTs.

TABLE 1 | Summary of drugs involved in DITMA.

Drug	Type	Specialty
Docetaxel Doxorubicin DCR-MYC Gemcitabine Oxaliplatin Pentostatin Vincristine	Chemotherapy	Hematology/Oncology
Carboplatin + Etoposide + Melphalan Cyclophosphamide + Thiotepa	Multiagent chemotherapy	
Bortezomib Carfilzomib Ixazomib	Proteasome inhibitors	
Bevacizumab Ramucirumab Cetuximab Imatinib Ipilimumab Pazopanib Ponatinib Palbociclib Ruxolitinib Sunitinib	VEGF, kinase and immune checkpoint inhibitors	
Cyclosporine Rapamycin Tacrolimus	Calcineurin and mTOR inhibitors	
Adalimumab Certolizumab pegol Emicizumab + aPCC Golimumab OKT3 Ustekinumab Moxetumomab pasudotox	Monoclonal antibodies	Hematology/Oncology/ Rheumatology
Cocaine/ Ecstasy Oxymorphone/Oxycodone Polyethylene oxide (PEO)	Opioids / Drugs of abuse	Toxicology
Interferon beta 1-a /1-b Alemtuzumab Fingolimod	Disease modifying treatments for Multiple Sclerosis	Neurology
Valproic acid	Anticonvulsive	
Tenofovir/Emtricitabine	Anti-infectives	Infectious diseases
Quinine/Hydroxychloroquine	Antimalarials	
Ciprofloxacin Fluoroquinolone Metronidazole Penicillin	Antibiotics	

INFECTIOUS DISEASES

An infection can be caused by a variety of organisms such as bacteria, viruses, parasites or fungi. Many anti-infectives agents

have been associated with DITMA in the past (2); quinine, the treatment of malaria, was the most commonly reported (81).

Novel studies implicate a number of different drugs in causing DITMA. First of all, a case report incriminates hydroxychloroquine, a synthetic derivative of quinine used for rheumatoid arthritis and systemic lupus erythematosus, as a possible cause of thrombotic thrombocytopenia purpura (TTP) (82). Disease progression was detrimental and patient died in spite of drug withdrawal and plasma exchange. Moreover, for the first time an antiretroviral treatment of human immunodeficiency virus consisting of tenofovir/emtricitabine was found to have a causality relationship with immune TTP (83). After the cessation of the drug and the initiation of corticosteroids and azathioprine the patient recovered. Last but not least, several antibiotics such as ciprofloxacin, penicillin, and metronidazole were reported with probable evidence to cause DITMA (2). A new case report implicates again ciprofloxacin in drug-induced TTP which resolved completely with plasma exchange (84). Another report, identified a highly effective and frequently prescribed fluoroquinolone, levofloxacin as a new potential suspect for DITMA (13). This case report described two patients who developed microangiopathic hemolysis and thrombocytopenia following levofloxacin treatment of respiratory tract infections. Both cases resolved after drug cessation; the first patient received also therapeutic plasma exchange. In conclusion, a wide variety of anti-infectives agents have been scarcely correlated with DITMA and unfortunately, no one could predict or prevent its appearance; hence, it is of paramount importance to be aware of that possibility in order to start the appropriate treatment promptly.

THERAPEUTIC POTENTIALS

The only proven intervention in the management of DITMA is discontinuation of the offending agent. Plasma exchange and immunosuppressive therapy may be a reasonable treatment option, especially when the diagnosis is uncertain. Although rarely described in DITMA, patients with severe ADAMTS13 deficiency respond to plasma exchange (7, 16–18, 82–84). However, these reports should be interpreted with caution, since severe ADAMTS13 deficiency indicates TTP as a more likely diagnosis. In true DITMA, plasma exchange is ineffective (85). On the other hand, numerous reports have now confirmed that complement inhibition with eculizumab shows efficacy in DITMA (22, 26, 44, 45, 50, 63, 80). Eculizumab is a first-in-class monoclonal antibody that blocks terminal complement activation with proven safety and efficacy in complement-mediated TMAs (86). Based on current literature, we would consider eculizumab administration in three instances: in patients with non-immune DITMA, in those who deteriorate despite discontinuation of the implicated drug and supportive care, and finally, in patients at risk of kidney failure (87).

CONCLUSIONS AND FUTURE PERSPECTIVES

Our state-of-the-art report categorizes drugs that have been associated with DITMA, summarized in **Table 1**. It also emphasizes on unique presentations and characteristics of DITMA, that require increased awareness by treating physicians of relevant specialties. Hematologists are largely involved in the administration of the majority of these drugs, along with other internal medicine specialties. Since many patients have presented with renal-limited complications, the role of nephrologists is also important. Therefore, our report highlights an unmet clinical need of increased recognition and better understanding of DITMA by treating physicians across different specialties.

Except for expanding the list of drugs associated with DITMA, future reports need to take into account potential mechanisms. Identification of underlying etiology is of utmost importance for proper management. Further mechanistic studies need to identify the drugs or pathways involved in complement activation in order to early select patients that would benefit from complement inhibition.

AUTHOR CONTRIBUTIONS

EG conceived the manuscript concept. TC and MG wrote the manuscript. AA and EG edited and approved the final manuscript.

REFERENCES

1. Gavriilaki E, Gkaliagkousi E, Grigoriadis S, Anyfanti P, Douma S, Anagnostopoulos A. Hypertension in hematologic malignancies and hematopoietic cell transplantation: an emerging issue with the introduction of novel treatments. *Blood Rev*. (2019) 35:51–8. doi: 10.1016/j.blre.2019.03.003
2. Al-Nouri ZL, Reese JA, Terrell DR, Vesely SK, George JN. Drug-induced thrombotic microangiopathy: a systematic review of published reports. *Blood*. (2015) 125:616–8. doi: 10.1182/blood-2014-11-611335
3. Saleem R, Reese JA, George JN. Drug-induced thrombotic microangiopathy: an updated systematic review, 2014-2018. *Am J Hematol*. (2018) 93:E241–43. doi: 10.1002/ajh.25208
4. Gavriilaki E, Sakellari I, Bousiou Z, Chatzikonstantinou T, Mallouri D, Masmanidou M, et al. Transplant-associated thrombotic microangiopathy is independently associated with ruxolitinib administration in patients with graft-versus-host-disease. *EBMT*. (2020). [Epub ahead of print].
5. Miller AJ, Chang A, Cunningham PN. Chronic Microangiopathy Due to DCR-MYC, a Myc-Targeted Short Interfering RNA. *Am J Kidney Dis*. (2019) 75:513–6. doi: 10.1053/j.ajkd.2019.09.011
6. Latifi Y, Moccetti F, Wu M, Xie A, Packwood W, Qi Y, et al. Thrombotic microangiopathy as a cause of cardiovascular toxicity from the BCR-ABL1 tyrosine kinase inhibitor ponatinib. *Blood*. (2019) 133:1597–606. doi: 10.1182/blood-2018-10-881557
7. Syed U, Wahlberg KJ, Douce DR, Sprague JR. Thrombotic thrombocytopenic purpura associated with pazopanib. *Case Rep Hematol*. (2018) 2018:4327904. doi: 10.1155/2018/4327904
8. Coles AJ, Twyman CL, Arnold DL, Cohen JA, Confavreux C, Fox EJ, et al. Alemtuzumab for patients with relapsing multiple sclerosis after

disease-modifying therapy: a randomised controlled phase 3 trial. *Lancet*. (2012) 380:1829–39. doi: 10.1016/S0140-6736(12)61768-1
9. Liou AA, Skiver BM, Yates E, Persad P, Meyer D, Farland AM, et al. Acute thrombotic microangiopathy and cortical necrosis following administration of Alemtuzumab: a case report. *Am J Kidney Dis*. (2019) 73:615–9. doi: 10.1053/j.ajkd.2018.09.013
10. George JN, Raskob GE, Shah SR, Rizvi MA, Hamilton SA, Osborne S, et al. Drug-induced thrombocytopenia: a systematic review of published case reports. *Ann Internal Med*. (1998) 129:886–90. doi: 10.7326/0003-4819-129-11_Part_1-199812010-00009
11. Edwards IR, Aronson JK. Adverse drug reactions: definitions, diagnosis, and management. *Lancet*. (2000) 356:1255–9. doi: 10.1016/S0140-6736(00)02799-9
12. Reese JA, Bougie DW, Curtis BR, Terrell DR, Vesely SK, Aster RH, et al. Drug-induced thrombotic microangiopathy: experience of the oklahoma registry and the bloodcenter of wisconsin. *Am J Hematol*. (2015) 90:406–10. doi: 10.1002/ajh.23960
13. Kazi S, Preston GC. Drug induced thrombotic microangiopathy caused by levofloxacin. *J R College Phys Edinburgh*. (2018) 48:127–9. doi: 10.4997/JRCPE.2018.206
14. Kavanagh D, McGlasson S, Jury A, Williams J, Scolding N, Bellamy C, et al. Type I interferon causes thrombotic microangiopathy by a dose-dependent toxic effect on the microvasculature. *Blood*. (2016) 128:2824–33. doi: 10.1182/blood-2016-05-715987
15. Hunt R, Yalamanoglu A, Tumlin J, Schiller T, Baek JH, Wu A, et al. A mechanistic investigation of thrombotic microangiopathy associated with IV abuse of Opana ER. *Blood*. (2017) 129:896–905. doi: 10.1182/blood-2016-08-736579
16. King J, de la Cruz J, Lutzky J. Ipilimumab-induced thrombotic thrombocytopenic purpura (TTP) *J Immunother Cancer*. (2017) 5:19. doi: 10.1186/s40425-017-0224-7
17. Zbaras B, Sam LN, Grimm MC. Thrombotic thrombocytopenic purpura associated with adalimumab (Humira) treatment in Crohn disease. *Internal Med J*. (2013) 43:216–7. doi: 10.1111/imj.12055
18. Cepeda J, Liedke C, Patnaik A, Yao Q. Development of thrombotic thrombocytopenic purpura in association with the monoclonal antibody, golimumab, used to treat rheumatoid arthritis, in a case with literature review. *J Clin Rheumatol*. (2018) 24:229–31. doi: 10.1097/RHU.0000000000000684
19. Izzedine H, Isnard-Bagnis C, Launay-Vacher V, Mercadal L, Tostivint I, Rixe O, et al. Gemcitabine-induced thrombotic microangiopathy: a systematic review. *Nephrol Dialysis Transpl*. (2006) 21:3038–45. doi: 10.1093/ndt/gfl507
20. Saif MW, Xyla V, Makrilia N, Bliziotis I, Syrigos K. Thrombotic microangiopathy associated with gemcitabine: rare but real. *Expert Opin Drug Saf*. (2009) 8:257–60. doi: 10.1517/14740330902942299
21. Price TM, Murgo AJ, Keveney JJ, Miller-Hardy D, Kasprisin DO. Renal failure and hemolytic anemia associated with mitomycin C. A case report. *Cancer*. (1985) 55:51–56. doi: 10.1002/1097-0142(19850101)55:1<51::AID-CNCR2820550109>3.0.CO;2-D
22. Hausberg M, Felten H, Pfeffer S. Treatment of chemotherapy-induced thrombotic microangiopathy with eculizumab in a patient with metastatic breast cancer. *Case Rep Oncol*. (2019) 12:1–6. doi: 10.1159/000495031
23. Leal F, Macedo LT, Carvalheira JB. Gemcitabine-related thrombotic microangiopathy: a single-centre retrospective series. *J Chemother*. (2014) 26:169–72. doi: 10.1179/1973947813Y.0000000122
24. Hasan A, Jain AG, Naim H, Munaf A, Everett G. Drug-induced thrombotic microangiopathy caused by gemcitabine. *Cureus*. (2018) 10:e3088. doi: 10.7759/cureus.3088
25. Daviet F, Rouby F, Poullin P, Moussi-Frances J, Sallee M, Burtey S, et al. Thrombotic microangiopathy associated with gemcitabine use: presentation and outcome in a national French retrospective cohort. *Br J Clin Pharm*. (2019) 85:403–12. doi: 10.1111/bcp.13808
26. Lopez Rubio ME, Rodado Martinez R, Illescas ML, Mateo Bosch E, Martinez Diaz M, de la Vara Inesta L, et al. Gemcitabine-induced hemolytic-uremic syndrome treated with eculizumab or plasmapheresis: two case reports. *Clin Nephrol*. (2017) 87:100–6. doi: 10.5414/CN108838
27. Cantrell JE Jr, Phillips TM, Schein PS. Carcinoma-associated hemolytic-uremic syndrome: a complication of mitomycin C chemotherapy. *J Clin Oncol*. (1985) 3:723–34. doi: 10.1200/JCO.1985.3.5.723
28. Zupancic M, Shah PC, Shah-Khan F. Gemcitabine-associated thrombotic thrombocytopenic purpura. *Lancet. Oncol*. (2007) 8:634–41. doi: 10.1016/S1470-2045(07)70203-6
29. Leach JW, Pham T, Diamandidis D, George JN. Thrombotic thrombocytopenic purpura-hemolytic uremic syndrome (TTP-HUS) following treatment with deoxycoformycin in a patient with cutaneous T-cell lymphoma (Sezary syndrome): a case report. *Am J Hematol*. (1999) 61:268–70. doi: 10.1002/(SICI)1096-8652(199908)61:4<268::AID-AJH9>3.0.CO;2-O
30. Kanchi H, Webb NJ, Eden OB. Hemolytic uremic syndrome secondary to the treatment of acute lymphoblastic leukemia. *J Pediatr Hematol Oncol*. (2000) 22:483–4. doi: 10.1097/00043426-200009000-00024
31. Shrestha A, Khosla P, Wei Y. Docetaxel-induced thrombotic thrombocytopenic purpura/hemolytic uremic syndrome-related complex in a patient with metastatic prostate cancer? *Am J Therap*. (2011) 18:e167–71. doi: 10.1097/MJT.0b013e3181cea0b3
32. Crouzet L, Edeline J, Le Du F, Boucher E, Audrain O, Raoul JL. Haemolytic uremic syndrome and gemcitabine: jaundice is not always progression in cholangiocarcinoma. *Acta Oncol*. (2012) 51:687–8. doi: 10.3109/0284186X.2012.661073
33. Shavit L, Lifschitz MD, Gabizon A, Kwa M, Muggia F, Slotki I. Pegylated liposomal doxorubicin and renal thrombotic microangiopathy: an under-recognized complication of prolonged treatment for ovarian cancer. *Kidney Internat*. (2014) 85:213. doi: 10.1038/ki.2013.408
34. Cheng G, Ozgonenel B, Bhambhani K, Kapur G, Smith RJ, Savasan S. Recurrent atypical hemolytic uremic syndrome in children with acute lymphoblastic leukemia undergoing maintenance chemotherapy. *J Pediatr Hematol Oncol*. (2018) 40:560–2. doi: 10.1097/MPH.0000000000000987
35. De Leonardis F, Koronica R, Daniele RM, Santoro N. Thrombotic thrombocytopenic purpura in a child treated for acute lymphoblastic leukemia: case report and review of literature. *J Pediatr Hematol Oncol*. (2018) 40:558–9. doi: 10.1097/MPH.0000000000001050
36. Ulas A, Silay K, Akinci S, Akinci MB, Sendur MA, Dede DS, et al. Thrombotic thrombocytopenic purpura following salvage chemotherapy with paclitaxel, ifosfamide and cisplatin in a patient with a refractory germ cell tumor: a case report and review of the literature. *Oncol Lett*. (2015) 10:2223–6. doi: 10.3892/ol.2015.3338
37. Muto J, Kishimoto H, Kaizuka Y, Kinjo M, Higashi H, Kishihara F. Thrombotic microangiopathy following chemotherapy with s-1 and cisplatin in a patient with gastric cancer: a case report. *In vivo*. (2017) 31:439–41. doi: 10.21873/invivo.11080
38. Jodele S, Dandoy CE, Myers K, Wallace G, Lane A, Teusink-Cross A, et al. High-dose Carboplatin/Etoposide/Melphalan increases risk of thrombotic microangiopathy and organ injury after autologous stem cell transplantation in patients with neuroblastoma. *Bone Marrow Transpl*. (2018) 53:1311–8. doi: 10.1038/s41409-018-0159-8
39. Tseng J, Citrin DE, Waldman M, White DE, Rosenberg SA, Yang JC. Thrombotic microangiopathy in metastatic melanoma patients treated with adoptive cell therapy and total body irradiation. *Cancer*. (2014) 120:1426–32. doi: 10.1002/cncr.28547
40. Cassol CA, Williams MPA, Caza TN, Rodriguez S. Renal and pulmonary thrombotic microangiopathy triggered by proteasome-inhibitor therapy in patient with smoldering myeloma: a renal biopsy and autopsy case report. *Medicine*. (2019) 98:e17148. doi: 10.1097/MD.0000000000017148
41. Yui JC, Dispenzieri A, Leung N. Ixazomib-induced thrombotic microangiopathy. *Am J Hematol*. (2017) 92:E53–5. doi: 10.1002/ajh.24662
42. Atallah-Yunes SA, Soe MH. Drug-induced thrombotic microangiopathy due to cumulative toxicity of ixazomib. *Case Rep Hematol*. (2018) 2018:7063145. doi: 10.1155/2018/7063145
43. Higuero Saavedra V, Gonzalez-Calle V, Sobejano E, Sebastia J, Cabrero M, Bastida JM, et al. Drug-induced thrombotic microangiopathy during maintenance treatment in a patient with multiple myeloma. *Hemasphere*. (2019) 3:e192. doi: 10.1097/HS9.0000000000000192
44. Gosain R, Gill A, Fuqua J, Volz LH, Kessans Knable MR, Bycroft R, et al. Gemcitabine and carfilzomib induced thrombotic microangiopathy: eculizumab as a life-saving treatment. *Clin Case Rep*. (2017) 5:1926–30. doi: 10.1002/ccr3.1214

45. Portuguese AJ, Lipe B. Carfilzomib-induced aHUS responds to early eculizumab and may be associated with heterozygous CFHR3-CFHR1 deletion. *Blood Adv*. (2018) 2:3443–6. doi: 10.1182/bloodadvances.2018027532
46. Eremina V, Jefferson JA, Kowalewska J, Hochster H, Haas M, Weisstuch J, et al. VEGF inhibition and renal thrombotic microangiopathy. *New Engl J Med*. (2008) 358:1129–36. doi: 10.1056/NEJMoa0707330
47. Frangie C, Lefaucheur C, Medioni J, Jacquot C, Hill GS, Nochy D. Renal thrombotic microangiopathy caused by anti-VEGF-antibody treatment for metastatic renal-cell carcinoma. *Lancet. Oncol*. (2007) 8:177–8. doi: 10.1016/S1470-2045(07)70037-2
48. Horino T, Ichii O, Shimamura Y, Terada Y. Renal thrombotic microangiopathy caused by bevacizumab. *Nephrology. (Carlton)*. (2018) 23:378–9. doi: 10.1111/nep.13072
49. Yilmaz S, Ozcakar ZB, Taktak A, Kiremitci S, Ensari A, Dincaslan H, et al. Anti-VEGF-related thrombotic microangiopathy in a child presenting with nephrotic syndrome. *Pediatr Nephrol*. (2016) 31:1029–32. doi: 10.1007/s00467-016-3355-z
50. Vakiti A, Singh D, Pilla R, Alhaj-Moustafa M, Fitzpatrick KW. Bevacizumab-induced atypical hemolytic uremic syndrome and treatment with eculizumab. *J Oncol Pharm Practice*. (2019) 25:1011–5. doi: 10.1177/1078155218774895
51. Yamada R, Okawa T, Matsuo K, Suzuki M, Mori N, Mori K. Renal-limited thrombotic microangiopathy after switching from bevacizumab to ramucirumab: a case report. *BMC Nephrol*. (2019) 20:14. doi: 10.1186/s12882-018-1194-9
52. Koizumi M, Takahashi M, Murata M, Kikuchi Y, Seta K, Yahata K. Thrombotic microangiopathy associated with cetuximab, an epidermal growth factor receptor inhibitor. *Clin Nephrol*. (2017) 87:51–4. doi: 10.5414/CN108901
53. Noronha V, Punatar S, Joshi A, Desphande RV, Prabhash K. Sunitinib-induced thrombotic microangiopathy. *J Cancer Res Ther*. (2016) 12:6–11. doi: 10.4103/0973-1482.172575
54. Kapiteijn E, Brand A, Kroep J, Gelderblom H. Sunitinib induced hypertension, thrombotic microangiopathy and reversible posterior leukencephalopathy syndrome. *Ann Oncol*. (2007) 18:1745–7. doi: 10.1093/annonc/mdm454
55. Ojeda-Uribe M, Merieau S, Guillon M, Aujoulat O, Hinschberger O, Eisenmann JC, et al. Secondary thrombotic microangiopathy in two patients with Philadelphia-positive hematological malignancies treated with imatinib mesylate. *J Oncol Pharm Practice*. (2016) 22:361–70. doi: 10.1177/1078155214568580
56. Raiss H, Peron J, Tartas S, Trillet-Lenoir V, Freyer G, Errihani H. Palbociclib-induced thrombotic microangiopathy in metastatic breast cancer patient surviving for 18 years: case report and review of the literature. *Clin Breast Cancer*. (2018) 18:e263–6. doi: 10.1016/j.clbc.2017.10.001
57. Gavriilaki E, Sakellari I, Anagnostopoulos A, Brodsky RA. Transplant-associated thrombotic microangiopathy: opening Pandora's box. *Bone Marrow Transpl*. (2017) 52:1355–60. doi: 10.1038/bmt.2017.39
58. Remuzzi G, Bertani T. Renal vascular and thrombotic effects of cyclosporine. *Am J Kidney Dis*. (1989) 13:261–72. doi: 10.1016/S0272-6386(89)80032-0
59. Gavriilaki E, Sakellari I. Kidney disease after allogeneic hematopoietic cell transplantation: in search of the truth. *Acta Haematol*. (2019) 2019:1–2. doi: 10.1159/000504523
60. Nava F, Cappelli G, Mori G, Granito M, Magnoni G, Botta C, et al. Everolimus, cyclosporine, and thrombotic microangiopathy: clinical role and preventive tools in renal transplantation. *Transpl Proceed*. (2014) 46:2263–8. doi: 10.1016/j.transproceed.2014.07.062
61. Garcia-Martin P, Alarcon-Payer C, Lopez-Fernandez E, Moratalla L, Romero A, Sainz J, et al. Transplantation-associated thrombotic microangiopathy in patients treated with sirolimus and cyclosporine as salvage therapy for graft-versus-host disease. *Ann Pharm*. (2015) 49:986–94. doi: 10.1177/1060028015593369
62. Shayani S, Palmer J, Stiller T, Liu X, Thomas SH, Khuu T, et al. Thrombotic microangiopathy associated with sirolimus level after allogeneic hematopoietic cell transplantation with tacrolimus/sirolimus-based graft-versus-host disease prophylaxis. *Biol Blood Marrow Transpl*. (2013) 19:298–304. doi: 10.1016/j.bbmt.2012.10.006
63. Cavero T, Rabasco C, Lopez A, Roman E, Avila A, Sevillano A, et al. Eculizumab in secondary atypical haemolytic uraemic syndrome. *Nephrol Dialysis Transpl*. (2017) 32:466–74. doi: 10.1093/ndt/gfw453
64. Gavriilaki E, Anagnostopoulos A, Mastellos DC. Complement in thrombotic microangiopathies: unraveling ariadne's thread into the labyrinth of complement therapeutics. *Front Immunol*. (2019) 10:337. doi: 10.3389/fimmu.2019.00337
65. Dussol B, Brunet P, Vacher-Coponat H, Saingra Y, Casanova P, Berland Y. Haemolytic uraemic syndrome in a renal transplant recipient during OKT3 therapy. *Nephrol Dialy Transpl*. (1994) 9:1191–3. doi: 10.1093/ndt/9.8.1191
66. Oldenburg J, Mahlangu JN, Kim B, Schmitt C, Callaghan MU, Young G, et al. Emicizumab prophylaxis in hemophilia A with inhibitors. *N Engl J Med*. (2017) 377:809–18. doi: 10.1056/NEJMoa1703068
67. Baysal M, Umit EG, Saritas F, Kodal NS, Demir AM. Drug induced thrombotic microangiopathy with certolizumab pegol. *Balkan Med J*. (2018) 35:398–9. doi: 10.4274/balkanmedj.2017.1224
68. Philippe L, Badie J, Faller JP, Krattinger E, Deconinck E, Aubin F. Fatal thrombotic thrombocytopenic purpura in a psoriasis patient treated with ustekinumab and methotrexate. *Acta Dermat Venereol*. (2015) 95:495–6. doi: 10.2340/00015555-1987
69. Wayne AS, Shah NN, Bhojwani D, Silverman LB, Whitlock JA, Stetler-Stevenson M, et al. Phase 1 study of the anti-CD22 immunotoxin moxetumomab pasudotox for childhood acute lymphoblastic leukemia. *Blood*. (2017) 130:1620–7. doi: 10.1182/blood-2017-02-749101
70. Volcy J, Nzerue CM, Oderinde A, Hewan-Iowe K. Cocaine-induced acute renal failure, hemolysis, and thrombocytopenia mimicking thrombotic thrombocytopenic purpura. *Am J Kidney Dis*. (2000) 35:E3. doi: 10.1016/S0272-6386(00)70321-0
71. Regner SR, Lerfald N. A case off TTP temporally associated with cocaine use: implications for treatment of suspected DITMA. *J Clin Apheresis*. (2019) 34:510–2. doi: 10.1002/jca.21687
72. Kayar Y, Kayar NB, Gangarapu V. Thrombotic thrombocytopenic purpura and focal segmental glomerulosclerosis associated with the use of ecstasy. *Indian J Crit Care Med*. (2015) 19:230–2. doi: 10.4103/0972-5229.154561
73. Feigin VL, Vos T, Nichols E, Owolabi MO, Carroll WM, Dichgans M, et al. The global burden of neurological disorders: translating evidence into policy. *Lancet. Neurol*. (2019) 19:255–65. doi: 10.1016/S1474-4422(19)30411-9
74. Rimmer K, Shah H, Thakur K. Expanding medicines for neurologic disorders on the WHO model list. *Neurology*. (2017) 88:e87–91. doi: 10.1212/WNL.0000000000003691
75. Faissner S, Gold R. Efficacy and safety of the newer multiple sclerosis drugs approved since 2010. *CNS Drugs*. (2018) 32:269–87. doi: 10.1007/s40263-018-0488-6
76. Osborne WL, Lennard AL. Acute renal failure and disseminated intravascular coagulation following an idiosyncratic reaction to Alemtuzumab (Campath 1H) or fludarabine. *Haematologica*. (2005) 90:ECR05.
77. Iwazu Y, Muto S, Ioka T, Watanabe Y, Iwazu K, Kusano E, et al. Multiple sclerosis drug fingolimod induces thrombotic microangiopathy in deoxycorticosterone acetate/salt hypertension. *Hypertension*. (2018) 72:776–84. doi: 10.1161/HYPERTENSIONAHA.117.10655
78. Yam C, Fok A, McLean C, Butler E, Kempster P. 035 Interferon beta induced thrombotic microangiopathy in multiple sclerosis: a clinical-pathological report. *J Neurol Neurosurg Psychiatry*. (2018) 89:A15. doi: 10.1136/jnnp-2018-ANZAN.34
79. Omoto S, Utsumi T, Matsuno H, Terasawa Y, Iguchi Y. Thrombotic Microangiopathy Presenting with Intestinal Involvement Following Long-term Interferon-beta1b Treatment for Multiple Sclerosis. *Internal Med*. (2018) 57:741–4. doi: 10.2169/internalmedicine.9326-17
80. Allinovi M, Cirami CL, Caroti L, Antognoli G, Farsetti S, Amato MP, et al. Thrombotic microangiopathy induced by interferon beta in patients with multiple sclerosis: three cases treated with eculizumab. *Clin Kidney J*. (2017) 10:625–31. doi: 10.1093/ckj/sfw143
81. Page EE, Little DJ, Vesely SK, George JN. Quinine-induced thrombotic microangiopathy: a report of 19 patients. *Am J Kidney Dis*. (2017) 70:686–95. doi: 10.1053/j.ajkd.2017.05.023
82. Fromm L. Suspected hydroxychloroquine-induced thrombotic thrombocytopaenic purpura. *J Pharm Practice Res*. (2017) 48:72–5. doi: 10.1002/jppr.1248
83. Howard MD, Sim S. A novel association between antiretroviral therapy and drug-induced immune thrombocytopenia purpura. *Acta Haematol*. (2019) 141:199–200. doi: 10.1159/000496325

84. Trasar L, Mirza N, Lisawat P, Raut R, Shih W. Thrombotic thrombocytopenic purpura and rhabdomyolysis associated with ciprofloxacin use. *Am J Kidney Dis*. (2016) 67:A109. doi: 10.1053/j.ajkd.2016.03.370
85. Schwartz J, Padmanabhan A, Aqui N, Balogun RA, Connelly-Smith L, Delaney M, et al. Guidelines on the use of therapeutic apheresis in clinical practice-evidence-based approach from the writing committee of the american society for apheresis: the seventh special issue. *J Clin Apher*. (2016) 31:149–62. doi: 10.1002/jca.21470
86. Gavriilaki E, Brodsky RA. Complementopathies and precision medicine. *J Clin Invest*. (2020) 130:2152–63. doi: 10.1172/JCI136094
87. de Francisco ALM, Macia M, Alonso F, Garcia P, Gutierrez E, Quintana LF, et al. Onco-nephrology: cancer, chemotherapy and kidney. *Nefrologia*. (2019) 39:473–81. doi: 10.1016/j.nefroe.2018.10.016

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CASE REPORT
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Diverse Clinical Presentations of C3 Dominant Glomerulonephritis

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C3 dominant immunofluorescence staining is present in a subset of patients with idiopathic immune complex membranoproliferative glomerulonephritis (iMPGN). It is increasingly recognized that iMPGN may be complement driven, as are cases of "typical" C3 glomerulopathy (C3G). In both iMPGN and C3G, a frequent membranoproliferative pattern of glomerular injury may indicate common pathogenic mechanisms via complement activation and endothelial cell damage. Dysregulation of the alternative complement pathway and mutations in certain regulatory factors are highly implicated in C3 glomerulopathy (which encompasses C3 glomerulonephritis, dense deposit disease, and cases of C3 dominant MPGN). We report three cases that demonstrate that an initial biopsy diagnosis of iMPGN does not exclude complement alterations similar to the ones observed in patients with a diagnosis of C3G. The first patient is a 39-year-old woman with iMPGN and C3 dominant staining, with persistently low C3 levels throughout her course. The second case is a 22-year-old woman with elevated anti-factor H antibodies and C3 dominant iMPGN findings on biopsy. The third case is a 25-year-old woman with C3 dominant iMPGN, dense deposit disease, and a crescentic glomerulonephritis on biopsy. We present the varied phenotypic variations of C3 dominant MPGN and review clinical course, complement profiles, genetic testing, treatment course, and peri-transplantation plans. Testing for complement involvement in iMPGN is important given emerging treatment options and transplant planning.

Keywords: complement mutations, membranoproliferative glomerulonephritis, alternative pathway, C3 glomerulonephritis, proteinuria

INTRODUCTION

C3 glomerulopathy (C3G) encompasses a group of diseases that result from abnormalities in the alternative pathway of complement regulation, and has been defined by C3 only or C3-dominant immunofluorescence staining seen on renal biopsy (1). In contrast to atypical Hemolytic Uremic Syndrome (aHUS) (2), the clinical course of C3G is more indolent. As such, C3G is less likely than aHUS to present clinically as a systemically active and rapidly progressive disease (3). The term C3G was introduced to differentiate glomerular diseases which result from alternative pathway

dysregulation from other immune complex mediated glomerular diseases. C3G includes C3 glomerulonephritis (C3GN) and dense deposit disease (DDD); the latter of which is characterized ultra-structurally by the presence of highly osmiophilic intramembranous deposits (4). Both C3GN and DDD often present with a membranoproliferative pattern of glomerular injury, a finding that can also be seen in thrombotic microangiopathy (TMA) (5). Also included under the "umbrella" term of C3G are a subset of cases which were historically diagnosed as immune complex mediated membranoproliferative glomerulonephritis (MPGN) of unknown etiology, but showed dominant staining for C3 by immunofluorescence staining, with lesser deposition of "typical" immune complex deposits such as IgG or IgA.

The pathogenic mechanism underlying C3G is uncontrolled production and deposition of the C3 breakdown product, C3b, along glomerular and sometimes tubular basement membranes (the latter which is most often seen in DDD) (6). While histologically the disease can appear quite heterogenous (7), pathogenically, there is a final common pathway leading to glomerular injury (8). There are important acquired forms of the disease such as autoantibodies against the regulatory proteins factor H (FH) and factor B (FB), as well as autoantibodies against the C3 convertases of the alternative and classical pathways (C3Nef and C4Nef, respectively)that can phenotypically mimic genetically acquired disorders (9–11). Patients with C5 nephritic factors (C5Nef) against downstream effectors in the final common pathway have also been reported (12). C3-5Nef and factor B antibodies have been observed in C3G patients with DDD as well as MPGN patterns on renal biopsy (9, 12–14), and can be treated with C3 and C5 blocking pharmacotherapy (15, 16).

As the understanding of the pathogenesis of C3G evolved, it became clear that some cases of immune complex mediated MPGN, including those with C3 dominant immunofluorescence staining and cases where there was also deposition of other immune complex deposits, were in fact complement-mediated, and represented a subset of C3G (1, 8, 16, 17). These cases could therefore be distinguished from cases displaying the more "typical" mixed complement and immunoglobulin deposition seen in MPGN secondary to infections and autoimmune disease, or MPGN associated with plasma cell dyscrasias and monoclonal immunoglobulin deposition disease (MIDD) (1, 10, 15, 16). In some cases, histologic features of TMA may co-exist with diagnostic features of C3G, also suggestive of abnormal complement regulation and activation as the source of glomerular disease.

Inherited or genetic causes of C3G include loss of function mutations that result in impaired self-protection from innate immunity (20), or uncontrolled activation of the alternative pathway (21–23). Mutations in Factor H, Factor I, C3, Factor B, Membrane cofactor protein (MCP), thrombomodulin (THBD), diacylglycerol kinase epsilon (DGKE) (24), and plasminogen are the more common mutations associated with DDD, C3GN, and C3 dominant iMPGN that form C3 GN (24). **Table 1** summarizes the pathological findings seen in C3G and idiopathic immune complex MPGN.

We report three cases of C3G with various clinical presentations and pathologic findings. The clinical course, complement profiles, and treatment plans are reviewed. In addition, the role of eculizumab and other immunosuppressive agents is discussed as it relates to each case. The chief aim of this case series is to provide examples of the importance of detecting and managing complement dysfunction in iMPGN for the purposes of treatment and planning for renal transplantation.

CASES

Case 1

The patient is a 39-year-old woman who presented 1 year ago with progressive proteinuria, renal failure, and hypertension. A biopsy was performed after development of significant renal dysfunction; serum creatinine was 3 mg/dL. The protein excretion rate was 1–2 g/24 h initially, which increased to 4–6 g/24 h with worsening renal function. The initial biopsy result at an outside institution was reported as non-diagnostic, with features of chronic autoimmune glomerulonephritis. The patient had a thorough infectious disease evaluation which was negative for hepatitis A, hepatitis B virus (HBV), and hepatitis C virus (HCV) serologies, negative for human immunodeficiency virus (HIV), negative for treponemal serologies, and with no evidence of tuberculosis, coccidiomycosis or other chronic infections. She did not have known diabetes, obesity, or malignancies.

Extensive serological evaluation was negative for lupus serologies [anti-nuclear antibody negative (<1:40 titer), anti-double stranded DNA (anti dsDNA) negative (<200 international units/ml), DNAase B antibody negative (<86 Units/ml), anti-histone antibody negative 0.4 units, anti-centromere <1, ribonucleoprotein <20 units, anti-smith antibodies <20 units, Sjogren's syndrome (SSA and SSB) antibodies <20 units, rheumatoid factor negative <1, scleroderma antibodies negative at 1 AU/ml, anti-citric citrulline peptide negative at 3 units, Cardiolipin IgA, IgG, IgM was negative, and ANCA panel (c-ANCA, p-ANCA, proteinase 3 and anti-myeloperoxidase were all negative <1:20 titer). Complement titers (CH 50) were near the lower limit of normal

Abbreviations: ADAMTS13, A Disintegrin and Metalloproteinase with a Thrombospondin Type 1 Motif, Member 13; aHUS, Atypical Hemolytic Uremic Syndrome; C1q, C1q Protein; C3, Complement Component 3; C4, Complement Component 4; C3G-C3, Glomerulopathy; C3GN-C3, Glomerulonephritis; CFB, Complement Factor B; CFH, Complement Factor H; CFHR1, Complement Factor H Related Protein 1; CFHR3, Complement Factor H Related Protein 3; CFHR5, Complement Factor H Related Protein 5; CFI, Complement factor I; C3Nef-C3, Nephritic Factor; C4Nef-C4, Nephritic Factor; C5Nef-C5, Nephritic Factor; C5b-9, Complement Factor 5b-9 Membrane Attack Complex; DGKE, Diacyl Glycerol Kinase Epsilon; DDD, Dense Deposit Disease; ELISA, Enzyme Linked Immunosorbent Assay; Factor B, Complement Factor B; HBV, Hepatitis B Virus; HCV, Hepatitis C Virus; IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M; iMPGN, diopathic, Idiopathic Immune Complex Membranoproliferative Glomerulonephritis MAC-Membrane Attack Complex; MCP, Membrane Cofactor Protein (CD-46); MMACHC, Methylmalonic Aciduria (Cobalamin Deficiency) CblC Type, with Homocystinuria; MLPA, Multiple Ligation Probe Amplification; MPGN, Membranoproliferative Glomerulonephritis; PLG, Plasminogen; THBD, Thrombomodulin; TMA, Thrombotic Microangiopathy. Note all abbreviations formatted according to standard complement nomenclature (18, 19).

TABLE 1 | Pathological findings in each subtype of C3 glomerulopathy and three cases of C3 dominant idiopathic MPGN.

Type	Age	Gender	Ethnicity	LM findings	IF findings	EM findings	Complement profile	Genetic mutations associated	Acquired	Peak UPC	Peak sCr
Classic C3 GN				MPGN, TMA, MP, DEP	C3 > Ig 2:1	Varies, may have deposits	Possible increased activation	FH, FB,FI, C3,C5, THBD, PLS, MCP, DAGKE	C3/C4/C5 Nef	Inc	Inc
DDD				MPGN, TMA, Crescentic GN	C3 > Ig 2:1	Numerous dense deposits	Possible increased activation	FH, FB,FI, C3,C5, THBD, PLS, MCP, DAGKE	C3/C4/C5 Nef	Inc	Inc
C3 Dom iMPGN				MPGN, TMA	C3 > Ig 2:1	Sub End, sub Ep deposits	Possible increased activation	FH, FB,FI, C3,C5, THBD, PLS, MCP, DAGKE	C3/C4/C5 Nef	Inc	Inc
Case 1 iMPGN	41	F	Hispanic	MPGN, Endothelial swelling, Crescents	C3 > Ig	Sub End, sub Ep deposits	Low C3, normal sC5b-9	None found	None	4–6 g protein/g creatinine	3mg/dL
Case 2 C3 Dom MPGN	22	F	Asian	MPGN	C3 only	Sub End, sub Ep deposits	120–160% of normal sC5b-9	Not done	Anti CFH Ab	1–2 g protein/g creatinine	0.5 mg/dL
Case 3 C3 Dom MPGN	25	F	Hispanic	MPGN, DDD, Crescents	C3 > Ig	Dense deposits	Initially normal then decreased C3, C4	None found	None	9 g protein/g creatinine	15 mg/dl

Anti CFH Ab, anti-complement Factor H antibody; C3G, C3 glomerulopathy; C3 Dom MPGN, C3 dominant membranoproliferative glomerulonephritis; CH50, total complement; DGKE, diacylglycerol kinase epsilon; DDD, dense deposit disease; DEP, diffuse endocapillary proliferative pattern; EM, electron microscopy; F, female; FH, factor H; FB, factor B; FI, factor I; g, gram; GN, glomerulonephritis; Inc, increased; iMPGN, idiopathic membranoproliferative glomerulonephritis; IF, immunofluorescence; Ig, immunoglobulin; iMPGN, idiopathic membranoproliferative glomerulonephritis; LM, light microscopy; M, male; MCP, membrane cofactor protein; mg/dL, milligram/deciliter; MP, mesangial proliferative pattern; MPGN, membranoproliferative glomerulonephritis [Type I]; NF, nephritic factor; PLS, plasminogen, sCr, serum creatinine [mg/dL]; Sub End, subendothelial; Sub Ep, subepithelial; SMAC, soluble membrane attack complex; THBD, thrombomodulin; TMA, thrombotic microangiopathy; UPC, urine protein/creatinine ratio [g/g creatinine].

at 43 units/mL [Reference range 42–95 Units/mL], and there was isolated depression of C3 at 67 mg/dL [Reference range 76–165 mg/dL] but with normal C4 levels at 23 mg/dL [Reference range 16–48 mg/dL]. Serum and urine electrophoretic studies and immunofixation studies did not reveal a monoclonal spike. Free light chain kappa to lambda ratio was also normal with a ratio of 1.66.

On renal biopsy (**Figure 1**), the dominant finding by light microscopy was an MPGN pattern of injury that was predominantly chronic with segmental sclerosis, extensive global glomerulosclerosis, and parenchymal scarring. However, there was residual activity including focal cellular-to-fibrocellular crescents, karyorrhexis, and diffuse, and mild endocapillary hypercellularity. Along with prominent glomerular basement membrane double contours, there was also some suggestion of mesangiolysis and marked endothelial cell swelling raising the possibility of endothelial cell injury, although definitive morphologic features of TMA were not identified. Immunofluorescence studies showed segmental to global granular staining for IgG (2+), IgA (trace), IgM (trace), C1q (2+), C3 (3+), and kappa/lambda light chains (both trace). At least two glomeruli exhibited segmental fibrinogen staining (2–3+) in areas of capillary loop necrosis. Electron microscopy revealed amorphous electron dense deposits present in a predominantly subendothelial and mesangial distribution with occasional subepithelial and intramembranous deposits. Endothelial cell cytoplasm was swollen. There were no tubuloreticular inclusions or extra-glomerular deposits. A diagnosis of immune-complex mediated membranoproliferative glomerulonephritis was rendered.

Given the negative evaluation for underlying infectious, autoimmune diseases, and neoplastic disorders, the possibility of a complement-mediated disorder was investigated. Complement profile testing did not reveal C3Nef, C4Nef, anti-factor H, or factor B antibodies. Soluble C5b-9 levels were not elevated (within the normal range of <250 ng/mL), a finding which was compatible with the patient's glomerular disease having entered a quiescent stage. No known genetic mutations or variants of uncertain significance were found at the time (Iowa complement laboratory) in the 2017 panel that was sent. The panel sent included CFH, CFI, MCP (CD46), CFB, CFHR5, THBD, C3, ADAMTS13, PLG, DGKE, MMACHC, G6PD. Multiple Ligation Probe Amplification (MLPA) was included in the testing to determine copy number variants over the complete complement Factor H related region (25).

It should be noted that the rate of capture of genetic mutations remains only 41–60%, as not all mutations leading to complement mediated disorders are known at this point (14).

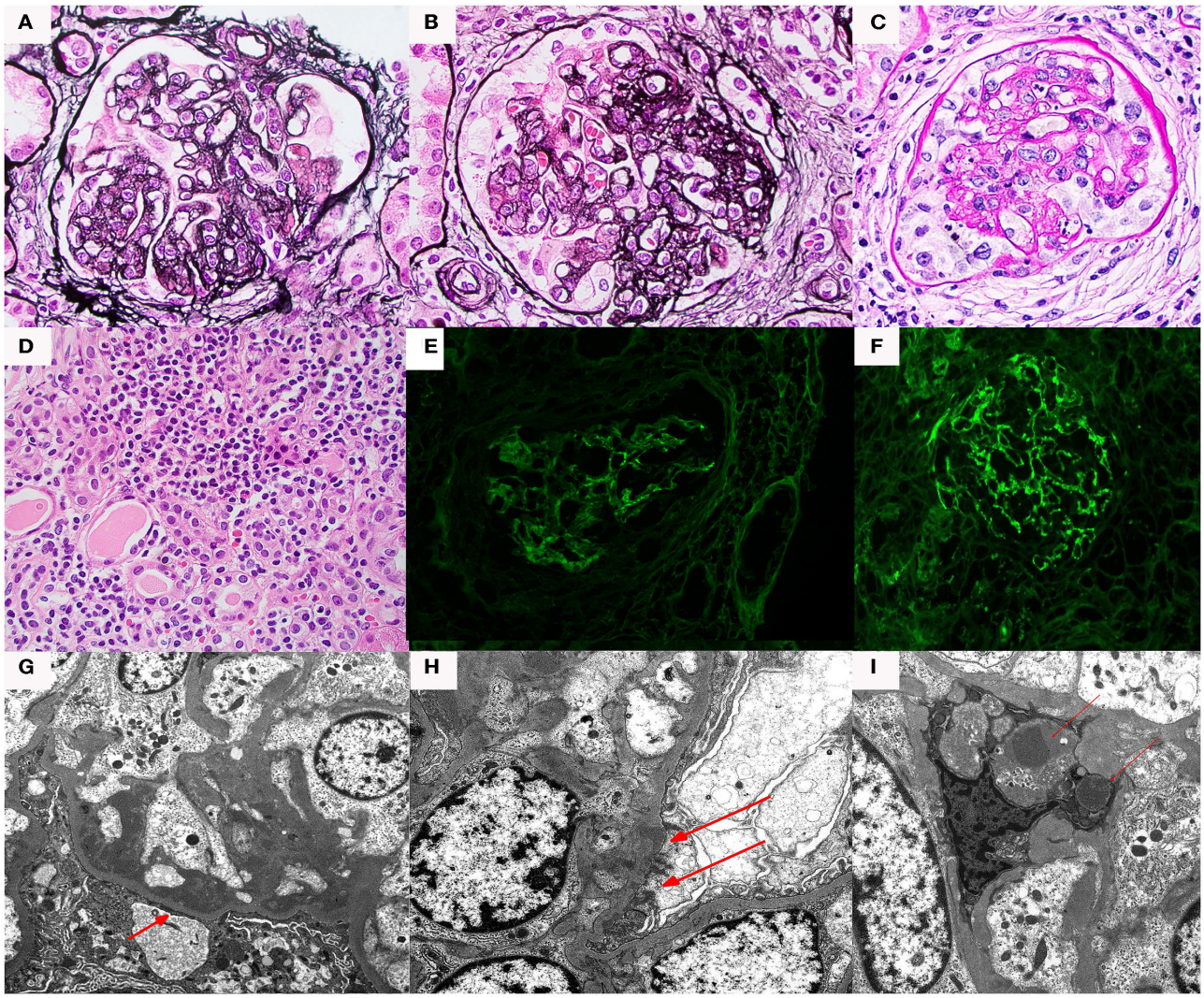

FIGURE 1 | Renal biopsy data for Case 1: **(A)** light microscopy 40× silver stain Double contours and segmental scar; **(B)** light microscopy 40× silver stain Double contours with endocapillary hypercellularity and segmental scar; **(C)** light microscopy 40× hematoxylin and eosin cellular crescent with segmental karyorrhexis; **(D)** light microscopy 40× hematoxylin and eosin tubulointerstitial inflammation; **(E)** immunofluorescence IgG staining; **(F)** immunofluorescence showed C3 staining > IgG staining. **(G)** Electron microscopy, subendothelial deposits (red arrows); **(H)** electron microscopy, subepithelial deposits (red arrows); **(I)** electron microscopy, mesangial deposits (red arrows).

However, C3 levels remained low at 67–75 mg/dL a year after the renal biopsy, supporting the presence of a complement-mediated disorder. The patient has remained off renal replacement therapy and is scheduled to undergo a preemptive renal transplant. Treatment with mycophenolate mofetil with the transplant may be able to prevent any complement mediated disease process recurrence; however, further therapy would depend on demonstration of recurrent glomerulonephritis in renal allograft biopsy (26, 27).

Case 2

The patient is a 22-year-old woman who was noted to have persistent non-nephrotic proteinuria of 2–3 g protein/g creatinine with preserved renal function; serum creatinine was 0.5 mg/dL. These parameters remained essentially unchanged for 2 years, at which time a renal biopsy (**Figure 2**) was performed. Serum C3, C4, and CH50 were within normal range.

By conventional light microscopy, a diffuse MPGN pattern of glomerular injury was observed, with diffuse mesangial and endocapillary hypercellularity associated with conspicuous glomerular basement membrane double contour formation. Prominent mesangial and capillary loop deposits were identified. There was focal segmental glomerulosclerosis. No crescents or necrotizing features were seen. Immunofluorescence studies demonstrated bright diffuse and global capillary wall and mesangial staining for C3 without significant staining for any other immunoglobulins or complement protein.

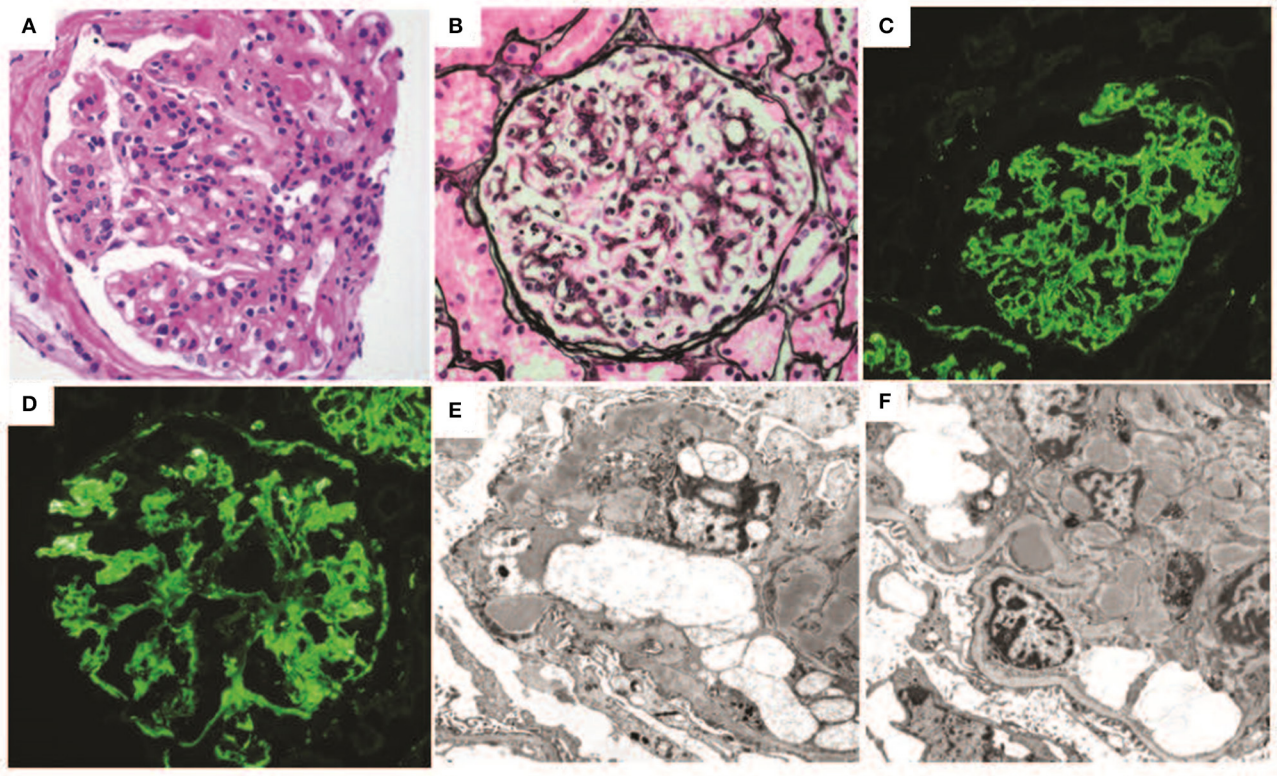

FIGURE 2 | Renal biopsy data for Case 2: **(A)** light micrographs demonstrating a diffuse and global membranoproliferative glomerulonephritis pattern of injury periodic acid Schiff (PAS) stain; **(B)** Jones silver stain (40× magnification) demonstrating glomerular basement membrane double contours; **(C,D)** immunofluorescence micrographs demonstrating diffuse global bright mesangial and capillary loop C3 staining; **(E,F)** electron micrographs demonstrating numerous mesangial, subepithelial, and intramembranous deposits. Several subepithelial deposits show "hump-like" morphology. Capillary loop double contours with cellular interpositions are also present.

Electron microscopic evaluation demonstrated numerous amorphous electron dense deposits throughout mesangial regions, intramembranous spaces, and subepithelial spaces (including several "hump-like" deposits), and less frequently in subendothelial locations. Bowman's capsular deposits were also seen. Glomerular basement membrane duplication with mesangial interposition was also present. No highly osmiophilic intramembranous or "sausage-like" deposits were seen.

Subsequent lab tests revealed evidence for alternative pathway activation (28). Specifically, an autoantibody against complement factor H was positive at a 1:200 titer(value of _1200_AU/mL) [Reference range < 150 AU/mL] (29). An elevated soluble C5b-9 membrane attack complex (MAC) was also detected. The titer was at 1.6 (160% reference range <1). C3NeF and C4NeF were negative by immunofixation and C3/C4 degradation tests and C3 CSAP [centriole-cilia-and spindle associated protein] were also negative. Genetic testing for Cockayne syndrome B protein [CSB protein] was normal. Deletions in CFHR3-CFHR1 were screened for and not found. This testing was done since some CFHR3-CFHR1 deletions maybe associated with development of anti CFH antibodies (30). This case illustrates what is likely an acquired form of C3G.

The patient was monitored closely by her nephrologist and started on renin-angiotensin-aldosterone system (RAAS) blockade with an angiotensin receptor blocker (ARB) and an aldosterone antagonist. Anti Factor H autoantibodies remained elevated at 1:200 and was found via an ELISA assay [Reference range < 150 AU/mL] (29). C5b-9 MAC levels remained elevated but improved at 1.2 (120% of reference range, <1). The patient's serum creatinine has remained normal at 0.5mg/dL, and current urinary protein-to-creatinine ratio is improved at 0.2 g protein/gram creatinine. The patient was not started on factor H inhibitor therapy or other immunosuppressive therapy given her remarkably stable renal parameters and lack of any other thrombotic manifestations. She is being monitored for deterioration at which time plasma exchange and anti CD20 monoclonal antibodies would be considered.

Case 3

The patient is a 25-year-old woman who presented at age 16 with lower extremity edema when she was approximately six months pregnant. Kidney biopsy at that time revealed DDD with 45% active crescents and marked acute tubulointerstitial injury (**Figures 3A–C**). On immunofluorescence, approximately

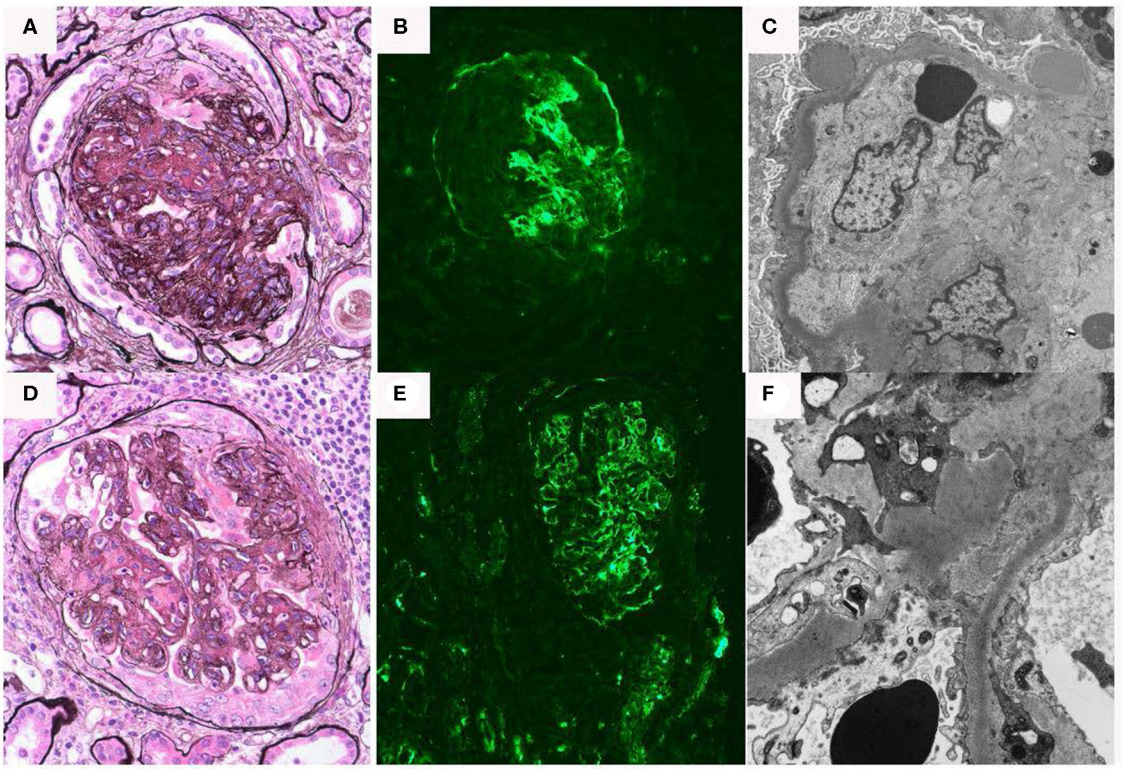

FIGURE 3 | Renal biopsy data for Case 3: **(A–C)** First renal biopsy demonstrating **(A)**; light microscopy with Jones silver stain demonstrating membranoproliferative pattern of injury 40×; **(B)** immunofluorescence demonstrating dominant C3 binding in a segmental confluent granular to pseudolinear pattern; **(C)** electron microscopy showing dense deposits within segments of the basement membrane, it also shows electron dense deposit-like aggregates in scattered subepithelial and intramembranous areas; **(D–F)** second renal biopsy re-demonstrates a membranoproliferative pattern of injury, C3 dominant binding on immunofluorescence and dense deposits.

half the glomeruli had capillary wall and/or mesangial staining for C3 (4+) in a segmental confluent granular to pseudolinear pattern. On electron microscopy, capillary walls were irregular and segmentally had thickened with osmiophilic deposits which segmentally transformed and replaced the lamina densa. There were scattered aggregates of electron dense deposits in a subepithelial and intramembranous distribution, and a pathological diagnosis of DDD was made. At that time the serum creatinine was 0.99 mg/dl, with normal C3 89.5 [76–165 normal range] and C4 80.1 (16–48 normal range). The patient declined treatment at that time because of concern about taking medications during her pregnancy.

Six days after a full-term delivery, she presented to a different hospital with abdominal pain, dysuria, and worsening edema. Labs were significant for creatinine 4.1 mg/dL, albumin 1.6 g/dL, and urine protein-to-creatinine ratio of 9.3 g/g. In addition, there was isolated depression of C3 at 54.1 [76–165 normal range] with normal C4 37.5 [16–48 normal range]. A repeat kidney biopsy again showed DDD with extensive crescent formation. The tubulointerstitium showed interstitial inflammation with focal intratubular neutrophils and cellular debris suggestive of acute pyelonephritis, as well as evolving parenchymal scarring (**Figures 3D–F**). On electron microscopy, few and large, "hump-like" subepithelial deposits were noted. She was treated with intravenous antibiotics, one dose of intravenous methylprednisolone 500 mg, and five sessions of plasma exchange. She also started hemodialysis at that time.

Genetic testing was performed as part of a transplant evaluation. A C3 glomerulonephritis (C3GN)/DDD genetic susceptibility panel, including C3, CFB, CFH, CFHR1, CFHR2, CFHR1, CFI, MCP (CD46) identified no known mutations. In addition, C3Nef, Factors B, H, and I, Factor H autoantibody, and soluble C5b-9 levels were within normal limits (reference range <1).

The patient was activated on the kidney transplant list. After 9 years on dialysis, the patient underwent a deceased donor kidney transplant (donor in 30 s, terminal creatinine 1 mg/dl). She received basiliximab for induction immunosuppression and tacrolimus, mycophenolate mofetil, and prednisone for maintenance immunosuppression. Her post-transplant course was significant for expected delayed graft function given cold ischemia time of 19 h requiring hemodialysis for two sessions post-transplant. She was monitored closely with no evidence of proteinuria or intermittent microscopic hematuria. She is now over 1-year post-transplant with a creatinine of 0.9 mg/dl

and no evidence of proteinuria or hematuria to indicate recurrent disease.

DISCUSSION

C3G was first described in 2007 (31, 32), and increased the awareness of the role of complement and complement dysregulation in renal disease. These diseases are continuing to be characterized in great molecular detail (17). C3G includes DDD (7, 33), C3 glomerulonephritis (34), and a subset of immune complex mediated MPGN which are thought to result from alternative pathway dysregulation. The wide variability in clinical presentation and pathology are shown in our three cases. Clinically, the presentation varied from mild to severe and progressive chronic kidney disease (CKD) requiring eventual hemodialysis. Histologically, the pathology manifestations included DDD as well as variably active and proliferative MPGN, sometimes with severe crescentic involvement. The latter finding is consistent with reports of more severe cases of C3G which have presented as crescentic and necrotizing rapidly progressive glomerulonephritis (RPGN) (27, 35), or as pulmonary renal syndromes (36). A low C3 level in iMPGN patients suggests a need for workup for alternative pathway dysfunction.

The three cases of C3G demonstrate the diversity of pathologic findings and the variability of clinical course. The first patient presented with a persistently low C3 level and had biopsy findings of MPGN and ultrastructural features suggestive of endothelial cell injury; with a more severe clinical course with significant CKD in the absence of genetic mutations in the alternative pathway. In contrast, the second patient also exhibited MPGN features on biopsy and was found to have anti-factor H autoantibodies, elevated C5b-9levels 120–160% of reference range, but with a milder clinical course not requiring immunosuppression. A limited genetic screen did not find CFHR1–3 deletions. The third patient presented with sporadic decreased C3 levels and demonstrated C3G biopsy findings with a DDD phenotype and extensive crescentic involvement. However, alternative pathway genetic testing was negative for known mutations or suspicious variants of unknown significance.

These cases highlight the etiologic, molecular, pathologic, genetic, and clinical features that demonstrate how a subset of immune complex mediated MPGN cases can overlap with diseases resulting from complement dysregulation as well as TMA (22). One important limitation of our three cases is that Pronase digestion was not used, hence, limiting ability to find "masked" immunoglobulin deposits (37). There is increasing evidence that a distinct group of C3G patients may be associated with monoclonal gammopathies, and can also manifest with histologic features consistent with C3G and MPGN (38).

Like aHUS, the genetic mutations leading to complement dysregulation in C3G are varied and interact in complex non-mendelian patterns (14, 39). There can be combinations of mutations or traits that coincide and ultimately lead to complement dysregulation. Less frequently observed are patients with mutations that are strongly heritable with a higher penetrance both in aHUS and in patients with C3G (21). It is recommended that potential organ donors be screened for complement mutations if C3G is suspected before transplant, analogous to a patient with aHUS having a family member who is a potential donor screened. This has real clinical implications as C3G has shown to frequently recur in renal allograft patients (40).

Acquired factors classically known as nephritic factors are autoantibodies that disrupt the function of various complement regulatory factors resulting in pathway dysfunction. This can ultimately lead to the uncontrolled production and deposition of complement components in the glomeruli (10, 11). The presence of nephritic factors can be found in the entire spectrum of the C3G family of disorders (10, 11, 22). In our patient group, however, we were not able to detect the presence of a nephritic factor.

It is imperative for the authors to mention that the complement testing that we did nearly 3 years ago was limited. It is now standard of genetic testing in MPGN to identify all the known genetic drivers in C3G (3). Studies have to include mutational screening of complement genes, copy number variations analyses to detect genomic rearrangements of CFHR genes, and screening of risk alleles of the CFH, MCP, and CFHR5 genes. In addition, immunological/biochemical evidence of complement involvement should be sought by measuring plasma levels of C3, C4, FH, FI, FB, and soluble C5b9 complexes, and by screening autoantibodies (anti-FH, anti-FB, C3Nef, C4Nef, C5Nef).

Treatment recommendations have been slowly evolving (26), but less targeted immunosuppression including glucocorticoids and rituximab are reportedly less effective (41, 42). There is great interest and an apparently increasing role of complement inhibition (i.e., off-label utilization of C5 convertase inhibitors such as eculizumab or other novel complement blocking agents) (43). The need to prevent recurrence post-transplant provides a compelling indication for effective treatment strategies in patients with C3G, as complement blockade has been postulated to allow an allograft to continue functioning and prevent recurrent TMA or C3G (44). Our patient with DDD who received a kidney transplant (case 3) did not require eculizumab and has not shown recurrence over 1-year post-transplant. Given the variable course of DDD, we opted not to administer eculizumab pre-transplant, but rather to clinically monitor for recurrence post-transplant. In addition, this patient remains on routine maintenance immunosuppression with mycophenolate mofetil, tacrolimus, and prednisone. It should be noted, however, that because this disease can present as a late recurrence, possibly due to an environmental trigger, vigilant screening of proteinuria and creatinine is warranted.

C3G as a persistent process related to complement dysregulation has potential analogs to aHUS beyond just diagnosis, genetics, molecular etiology, and therapy. The importance of both genetic and environmental risk factors in both diseases can explain the low penetrance and periods of quiescence seen in many cases. The finding that complement inhibition in C3G is only effective when the disease is more active might be explained by the fact that complement inhibition fails to affect quiescent or smoldering disease (44). Similarly,

an environmental trigger is often needed to activate an episode or relapse of aHUS (such as pregnancy, medications, infection, multiple myeloma, and diverse rheumatological diseases) (45–47). Therefore, it is possible that C3G follows an analogous pattern where complement activating conditions can also trigger the development of C3G in genetically susceptible individuals.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

RH: writing of manuscript. JH: extensive editing of manuscript. HH: report for Case 2. FA, US, JW, SO, MB, and IK: editing and writing manuscript. JZ: pathology figures and slides. JH: editing pathology figures and slides. JY: senior author. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Hou J, Markowitz GS, Bomback AS, Appel GB, Herlitz LC, Barry Stokes M, et al. Toward a working definition of C3 glomerulopathy by immunofluorescence. *Kidney Int.* (2014) 85:450–6. doi: 10.1038/ki.2013.340
2. Zipfel PF, Skerka C, Chen Q, Wiech T, Goodship T, Johnson S, et al. The role of complement in C3 glomerulopathy. *Mol Immunol.* (2015) 67:21–30. doi: 10.1016/j.molimm.2015.03.012
3. Goodship TH, Cook HT, Fakhouri F, Fervenza FC, Fremeaux-Bacchi V, Kavanagh D, et al. Atypical hemolytic uremic syndrome and C3 glomerulopathy: conclusions from a "kidney disease: improving global outcomes" (KDIGO) controversies conference. *Kidney Int.* (2017) 91:539–51. doi: 10.1016/j.kint.2016.10.005
4. Smith RJ, Alexander J, Barlow PN, Botto M, Cassavant TL, Cook HT, et al. New approaches to the treatment of dense deposit disease. *J Am Soc Nephrol.* (2007) 18:2447–56. doi: 10.1681/ASN.2007030356
5. Brackman D, Sartz L, Leh S, Kristoffersson AC, Bjerre A, Tati R, et al. Thrombotic microangiopathy mimicking membranoproliferative glomerulonephritis. *Nephrol Dial Transplant.* (2011) 26:3399–403. doi: 10.1093/ndt/gfr422
6. Barbour TD, Ruseva MM, Pickering MC. Update on C3 glomerulopathy. *Nephrol Dial Transplant.* (2016) 31:717–25. doi: 10.1093/ndt/gfu317
7. Bomback AS, Santoriello D, Avasare RS, Regunathan-Shenk R, Canetta PA, Ahn W, et al. C3 glomerulonephritis and dense deposit disease share a similar disease course in a large United States cohort of patients with C3 glomerulopathy. *Kidney Int.* (2018) 93:977–85. doi: 10.1016/j.kint.2017.10.022
8. Master Sankar Raj V, Gordillo R, Chand DH. Overview of C3 Glomerulopathy. *Front Pediatr.* (2016) 4:45. doi: 10.3389/fped.2016.00045
9. Marinozzi MC, Roumenina LT, Chauvet S, Hertig A, Bertrand D, Olagne J, et al. Anti-factor B and anti-C3b autoantibodies in C3 glomerulopathy and Ig-associated membranoproliferative GN. *J Am Soc Nephrol.* (2017) 28:1603–13. doi: 10.1681/ASN.2016030343
10. Schena FP, Pertosa G, Stanziale P, Vox E, Pecoraro C, Andreucci VE. Biological significance of the C3 nephritic factor in membranoproliferative glomerulonephritis. *Clin Nephrol.* (1982) 18:240–6.
11. Zhang Y, Meyer NC, Fervenza FC, Lau W, Keenan A, Cara-Fuentes G, et al. C4 Nephritic factors in C3 glomerulopathy: a case series. *Am J Kidney Dis.* (2017) 70:834–43. doi: 10.1053/j.ajkd.2017.07.004
12. Marinozzi MC, Chauvet S, Le Quintrec M, Mignotet M, Petitprez F, Legendre C, et al. C5 nephritic factors drive the biological phenotype of C3 glomerulopathies. *Kidney Int.* (2017) 92:1232–41. doi: 10.1016/j.kint.2017.04.017
13. Strobel S, Zimmering M, Papp K, Prechl J, Jozsi M. Anti-factor B autoantibody in dense deposit disease. *Mol Immunol.* (2010) 47:1476–83. doi: 10.1016/j.molimm.2010.02.002
14. Xiao X, Pickering MC, Smith RJ. C3 glomerulopathy: the genetic and clinical findings in dense deposit disease and C3 glomerulonephritis. *Semin Thromb Hemost.* (2014) 40:465–71. doi: 10.1055/s-0034-1376334
15. Nozal P, Lopez-Trascasa M. Autoantibodies against alternative complement pathway proteins in renal pathologies. *Nefrologia.* (2016) 36:489–95. doi: 10.1016/j.nefro.2016.01.014
16. Salvadori M, Rosso G. Reclassification of membranoproliferative glomerulonephritis: identification of a new GN: C3GN. *World J Nephrol.* (2016) 5:308–20. doi: 10.5527/wjn.v5.i4.308
17. Heeringa SF, Cohen CD. Kidney diseases caused by complement dysregulation: acquired, inherited, and still more to come. *Clin Dev Immunol.* (2012) 2012:695131. doi: 10.1155/2012/695131
18. Bohlson SS, Garred P, Kemper C, Tenner AJ. Complement nomenclature-deconvoluted. *Front Immunol.* (2019) 10:1308. doi: 10.3389/fimmu.2019.01308
19. Corvillo F, Okroj M, Nozal P, Melgosa M, Sanchez-Corral P, Lopez-Trascasa M. Nephritic factors: an overview of classification, diagnostic tools and clinical associations. *Front Immunol.* (2019) 10:886. doi: 10.3389/fimmu.2019.00886
20. Thomas S, Ranganathan D, Francis L, Madhan K, John GT. Current concepts in C3 glomerulopathy. *Indian J Nephrol.* (2014) 24:339–48. doi: 10.4103/0971-4065.134089
21. Chauvet S, Roumenina LT, Bruneau S, Marinozzi MC, Rybkine T, Schramm EC, et al. A Familial C3GN secondary to defective C3 regulation by complement receptor 1 and complement factor H. *J Am Soc Nephrol.* (2016) 27:1665–77. doi: 10.1681/ASN.2015040348
22. Iatropoulos P, Noris M, Mele C, Piras R, Valoti E, Bresin E, et al. Complement gene variants determine the risk of immunoglobulin-associated MPGN and C3 glomerulopathy and predict long-term renal outcome. *Mol Immunol.* (2016) 71:131–42. doi: 10.1016/j.molimm.2016.01.010
23. Kawasaki Y, Kanno S, Ono A, Suzuki Y, Ohara S, Sato M, et al. Differences in clinical findings, pathology, and outcomes between C3 glomerulonephritis and membranoproliferative glomerulonephritis. *Pediatr Nephrol.* (2016) 31:1091–9. doi: 10.1007/s00467-015-3307-z
24. Mele C, Lemaire M, Iatropoulos P, Piras R, Bresin E, Bettoni S, et al. Characterization of a new DGKE intronic mutation in genetically unsolved cases of familial atypical hemolytic uremic syndrome. *Clin J Am Soc Nephrol.* (2015) 10:1011–9. doi: 10.2215/CJN.08520814
25. Smith R. *Molecular Otolaryngology and Renal Research Laboratories*. (2020). Available online at: https://morl.lab.uiowa.edu/clinical-diagnostics/kidney-disease-genetic-renal-panel/genetic-renal-panel-testing/genes-included (updated April 24, 2020).
26. Caliskan Y, Torun ES, Tiryaki TO, Oruc A, Ozluk Y, Akgul SU, et al. Immunosuppressive treatment in C3 glomerulopathy: is it really effective? *Am J Nephrol.* (2017) 46:96–107. doi: 10.1159/000479012

27. Pinho A, Ferreira G, Mota C. Successful management of a patient with a C3 Glomerulonephritis and crescentic pattern: a case report. *BMC Res Notes.* (2014) 7:792. doi: 10.1186/1756-0500-7-792
28. Bu F, Meyer NC, Zhang Y, Borsa NG, Thomas C, Nester C, et al. Soluble c5b-9 as a biomarker for complement activation in atypical hemolytic uremic syndrome. *Am J Kidney Dis.* (2015) 65:968–9. doi: 10.1053/j.ajkd.2015.02.326
29. Puraswani M, Khandelwal P, Saini H, Saini S, Gurjar BS, Sinha A, et al. Clinical and immunological profile of anti-factor H antibody associated atypical hemolytic uremic syndrome: a nationwide database. *Front Immunol.* (2019) 10:1282. doi: 10.3389/fimmu.2019.01282
30. Blanc C, Togarsimalemath SK, Chauvet S, Le Quintrec M, Moulin B, Buchler M, et al. Anti-factor H autoantibodies in C3 glomerulopathies and in atypical hemolytic uremic syndrome: one target, two diseases. *J Immunol.* (2015) 194:5129–38. doi: 10.4049/jimmunol.1402770
31. Fakhouri F, Fremeaux-Bacchi V, Noel LH, Cook HT, Pickering MC. C3 glomerulopathy: a new classification. *Nat Rev Nephrol.* (2010) 6:494–9. doi: 10.1038/nrneph.2010.85
32. Servais A, Fremeaux-Bacchi V, Lequintrec M, Salomon R, Blouin J, Knebelmann B, et al. Primary glomerulonephritis with isolated C3 deposits: a new entity which shares common genetic risk factors with haemolytic uraemic syndrome. *J Med Genet.* (2007) 44:193–9. doi: 10.1136/jmg.2006.045328
33. Medjeral-Thomas NR, O'Shaughnessy MM, O'Regan JA, Traynor C, Flanagan M, Wong L, et al. C3 glomerulopathy: clinicopathologic features and predictors of outcome. *Clin J Am Soc Nephrol.* (2014) 9:46–53. doi: 10.2215/CJN.04700513
34. Pickering MC, D'Agati VD, Nester CM, Smith RJ, Haas M, Appel GB, et al. C3 glomerulopathy: consensus report. *Kidney Int.* (2013) 84:1079–89. doi: 10.1038/ki.2013.377
35. Ravindran A, Fervenza FC, Smith RJH, Sethi S. C3 glomerulonephritis with a severe crescentic phenotype. *Pediatr Nephrol.* (2017) 32:1625–33. doi: 10.1007/s00467-017-3702-8
36. Nada R, Kumar A, Agrawal P, Ramachandran R, Sethi S. Renal and pulmonary dense deposit disease presenting as pulmonary-renal syndrome. *Kidney Int Rep.* (2018) 3:755–61. doi: 10.1016/j.ekir.2018.01.005
37. Larsen CP, Messias NC, Walker PD, Fidler ME, Cornell LD, Hernandez LH, et al. Membranoproliferative glomerulonephritis with masked monotypic immunoglobulin deposits. *Kidney Int.* (2015) 88:867–73. doi: 10.1038/ki.2015.195
38. Ravindran A, Fervenza FC, Smith RJH, Sethi S. C3 glomerulopathy associated with monoclonal Ig is a distinct subtype. *Kidney Int.* (2018) 94:178–86. doi: 10.1016/j.kint.2018.01.037
39. Zhang T, Lu J, Liang S, Chen D, Zhang H, Zeng C, et al. Comprehensive analysis of complement genes in patients with atypical hemolytic uremic syndrome. *Am J Nephrol.* (2016) 43:160–9. doi: 10.1159/000445127
40. Regunathan-Shenk R, Avasare RS, Ahn W, Canetta PA, Cohen DJ, Appel GB, et al. Kidney transplantation in C3 glomerulopathy: a case series. *Am J Kidney Dis.* (2019) 73:316–23. doi: 10.1053/j.ajkd.2018.09.002
41. Rudnicki M. Rituximab for treatment of membranoproliferative glomerulonephritis and C3 glomerulopathies. *Biomed Res Int.* (2017) 2017:2180508. doi: 10.1155/2017/2180508
42. Tesar V, Hruskova Z. Immunosuppressive treatment in C3 glomerulopathy: time to reconsider our approach. *Am J Nephrol.* (2017) 46:93–5. doi: 10.1159/000479014
43. Welte T, Arnold F, Kappes J, Seidl M, Haffner K, Bergmann C, et al. Treating C3 glomerulopathy with eculizumab. *BMC Nephrol.* (2018) 19:7. doi: 10.1186/s12882-017-0802-4
44. Garg N, Zhang Y, Nicholson-Weller A, Khankin EV, Borsa NG, Meyer NC, et al. C3 glomerulonephritis secondary to mutations in factors H and I: rapid recurrence in deceased donor kidney transplant effectively treated with eculizumab. *Nephrol Dial Transplant.* (2018) 33:2260–5. doi: 10.1093/ndt/gfx369
45. Olson SR, Lu E, Sulpizio E, Shatzel JJ, Rueda JF, DeLoughery TG. When to stop eculizumab in complement-mediated thrombotic microangiopathies. *Am J Nephrol.* (2018) 48:96–107. doi: 10.1159/000492033
46. Park MH, Caselman N, Ulmer S, Weitz IC. Complement-mediated thrombotic microangiopathy associated with lupus nephritis. *Blood Adv.* (2018) 2:2090–4. doi: 10.1182/bloodadvances.2018019596
47. Yin G, Cheng Z, Zeng CH, Liu ZH. C3 glomerulonephritis in multiple myeloma: a case report and literature review. *Medicine (Baltimore).* (2016) 95:e4843. doi: 10.1097/MD.0000000000004843

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Validation of Early Increase in Complement Activation Marker sC5b-9 as a Predictive Biomarker for the Development of Thrombotic Microangiopathy After Stem Cell Transplantation

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Hematopoietic stem cell transplantation (HSCT)-associated thrombotic microangiopathy (TA-TMA) is a multifactorial complication. Complement dysregulation may play an important role in the pathogenesis of TA-TMA. Our previous observations suggested that early increase of soluble C5b-9 (sC5b-9), before the development of other complications, can predict the development of later TA-TMA. The present study aims to validate our earlier findings in an independent cohort enrolling 67 pediatric patients who underwent allogeneic HSCT during the study period (October 2015–January 2019). Five different TA-TMA diagnostic criteria were applied, and all important clinical and laboratory parameters of TA-TMA activity were registered. Complement pathway activities, components and sC5b-9 levels were systematically measured before transplantation and on days 28, 56, and 100 after HSCT. A strong and remarkable association still have been found between early increase of sC5b-9 (10 of 10 patients with TA-TMA vs. 27 of 57 without TA-TMA; $P = 0.002$) and the development of TA-TMA during 100 days post-transplantation. An increase in sC5b-9 concentration had 100% sensitivity and 53% specificity for TA-TMA in the cohort. All TA-TMA cases have been observed during cyclosporine immunosuppression, no TA-TMA was diagnosed during tacrolimus or mycophenolat mofetil therapy. In the majority of patients TA-TMA was mild and self-limiting, without any signs of organ damage. No additional complement parameters were closely associated with the development of TA-TMA. Early raise of the sC5b-9 activation marker was predictive for later development of TA-TMA throughout the whole study period. In patients

with a marked increase, early and frequent monitoring of TA-TMA activity markers should be attempted, to facilitate subsequent therapy decisions in time. However, patients with TA-TMA were only identified during or after cyclosporine immunosuppression. Further studies enrolling higher number of patients are necessary to determine the role of immunosuppression in the pathogenesis of TA-TMA.

Keywords: hematopoietic stem cell transplantation, HSCT, transplant-associated thrombotic microangiopathy, TA-TMA, complement, pediatric, sC5b-9, thrombotic microangioapathy

INTRODUCTION

Transplant-associated thrombotic microangiopathy (TA-TMA) is a severe complication of hematopoietic stem cell transplantation (HSCT), characterized by microangiopathic hemolytic anemia, elevated serum lactate dehydrogenase level, thrombocytopenia, and multiorgan injury (1). Earlier studies reported incidence rates of TA-TMA between 0.5 and 63.6% (2), while more recent studies have shown the rate of 2.3 to 39% (1, 3). Although the exact pathophysiology is still unclear, endothelial damage appears to be central. Numerous risk factors have been identified including non-modifiable factors such as older age, number of prior transplantations, complement gene variations and CMV seropositivity of the recipient (4, 5). Multiple studies determined various transplant-associated risk factors like the use of HLA-mismatched donors and peripheral blood stem cells, infections, conditioning regimens and high-dose chemotherapy, graft-vs.-host disease (GVHD), and calcineurin inhibitors (6, 7). Recent research demonstrated that the presence of TA-TMA with concurrent infection or GVHD associates with worse survival than TA-TMA alone, furthermore patients who develop TA-TMA are also at increased risk of developing GVHD, infections and other complications (8, 9).

However, there is growing evidence that the overactivation of the complement is involved in the pathophysiology of TA-TMA. Jodele et al. reported that HSCT recipients with proteinuria and elevated soluble terminal complement complex sC5b-9 levels in blood at the time of TMA diagnosis were associated with very poor survival (10). In recent years, numerous studies have demonstrated the elevated level of sC5b-9 may aid in the diagnosis of TA-TMA, while patients with increased sC5b-9 levels are at higher risk of mortality, although not necessarily led to the development of TA-TMA (7, 11).

Recent studies suggest that elevated levels of other proteins and activity of the complement, like the activation product Ba, C3b and the total classical pathway activity (CH50) could serve as a marker of TA-TMA, supporting the role of the complement system in the pathogenesis of TA-TMA (11, 12).

These findings suggest that activation of the complement via the alternative pathway may lead to terminal pathway activation in TA-TMA and may help to identify patients who are at higher risk for the development of TA-TMA after HSCT.

In our previous pediatric cohort the early increase in level of sC5b-9 activation marker (from baseline until 28 days after HSCT) was proved to be predictive for the development of TA-TMA during the first 100 days post-transplantation with 100% sensitivity and 61% specificity. In the present study our aim was to validate the earlier observations in an independent, pediatric cohort, enrolling higher number of patients.

PATIENTS AND METHODS

The sample and data collection as well as the methods used for the detection and analysis of complement parameters were described with full details in our previously published paper (13).

Patient Population, Sample, and Data Collection

All patients from July 2015 to February 2019 who underwent allogeneic HSCT at United St. István and St. László Hospital in Budapest were approached to participate in this study. In total, 67 subjects of the 84 fulfilled inclusion criteria and had adequate material for complement assessments. Individuals with weight below 10 kg (seven patients) and death <30 days after transplantation (one patient) were excluded from this prospective cohort. Seven patients were excluded due to technical problems during sample collection. Moreover, two patients underwent transplantation in both study periods, their complement data were included in the first cohort, and excluded from validation cohort. Detailed flow chart is presented on **Figure 1**. The study was conducted in accordance with the Declarations of Helsinki and approved by the institutional Ethics Committee on Human Research.

Sample and data collection were performed in the same way as in our previous paper. Briefly, samples (serum, EDTA-anticoagulated plasma, and sodium-citrate anticoagulated plasma) were collected at four time points: before transplantation, 28, 56, and 100 days after HSCT. Coombs test, hypertension, proteinuria, serum lactate dehydrogenase, haptoglobin and creatinine levels, new onset anemia, thrombocytopenia, and fragmentocytes as markers of TMA activity were also determined.

Detection of Complement Parameters

Complement measurements were performed after the 100 days follow-up. All parameters of a subject were determined at a single time point. Serum concentration of complement C3 (reference range: 0.9–1.8 g/L) and C4 (reference range: 0.15–0.55 g/L) were determined by turbidimetry. CH50 (reference range: 48–103 CH50/ml) was determined by an in-house hemolytic-titration assay while an enzyme-linked immunosorbent assay

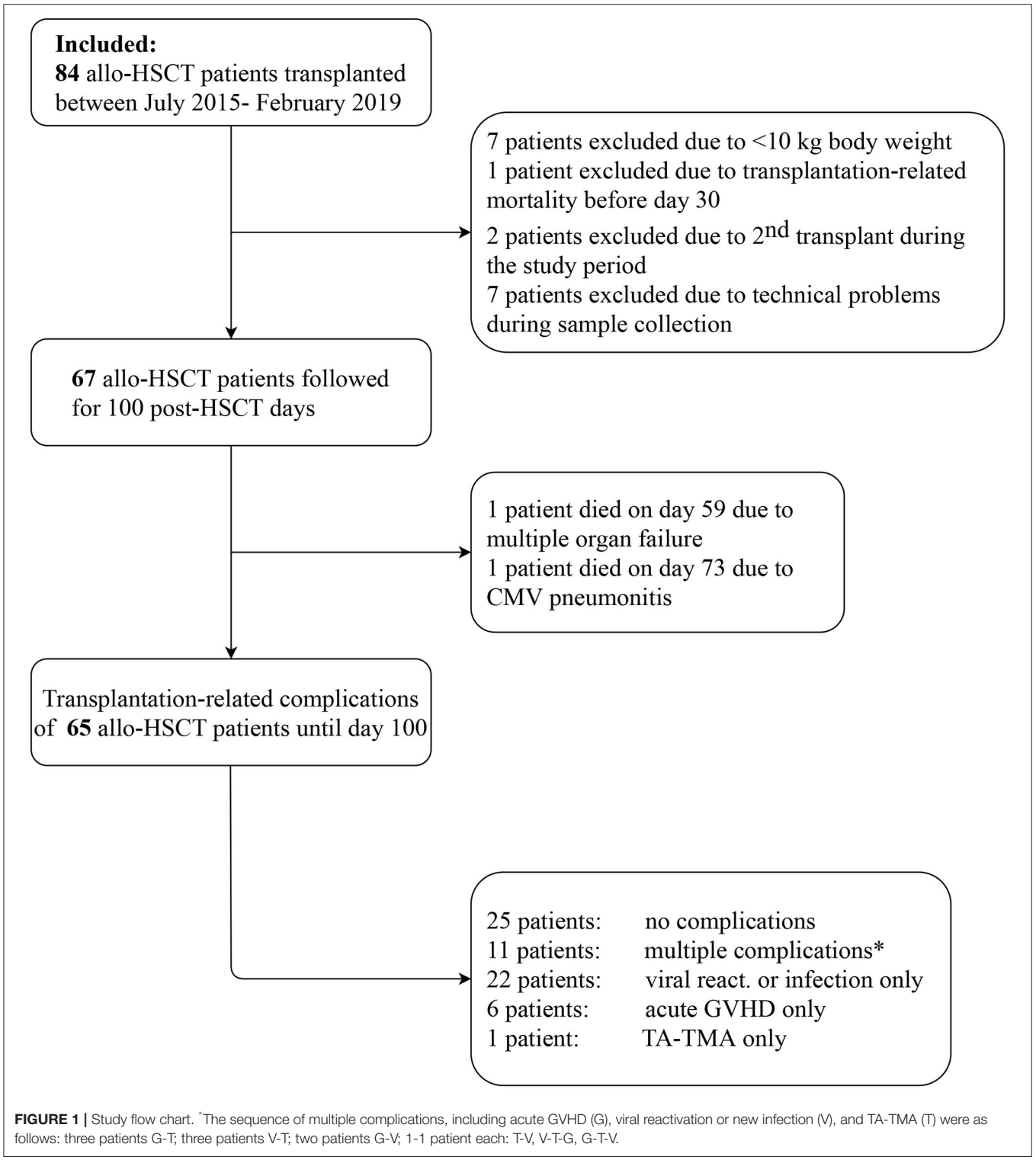

FIGURE 1 | Study flow chart. *The sequence of multiple complications, including acute GVHD (G), viral reactivation or new infection (V), and TA-TMA (T) were as follows: three patients G-T; three patients V-T; two patients G-V; 1-1 patient each: T-V, V-T-G, G-T-V.

was applied to measure C1q (reference range: 60–180 mg/L), terminal pathway activation complex sC5b-9 (reference range: 110–252 ng/ml) as well as factor H (FH) levels (reference range: 250–880 mg/L), and the activity of the lectin (Wieslab Comp AP320 kit, reference range: 35–130%, EuroDiagnostica, Malmo, Sweden) and alternative pathways (reference range: 70–125%). Factor I (FI) and factor B (FB) levels were measured by radial immunodiffusion (reference range: 70–130%). FRET-VWF73 substrate was used to detect the ADAMTS13 enzyme activity (reference range: 67–151%).

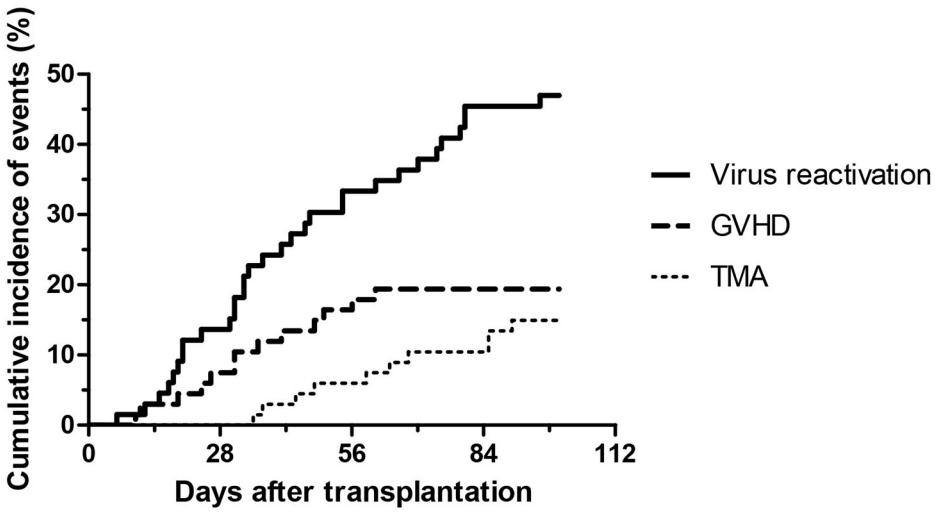

FIGURE 2 | Development of virus reactivation, GVHD and thrombotic microangiopathy after HSCT from baseline to day 100.

Definition of Transplant-Related Complications

Glucksberg criteria was used to grade the severity of acute GVHD (14). Quantitative PCR assays were performed to detect viral reactivation or primary infection. During the first 100 days of the post-transplantation period, viral reactivation (CMV, EBV or adenovirus) have occurred in 32 cases. Acyclovir was started on the first day of conditioning therapy. As preemptive therapy rituximab, foscarnet, gancyclovir, and cidofovir were used.

For the determination of TA-TMA, five sets of diagnostic criteria were used (15–19) and the date of diagnosis was defined as the day when all of the TA-TMA diagnostic criteria introduced by Jodele et al. were fulfilled.

None of the patients who underwent complement evaluation received eculizumab treatment in the whole study period.

Statistical Methods

Continuous data were expressed as the median and IQR (range from the 25th to the 75th percentile) and categorical variables as frequencies. Fisher exact tests were used to compare categorical data and Mann-Whitney, Wilcoxon or Friedman tests to compare continuous data.

Kaplan-Meier analysis was applied for calculation of TA-TMA even-free survival and Log-rank test was used for the comparison of survival curves. Two-sided P-value <0.05 was considered statistically significant. For analyzes GraphPad Prism 6.03 (GraphPad Software Inc., La Jolla, CA) and IBM SPSS Statistics 24 (IBM Corporation, Armonk, NY) were used.

RESULTS

Baseline Characteristics

The study population included 67 pediatric patients undergoing allogeneic stem cell transplantation. The study flow chart is shown on **Figure 1**. The median age of the recipients at the time of HSCT was 8 years (range, 2.6–12.1 years), the majority of patients were male gender (55%). Malignant diseases were the primary indication of transplantation (55%), including acute lymphoblastic leukemia (46%), acute myeloid leukemia (22%) and juvenile myelomonocytic leukemia (11%) as the most common diseases. The non-malignant disorders (45%) involved were bone marrow failure syndromes, immunodeficiencies and metabolic diseases in 19 (63%), 8 (27%) and 3 (10%) patients, respectively. The conditioning regimen was myeloablative in 42 subjects (63%), the majority were treosulfan-based (52%) and busulfan was also used in 20 cases (48%). The most common graft source for HSCT was bone marrow (73%), particularly from matched unrelated donors. Patients were followed for 100 days after transplantation and transplantation related complications were monitored.

During the first 100 days of the post-transplantation period acute GVHD was observed in 13 cases (19%), out of which 8 was graded as I-II, and 5 was graded as severe III-IV GVHD. Interestingly, out of the five severe acute GVHD cases, only two patient developed TA-TMA. Sixty-four of the 67 subjects received at least one of the calcineurin inhibitors as GVHD prophylaxis, including cyclosporine ($n = 54$) and tacrolimus ($n = 13$), furthermore mycofenolate mofetil was used in two cases, and one patient received no immunosuppression due to an identical twin donor. Sirolimus was used in two cases as GVHD prophylaxis or treatment. Furthermore, the most frequent transplant-related complication was virus reactivation which was detected in 33 patients (49%). Twenty-five patients had no complications, while most of the TA-TMA events were preceded by GVHD or viral reactivation ($n = 9$). Incidence of transplantation-related complications is shown on **Figures 1**, **2**.

All components of the five various diagnostic systems for TA-TMA were monitored consecutively. Depending on the different diagnostic systems, 6 (9%)(BMT = 26), 5 (7%) (IWG = 27), 5

(7%)(Cho = 28), 7 (10%)(CoH = 29), and 10 (15%)(Jodele) of 67 subjects met the criteria for TA-TMA. All of the 10 patients identified by the criteria system of Jodele et al. fulfilled the requirements of at least three TA-TMA diagnostic systems.

Of 67 patients, 10 (15%) met the criteria for TA-TMA with the median occurrence of 62 days post-transplantation (range, 42–85 days). Characteristics of patient groups with or without TA-TMA are summarized in **Table 1**. No differences were found in relation to age, gender, previous transplantations, donor type, stem cell source, conditioning therapy, time of engraftment, and viral infections between the two groups. All TA-TMA cases have been observed during cyclosporine immunosuppression and no TA-TMA was diagnosed during tacrolimus (0 of 13 patients) or mycophenolat mofetil (0 of 2 patients) therapy. As a consequence of reduced toxicity conditioning regimen, in majority of patients TA-TMA was mild and self-limiting, without any signs of organ damage. Out of the 10 TA-TMA cases, three patients required intensive therapy due to organ damage during GVHD and TA-TMA treatment. In case of early signs of a developing TA-TMA, withdrawal or change of calcineurin inhibitors was the initial step to control the process in our clinical practice. Calcineurin inhibitor was discontinued or changed by the treating physicians if hypertension, posterior reversible encephalopathy syndrome, relapse, minimal residual disease or rapidly elevating LDH occurred. One patient received defibrotide therapy as TA-TMA treatment.

However, individuals with acute GVHD during the first 100 days post-HSCT were at higher risk for the development of TA-TMA ($P = 0.019$). Although the difference in the incidence of viral infections was statistically not significant between the TA-TMA positive and negative groups (60 vs. 46.6%, $P = 0.5$), the co-occurrence of acute GVHD and virus reactivation was more frequently registered for subjects with TA-TMA.

TABLE 1 | Patient characteristics.

	Patients without TA-TMA ($n = 57$)	Patients with TA-TMA ($n = 10$)	*P*-value
Age, years (median; IQR)	7.1 (2.5–11.8)	9.4 (6.7–14.3)	0.07
Male recipient sex	31 (54.4)	6 (60)	1
Indication of transplantation			
Malignancy	31 (54.39)	6 (60)	
Bone marrow failure	16 (28.1)	3 (30)	0.84
Other (immune deficiency or metabolic disease)	10 (17.5)	1 (10)	
Recent transplantation			
0	53 (93)	10 (100)	1
1	4 (7)	0	
Donor type			
Related identical or haploidentical	21 (36.8)	2 (20)	0.47
Unrelated	36 (63.2)	8 (80)	
Stem cell source			
Bone marrow	42 (73.7)	7 (70)	
PBCS	11 (19.3)	3 (30)	0.55
Cord blood	4 (7)	0	
Conditioning therapy			
Myeloablative	34 (59.6)	8 (80)	0.3
Reduced intensity	23 (40.4)	2 (20)	
Engraftment, days	20.5 (15–26)	26 (13–48)	0.41
HSCT-related complications			
Acute GVHD before day 100	8 (14.1)	5 (50)	0.019
GVHD before day 28	3 (5.3)	2 (20)	0.16
Viral infection before day 100	27 (46.6)	6 (60)	0.5
Viral infection before day 28	8 (14)	2 (20)	0.64
None of the 2	25 (43.9)	1 (10)	
GVHD or viral reactivation	30 (52.6)	7 (70)	0.012
GVHD and viral reactivation	2 (3.5)	2 (20)	
Relapse-related mortality	10 (17.5)	0	0.34
TRM	7 (12.3)	2 (20)	0.28

Data presented are n (%) unless otherwise indicated.
PBSC, peripheral blood stem cell; IQR, interquartile range.

Complement Activation During the First 100 Days After HSCT

Our first goal was to identify the associations between various measures of complement profile and TA-TMA, by measuring the activities of the classical-, lectin- and alternative pathways, plasma/serum levels of C1q, C4, C3, FH, FI, FB, and sC5b-9, as well as the activity of ADAMTS13 during the first 100 days after transplantation (**Table 2**). The levels of complement proteins were considered to be elevated when an increase from baseline was observed.

Among these parameters, complement terminal pathway activation complex sC5b-9 and C4 levels showed association with incident TA-TMA.

We observed a significant association between elevated sC5b-9 levels and TA-TMA development. Baseline sC5b-9 levels did not differ in patients with (interquartile range, 128–202 ng/ml), or without (interquartile range, 113–220 ng/ml) subsequent TA-TMA, furthermore the median levels remained within the reference range of the assay for both groups. However, on day 28, sC5b-9 levels of 4 of 10 subjects with TA-TMA exceeded 252 ng/ml, the upper limit of the reference range, while 27 recipients showed an increase in the group of patients without TA-TMA, and among them 6 reached the upper limit ($P = 0.037$). Peak sC5b-9 levels were detected on day 28 in both groups, however, concentrations were significantly higher in patients who later developed TA-TMA, than recipients without: 240 ng/ml (interquartile range, 219 to 376) vs. 160 (interquartile range, 110–201; $P = 0.0008$). Furthermore, this difference remained on day 56 ($P = 0.001$), while sC5b-9 levels measured before HSCT and on day 100 did not differ between them. Moreover, all patients with later development of TA-TMA showed and early increase (until day 28) of sC5b-9 (**Table 3**).

When absolute difference between day 28 and day 0 sC5b-9 values were calculated, ROC analysis identified 66 ng/ml increase, as the optimum cut-off point to identify

TABLE 2 | Complement pathway activities and activation product levels at different time points before and after transplantation.

Parameter	Before HSCT	Day 28	Day 56	Day 100	*P*-value
Classical pathway activity, CH50/ml					
Reference range: 48–103					
TA-TMA ($n = 10$)	73 (56–95)	90 (74–110)	87 (77–106)	88 (72–120)	ns
No TA-TMA ($n = 57$)	70 (63–96)	77 (69–100)	79 (66–92)	72 (61–84)	
Alternative pathway activity, %					
Reference range: 70–125					
TA-TMA ($n = 10$)	87 (75–106)	105 (42–119)	92 (80–102)	106 (99–109)	ns
No TA-TMA ($n = 57$)	89 (69–107)	94 (72–109)	92 (63–102)	89 (70–99)	
MBL pathway activity, %					
Reference range: 35–130					
TA-TMA ($n = 10$)	117 (80–119)	115 (62–118)	105 (102–128)	122 (90–126)	ns
No TA-TMA ($n = 57$)	84 (18–145)	105 (20–150)	99 (12–162)	95 (21–120)	
Complement C3, g/L					
Reference range: 0.9–1.8					
TA-TMA ($n = 10$)	1.42 (1.26–1.44)	1.51 (0.95–1.79)	1.5 (1.28–1.96)	1.43 (1.33–1.73)	ns
No TA-TMA ($n = 57$)	1.35 (1.16–1.58)	1.37 (1.2–1.64)	1.31 (1.15–1.49)	1.25 (1.06–1.36)	
Complement C4, g/L					
Reference range: 0.15–0.55					
TA-TMA ($n = 10$)	0.38 (0.29–0.44)	0.38 (0.36–0.53)	0.45 (0.36–0.5)	0.5 (0.42–0.56)	0.009 for TA-TMA
No TA-TMA ($n = 57$)	0.36 (0.26–0.45)	0.39 (0.3–0.51)	0.35 (0.27–0.43)	0.33 (0.26–0.4)	
FH, mg/L					
Reference range: 250-880					
TA-TMA ($n = 10$)	690 (456–758)	564 (440–715)	479(369–897)	535 (419–761)	ns
No TA-TMA ($n = 57$)	448 (316–605)	468 (347–612)	449 (296–614)	444 (313.9–531)	
FI, %					
Reference range: 70–130					
TA-TMA ($n = 10$)	90 (83–102)	76 (73–83)	79 (70–89)	77 (72–94)	ns
No TA-TMA ($n = 57$)	88 (78–102)	86 (76–103)	88 (76–105)	81 (70–97)	
FB, %					
Reference range: 70–130					
TA-TMA ($n = 10$)	98 (89–102)	89 (85–102)	106 (67–122)	84 (74–98)	ns
No TA-TMA ($n = 57$)	90 (74–102)	84 (67–110)	82 (67–100)	85 (68–104)	
ADAMTS13 activity, %					
Reference range: 67–151					
TA-TMA ($n = 10$)	99 (81–116)	68 (60–96)	84 (65–117)	92 (58–117)	ns
No TA-TMA ($n = 57$)	91 (70–112)	78 (55–102)	93 (58–121)	96 (62–120)	
sC5b-9, ng/ml					
Reference range: 110–252					
TA-TMA ($n = 10$)	157 (128–202)	240 (219–376)	210 (161–272)	137 (126–148)	0.003 for TA-TMA
No TA-TMA ($n = 57$)	153 (113–220)	164 (110–201)	147 (120–189)	149 (108–201)	
C1q, mg/L					
Reference range: 60–180					
TA-TMA ($n = 10$)	90.9 (77.9-112)	101.6 (63-116.1)	102.6 (90-120)	93.1 (35-125.7)	ns
No TA-TMA ($n = 57$)	89.9 (73.2–105.5)	78.1 (68.5–95.5)	78.7 (64.2–91.1)	66.3 (50.7–84.8)	

Data are presented as median (interquartile range). P-values were obtained with two-way analysis of variance.
ns, not significant.

TA-TMA patients with 90% sensitivity and 84% specificity (AUC: 0.869; 95% CI 0.776–0.963).

Changes in C4 levels after HSCT showed moderate, significant differences between patients with or without TA-TMA. Patients with TA-TMA showed increasing C4 on 56 and 100 days after HSCT, whereas in patients without TA-TMA C4 levels peaked on day 28. We have no formal explanation for this observation, but since multiple complications necessitating combined/long drug treatment coincided with development of TMA, cumulative effects drug- or inflammation related factors might be in the background of elevated C4. However, a direct relationship between elevated C4 levels and development of

TABLE 3 | Complications and activity markers with or without early elevation of sC5b-9 until day 28.

	sC5b-9 elevation from baseline to day 28 ($n = 37$)	No change or decrease of sC5b-9 from baseline to day 28 ($n = 29$)	*P*-value
Engraftment day	23 (17–27)	18 (13–24.5)	0.022
TA-TMA before day 100	10 (27)	0 (0)	0.002
TA-TMA before day 28	3 (8.1)	0 (0)	
Acute GVHD before day 100	7 (18.9)	6 (20.7)	1
Acute GVHD before day 28	3 (8.1)	2 (6.9)	1
Viral reactivation before day 100	18 (48.6)	14 (48.3)	1
Viral reactivation before day 28	8 (21.6)	7 (24.1)	0.31
Fever on day 28	3 (8.1)	1 (3.5)	0.62
Bloodstream infection	11 (29.7)	8 (27.6)	1
Transplant failure before 100 days	3 (8.1)	1 (3.5)	0.62
Diagnostic parameters for TA-TMA			
Elevated LDH until day 28	5 (13.5)	5 (17.2)	0.74
Elevated LDH until day 100	24 (64.9)	13 (44.8)	0.14
New onset anemia until day 28	1 (2.7)	2 (6.9)	0.58
New onset anemia until day 100	18 (48.6)	13 (44.8)	0.8
New onset thrombocytopenia until day 28	0	0	
New onset thrombocytopenia until day 100	15 (40.5)	10 (34.5)	0.8
Low haptoglobin until day 28	10 (27)	8 (27.6)	1
Low haptoglobin until day 100	13 (35.1)	12 (41.4)	0.62
Fragmentocytes until day 28	13 (35.1)	12 (41.4)	0.62
Fragmentocytes until day 100	20 (54)	15 (51.7)	1
Proteinuria until day 28	6 (16.2)	5 (17.2)	1
Proteinuria until day 100	13 (35.1)	5 (17.2)	0.16
Hypertension until day 28	17 (45.9)	7 (24.1)	0.078
Hypertension until day 100	22 (59.5)	8 (27.6)	0.013
None of the seven	11 (29.7)	4 (13.8)	
One of the seven	12 (32.4)	13 (44.8)	0.28
At least two of the seven until day 28	14 (37.8)	12 (41.4)	

Data presented are median (interquartile range) or n (%). P-values were obtained by Fisher's exact test.

TMA cannot be excluded, requiring further studies to clear this association.

There was no significant association of TA-TMA with changes in classical, lectin and alternative pathway activities, and other complement parameters.

The relationship between the early increase of sC5b-9 and transplantation related complications and early laboratory or clinical markers of TA-TMA were also analyzed (**Table 3**). Based on the changes in sC5b-9 levels from baseline to day 28, two groups were defined, including patients with elevated, or with decreased or unchanging levels of sC5b-9.

TA-TMA showed a remarkable association with early increase of terminal pathway activation (**Figure 3**). Ten patients of 37 with early increase in sC5b-9 levels developed later TA-TMA, while none of the 29 recipients without an elevation. Furthermore, the day of engraftment was delayed in patients with early increase in sC5b-9 levels. Acute GVHD, viral reactivation and/or infection, severe mucositis, fever, blood stream infection, transplant failure, elevated LDH, new onset anemia and thrombocytopenia showed no significant association with early increase of sC5b-9 levels (**Table 3**).

Finally, we analyzed if early increase of sC5b-9 levels could help in the clinical management of HSCT patients and calculated sensitivity and specificity values to predict development of TA-TMA. Early sC5b-9 elevation was a 100% sensitive predictive marker for the later development of TA-TMA (10 of 10 patients with TA-TMA had early elevation of sC5b-9; **Figure 3**), but it was less specific (10 of 37 patients with early sC5b-9 increase had TA-TMA events translating to 53% specificity). Positive predictive value of having early elevation in sC5b-9 for the development of TA-TMA was low, 27%, but the negative predictive value for the same (not having sC5b-9 elevation, and lack of development of TA-TMA) was 100%. Therefore, since 44% of our cohort had no early elevation of sC5b-9 levels we consider checking of sC5b-9 levels clinically useful to stratify patients for later development of TA-TMA.

DISCUSSION

Transplantation associated TMA is a frequent cause of organ damage, and is associated with increased morbidity and mortality after HSCT (20). Modifiable risk factors of TA-TMA are related to transplantation-associated factors like HLA- or minor ABO mismatched donor, use of PBSC, and lack of ATG- or presence of myeloablative conditioning regimen. In addition, several post transplantation events including infections, viral reactivation, acute graft vs. host disease and use of calcineurin inhibitors may also increase the risk of post-HSCT TMA (7). HSCT is planned process with strict protocols for biological sampling and monitoring of various organ functions, therefore, it is feasible to search for, and to identify predictive biomarkers of various post-TA-TMA complications. In our previous study we utilized the advantages of our institutional protocol and conducted an explorative, consecutive, comprehensive analysis of complement biomarkers to search for TA-TMA associated, predictive biomarkers. Early increase of terminal pathway activation marker was identified as a sensitive marker of later development of TA-TMA (13). To decrease the risk of a false positive conclusion that may be related to the small size of our previous observational study, we found it of utmost importance to validate our initial observations in an independent cohort, before making any firm conclusions based on the initial data.

Accordingly, the aim of this study was to enroll a new prospective cohort of pediatric patients undergoing HSCT, and to consecutively collect appropriate plasma and serum samples for complement analysis with relevant clinical information, in order to validate our previous results. Here we show, that early elevation of complement terminal pathway activation complex sC5b-9, is a predictive biomarker for the development

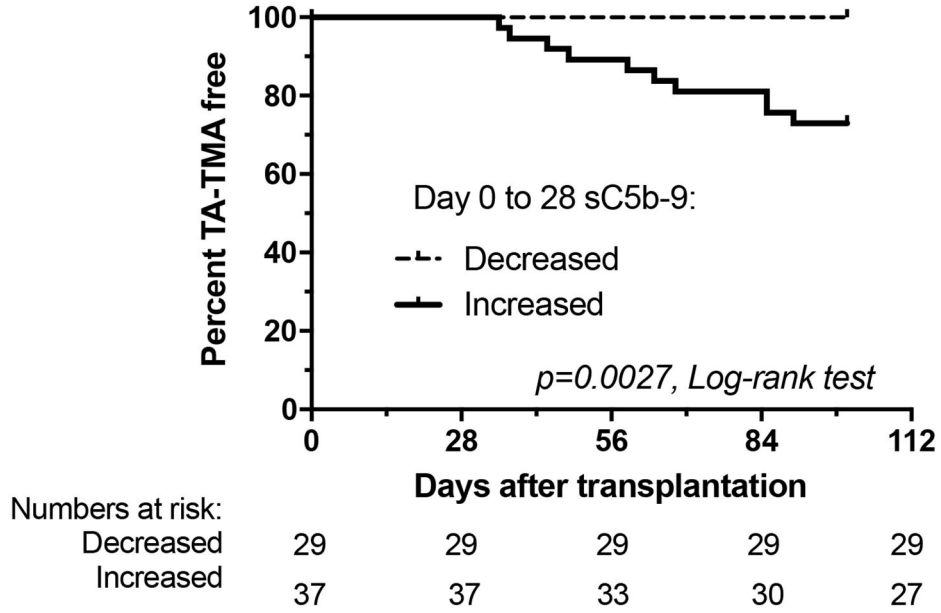

FIGURE 3 | Development of thrombotic microangiopathy after HSCT in relation to changes of terminal pathway activation marker sC5b-9 from baseline to day 28. Kaplan-Meier plot showing percentage of TA-TMA event-free patients.

of TA-TMA after HSCT (**Figure 3** and **Table 2**). The current findings are fully supporting and validating our previous results, demonstrating 66 ng/ml increase, as the optimum cut-off point to identify TA-TMA patients with 90% sensitivity and 84% specificity (AUC: 0.869; 95% CI 0.776–0.963). The association between the early increase of sC5b-9 (binary variable) and later development of TA-TMA translates to remarkably high negative predictive value (100%) with 27% positive predictive value.

Most patients in our cohort had mild to moderate severity of TA-TMA (**Tables 1**, **3**) and were managed by modification of immunosuppressive therapy, and by treatments for GVHD and infections. All TA-TMA cases have been observed during cyclosporine immunosuppression and no TA-TMA was diagnosed during tacrolimus or mycophenolat mofetil therapy. There was no patient in this cohort who required plasmapheresis or complement inhibitory therapy to manage TA-TMA. Accordingly, the mortality in this cohort was not associated with TA-TMA (**Table 1**). All, but one, of the 10 TA-TMA events were preceded by development of acute GVHD or viral reactivation, or both (**Figures 1**, **2**). This observation is supporting the current "multiple hits" pathogenesis model of TA-TMA.

Currently, there are no universally accepted biomarkers for the prediction of TA-TMA, although platelet activation, neutrophil extracellular traps and complement activation are suggested to play key roles (21). The relationship between elevated sC5b-9 levels at the time of TA-TMA diagnosis and poor survival was reported a few years ago (10), which has been followed by several studies demonstrating the possible link between complement activation and TA-TMA, and facilitated subsequent therapeutic decisions and analysis to predict response to treatment (7, 22). Importantly, according to recent observations in a large pediatric cohort use of anti-C5 complement inhibitory drug seems to be an effective therapeutic strategy for high-risk TA-TMA patients after HSCT (22). In addition, Jodele and associates observed that subjects with higher complement activation marker sC5b-9 before initiation of anti-C5 treatment were less likely to respond and required more doses of the drug. Our results are in line of these observations and provide further support to complement activation markers, specifically terminal pathway activation marker sC5b-9, as clinically useful biomarkers to manage post HSCT TA-TMA patients.

In addition to sC5b-9 marker, our study identified development of hypertension and acute GVHD, as signs, and clinical factors associated with the development of TA-TMA. Additional risk factors for the development of TA-TMA after HSCT include conditioning agents and regimens (23). As a result of modifications in the institutional conditioning therapy (according to an international clinical study in patients with acute lymphoblastic leukemia a less toxic treosulphan based conditioning was used) and prophylactic immunosuppression protocol, the incidence of TA-TMA was lower in the current study (10/67) when compared to our original cohort (10/33; $P = 0.05$). As a consequence of reduced toxicity conditioning regimen, in majority of patients TA-TMA was mild and self-limiting, without any signs of organ damage and may contribute to smaller extent of sC5b-9 formation compared to sC5b-9 levels measured in our previous study. These observations indicate that the more aggressive or toxic conditioning regimens could lead increased TA-TMA incidence, although further, larger studies or pooled analysis, allowing multivariable analysis, would be necessary to identify and

validate the exact composition and role of clinical risk factors for TA-TMA.

In conclusion, based on our original and current results we consider early elevation of sC5b-9, the terminal complement pathway activation marker, as a clinically useful predictive marker for the development of mild to moderate TA-TMA in pediatric HSCT patients. We suggest to include regular monitoring of complement biomarkers into additional studies and possibly also into institutional protocols. Studies on complement targeting drugs are very limited in this setting at this time, but interest is increasing after the first supportive clinical observations (22, 24). Accumulating data and evidence about the role and clinical utility of complement biomarkers, and advanced understanding of the exact role of complement activation in the post-transplantation setting may facilitate the development of biomarker-stratified therapy schemes, and improved management of the affected patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Scientific and Research Ethics Committee of the Medical Research Council (ETT TUKEB) in Budapest, Hungary. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ZP and GK: study concept and design. BM, OH, GS, and NV: experimental procedures. OH and GK: acquisition of data. BM and ZP: critical writing of the manuscript. ZP and GK: study supervision. GK, OH, and ZP: acquisition of funding. All authors: data analysis, interpretation of data, and critical revision of the manuscript for important intellectual content.

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REFERENCES

1. Yamada R, Nemoto T, Ohashi K, Tonooka A, Horiguchi SI, Motoi T, et al. Distribution of transplantation-associated thrombotic microangiopathy (ta-tma) and comparison between renal ta-tma and intestinal ta-tma: autopsy study. *Biol Blood Marrow Transplant.* (2020) 26:178–88. doi: 10.1016/j.bbmt.2019.08.025
2. Obut F, Kasinath V, Abdi R. Post-bone marrow transplant thrombotic microangiopathy. *Bone Marrow Transplant.* (2016) 51:891–7. doi: 10.1038/bmt.2016.61
3. Wanchoo R, Bayer RL, Bassil C, Jhaveri KD. Emerging concepts in hematopoietic stem cell transplantation-associated renal thrombotic microangiopathy and prospects for new treatments. *Am J Kidney Dis.* (2018) 72:857–65. doi: 10.1053/j.ajkd.2018.06.013
4. Hildebrandt GC, Chao N. Endothelial cell function and endothelial-related disorders following haematopoietic cell transplantation. *Br J Haematol.* (2020) 4:508–19. doi: 10.1111/bjh.16621
5. Postalcioglu M, Kim HT, Obut F, Yilmam OA, Yang J, Byun BC, et al. Impact of thrombotic microangiopathy on renal outcomes and survival after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant.* (2018) 24:2344–53. doi: 10.1016/j.bbmt.2018.05.010
6. Epperla N, Li A, Logan B, Fretham C, Chhabra S, Aljurf M, et al. Incidence, risk factors for and outcomes of transplant-associated thrombotic microangiopathy. *Br J Haematol.* (2020) 189: 1171–81. doi: 10.1111/bjh.16457
7. Dvorak CC, Higham C, Shimano KA. Transplant-associated thrombotic microangiopathy in pediatric hematopoietic cell transplant recipients: a practical approach to diagnosis and management. *Front Pediatr.* (2019) 7:133. doi: 10.3389/fped.2019.00133
8. Kraft S, Bollinger N, Bodenmann B, Heim D, Bucher C, Lengerke C, et al. High mortality in hematopoietic stem cell transplant-associated thrombotic microangiopathy with and without concomitant acute graft-versus-host disease. *Bone Marrow Transplant.* (2019) 54:540–8. doi: 10.1038/s41409-018-0293-3
9. Li A, Wu Q, Davis C, Kirtane KS, Pham PD, Sorror ML, et al. Transplant-associated thrombotic microangiopathy is a multifactorial disease unresponsive to immunosuppressant withdrawal. *Biol Blood Marrow Transplant.* (2019) 25:570–6. doi: 10.1016/j.bbmt.2018.10.015
10. Jodele S, Davies SM, Lane A, Khoury J, Dandoy C, Goebel J, et al. Diagnostic and risk criteria for HSCT-associated thrombotic microangiopathy: a study in children and young adults. *Blood.* (2014) 124:645–53. doi: 10.1182/blood-2014-03-564997
11. Qi J, Wang J, Chen J, Su J, Tang Y, Wu X, et al. Plasma levels of complement activation fragments C3b and sC5b-9 significantly increased in patients with thrombotic microangiopathy after allogeneic stem cell transplantation. *Ann Hematol.* (2017) 96:1849–55. doi: 10.1007/s00277-017-3092-9
12. Sartain S, Shubert S, Wu MF, Wang T, Martinez C. The alternative complement pathway activation product Ba as a marker for transplant-associated thrombotic microangiopathy. *Pediatr Blood Cancer.* (2020) 67:e28070. doi: 10.1002/pbc.28070
13. Horvath O, Kallay K, Csuka D, Mezo B, Sinkovits G, Kassa C, et al. Early increase in complement terminal pathway activation marker sc5b-9 is predictive for the development of thrombotic microangiopathy after stem cell transplantation. *Biol Blood Marrow Transplant.* (2018) 24:989–96. doi: 10.1016/j.bbmt.2018.01.009
14. Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation.* (1974) 18:295–304. doi: 10.1097/00007890-197410000-00001
15. Ho VT, Cutler C, Carter S, Martin P, Adams R, Horowitz M, et al. Blood and marrow transplant clinical trials network toxicity committee consensus summary: thrombotic microangiopathy after hematopoietic stem

cell transplantation. *Biol Blood Marrow Transplant.* (2005) 11:571–5. doi: 10.1016/j.bbmt.2005.0+6.001
16. Jodele S, Laskin BL, Dandoy CE, Myers KC, El-Bietar J, Davies SM, et al. A new paradigm: diagnosis and management of HSCT-associated thrombotic microangiopathy as multi-system endothelial injury. *Blood Rev.* (2015) 29:191–204. doi: 10.1016/j.blre.2014.11.001
17. Ruutu T, Barosi G, Benjamin RJ, Clark RE, George JN, Gratwohl A, et al. Diagnostic criteria for hematopoietic stem cell transplant-associated microangiopathy: results of a consensus process by an International Working Group. *Haematologica.* (2007) 92:95–100. doi: 10.3324/haematol.10699
18. Cho BS, Yahng SA, Lee SE, Eom KS, Kim YJ, Kim HJ, et al. Validation of recently proposed consensus criteria for thrombotic microangiopathy after allogeneic hematopoietic stem-cell transplantation. *Transplantation.* (2010) 90:918–26. doi: 10.1097/TP.0b013e3181f24e8d
19. Garcia-Martin P, Alarcon-Payer C, Lopez-Fernandez E, Moratalla L, Romero A, Sainz J, et al. Transplantation-associated thrombotic microangiopathy in patients treated with sirolimus and cyclosporine as salvage therapy for graft-versus-host disease. *Ann Pharmacother.* (2015) 49:986–94. doi: 10.1177/1060028015593369
20. Jodele S, Dandoy CE, Myers KC, El-Bietar J, Nelson A, Wallace G, et al. New approaches in the diagnosis, pathophysiology, and treatment of pediatric hematopoietic stem cell transplantation-associated thrombotic microangiopathy. *Transfus Apher Sci.* (2016) 54:181–90. doi: 10.1016/j.transci.2016.04.007
21. Seaby EG, Gilbert RD. Thrombotic microangiopathy following haematopoietic stem cell transplant. *Pediatr Nephrol.* (2018) 33:1489–500. doi: 10.1007/s00467-017-3803-4
22. Jodele S, Dandoy CE, Lane A, Laskin BL, Teusink-Cross A, Myers KC, et al. Complement blockade for TA-TMA: lessons learned from a large pediatric cohort treated with eculizumab. *Blood.* (2020) 135:1049–57. doi: 10.1182/blood.2019004218
23. Castagnoli R, Delmonte OM, Calzoni E, Notarangelo LD. Hematopoietic stem cell transplantation in primary immunodeficiency diseases: current status and future perspectives. *Front Pediatr.* (2019) 7:295. doi: 10.3389/fped.2019.00295
24. Jodele S, Fukuda T, Vinks A, Mizuno K, Laskin BL, Goebel J, et al. Eculizumab therapy in children with severe hematopoietic stem cell transplantation-associated thrombotic microangiopathy. *Biol Blood Marrow Transplant.* (2014) 20:518–25. doi: 10.1016/j.bbmt.2013.12.565

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CASE REPORT
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Refractory Auto-Immune Thrombotic Thrombocytopenic Pupura Successfully Treated With Caplacizumab

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Thrombotic thrombocytopenic purpura (TTP) is a rare thrombotic microangiopathy characterized by mechanical hemolytic anemia, profound thrombocytopenia, and neurological manifestations. Acquired auto-immune TTP, the most prevalent cause of TTP, is induced by the presence of inhibitory anti-ADAMTS13 auto-antibodies. Modern treatment of acquired TTP relies on plasma exchange, rituximab, and steroids. Caplacizumab (Cablivi®), a humanized single-variable domain immunoglobulin that targets the A1 domain of the ultra-large von Willebrand factor, inhibits the interaction between ultra-large vWFand platelets. In two clinical trials, caplacizumab, in addition to conventional treatment, shortened the delay to platelet count normalization in comparison to conventional treatment plus placebo, without increasing significantly hemorrhagic complications. Moreover, caplacizumab was associated with reduced occurrence of a secondary endpoint associating death, TTP recurrence, and major thromboembolic events. Here, we report the off-label use of caplacizumab in a 68-year-old patient with confirmed acquired TTP, severe thrombocytopenia, and generalized tonic–clonic seizures requiring mechanical ventilation and admission in the intensive care unit. Conventional treatment was rapidly started. Despite the intensification of plasma exchange treatment with twice-daily sessions, steroid continuation, and a second rituximab infusion on day 6, thrombotic microangiopathy worsened with thrombocytopenia at 21 g/L on day 8 from admission. We also considered using caplacizumab, which we could obtain and start on day 12 from admission, as it was available under a temporary authorization use in France. As soon as 12 h after caplacizumab initiation, we observed a significant increase of platelet count and improvement of other hemolytic parameters. We observed resolution of encephalopathy and complete recovery of motor paralysis, allowing us to stop mechanical ventilation on day 14. Caplacizumab was maintained for 128 days until day 139 from initial admission. The patient is going well 10 months after initial admission, without any neurological

sequelae, and TTP did not relapse. To the best of our knowledge, this is the first reported use of caplacizumab in such a condition. This case report suggests that caplacizumab use may help to reduce the rate of refractory TTP episodes.

Keywords: thrombotic thrombocitopenic purpura, caplacizumab, refractor, case report, platelet

BACKGROUND

Thrombotic thrombocytopenic purpura (TTP) is a rare thrombotic microangiopathy characterized by mechanical hemolytic anemia, profound thrombocytopenia, and neurological manifestations (1). Acquired auto-immune TTP, the most prevalent cause of TTP, is induced by the presence of inhibitory anti-ADAMTS13 auto-antibodies. At the pathophysiological level, auto-antibodies induce severe ADAMTS13 deficiency (typically below 10%) and the inability to cleave von Willebrand factor (vWF), the ADAMTS13 substrate, leading to the expression of prothrombotic ultra-large vWF in the microcirculation. Interactions between ultra-large vWF and platelets induce microthrombi formation, which is responsible for tissue injury and organ dysfunction (1).

Modern treatment of acquired TTP relies on plasma exchange (PE), rituximab (RTX), and steroids (2). PE allows to remove autoantibodies and to replenish ADAMTS13 concentration using plasma from healthy donors, whereas RTX targets B cells, including autoreactive B cells that produce pathogenic autoantibodies. Using this regimen, up to 85% of patients achieve disease remission by 5–15 days, the latter being classically defined as organ dysfunction recovery and sustained platelet count normalization. In a minority of patients, resistance to first-line treatment is observed, leading to consider the use of intensive PE (twice daily), cytotoxic agents (i.e., cyclophosphamide), and/or splenectomy (3). It is important to underline that despite significant therapeutic signs of progress, acquired TTP remains a life-threatening disease with a mortality rate ranging from 5 to 15% occurring predominantly within the 2 weeks after hospital admission (4).

Caplacizumab (Cablivi®), a humanized single-variable domain immunoglobulin that targets the A1 domain of the ultra-large vWF, inhibits the interaction between ultra-large vWF and platelets (5). In two clinical trials, caplacizumab, in addition to conventional treatment, allowed to shorten the delay to platelet count normalization in comparison with conventional treatment plus placebo, without increasing significantly hemorrhagic complications (6, 7). Moreover, caplacizumab was associated with reduced occurrence of a secondary endpoint associating death, TTP recurrence, and major thromboembolic events (7). After these trials, caplacizumab was approved for TPP treatment and conventional treatment in the US and Europe.

Here, we report the off-label use of caplacizumab in a patient with acquired TTP resistant to conventional therapy. To the best of our knowledge, this is the first reported use of caplacizumab in such a condition.

CASE PRESENTATION

A 68-year-old woman was admitted to the emergency department of a local hospital after a 15-min transient neurological attack. She had a history of essential hypertension treated with lercanidipine and Sjögren's syndrome, which was recently diagnosed. On examination, arterial pressure was 148/85 mmHg; she had purpuric lesions of the upper limbs, petechia of the mucosa, and diffuse ecchymoses. The neurological examination was first normal. Biological examination revealed thrombotic microangiopathy (TMA), with mechanical microangiopathic hemolytic anemia (hemoglobin 8.9 g/dL, haptoglobin below 0.2 g/L, free bilirubin at 57 μmol/L, lactate dehydrogenase 1,245 UI/L, schistocytes between 5 and 10%, and thrombocytopenia at 10 g/L). Serum creatinine was 94 μmol/L, without significant proteinuria. The diagnosis of TTP was rapidly considered, and she was transferred to the proximity university hospital for therapeutic management.

Confirming acquired TTP, ADAMTS13 activity was below 5%, and anti-ADAMTS13 antibodies (titer $>$ 100 UI/ml) were detected. Positive antinuclear antibodies (1/200) were also detected with anti-Sm-RNP antibodies. The search for antiphospholipid antibody syndrome was negative.

After the first PE session on day 1, the patient presented generalized tonic–clonic seizures requiring mechanical ventilation and admission to the intensive care unit. Steroids (1-mg/kg/day prednisone) and the first infusion of 375-mg/m² RTX were administered. Her clinical and biological condition improved on day 3, allowing to stop sedative drugs and weaning from mechanical ventilation. However, despite PE treatment, steroid continuation, and a second RTX infusion on day 6, TMA worsened with thrombocytopenia at 21 g/L on day 8 from admission. At that time, we decided to intensify PE treatment to twice-daily sessions. However, her clinical and neurological condition worsened. Indeed, TMA was not controlled as attested by the platelet count remaining below 10 g/L. On day 9, she developed encephalopathy with repetitive partial seizures necessitating profound sedation and mechanical ventilation. At clinical examination, persistent motor paralysis of the upper left limb was observed. The CT scan was normal, excluding brain hemorrhage, and the MRI showed two micro-infarctions. A third RTX infusion was done on day 10. On day 11, platelet count was 7 g/L, and encephalopathy with motor paralysis was persistent. Given that complement activation has been observed in TMA, including in TTP, and based on some literature case reports (8), we decided to administer eculizumab to our patient as a rescue therapy on day 11. Despite complement blockade, we did not observe any improvement of TMA activity and neurological involvement.

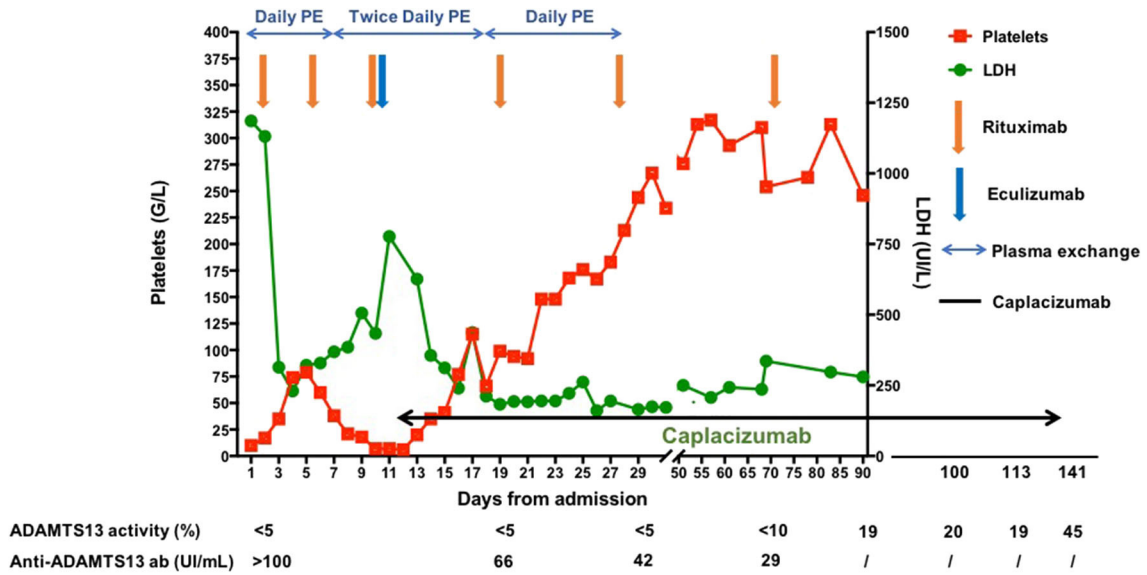

FIGURE 1 | Treatment management and evolution of the platelet count and the lactate dehydrogenase level from diagnosis and day 90. The patient gave her written consent for publication of the case report.

Concomitantly, we also considered using caplacizumab, which we could obtain and start on day 12 from admission, as it was just available under a temporary authorization use in France. The first injection of caplacizumab was done intravenously at 10-mg dose, followed by 10 mg twice-daily subcutaneous administration after each PE session, as recommended by the manufacturer. As soon as 12 h after caplacizumab initiation, we observed a significant increase of platelet count and improvement of other hemolytic parameters. We observed a resolution of encephalopathy and complete recovery of motor paralysis, allowing to stop mechanical ventilation on day 14. A drop of platelet count to 66 g/L without other biological signs of TMA occurred on day 18, which we attributed to a hypersensibility reaction to plasma infusion. Complete resolution of biological TMA signs was observed on day 24 from admission.

PE sessions were continued twice daily until day 20 from admission and could be stopped on day 31 (a total of 41 PE sessions performed). Rituximab infusions were administered on day 19 (CD19 count 17 cells/μl, fourth infusion), day 28 (CD19 count 2 cells/μl, fifth infusion), and day 71 (CD19 count 1 cell/μl, sixth infusion) to achieve complete B cell depletion. In parallel, we closely monitored ADAMTS13 activity and decided to continue caplacizumab until ADAMTS13 activity returned above 20%. Caplacizumab was maintained for 128 days until day 139 from initial admission. Apart from a benign palpebral hematoma, no other adverse event occurred under caplacizumab treatment. The treatment management and the evolution of TMA parameters are summarized in **Figure 1**. The patient was going well 20 months after initial admission, without any neurological sequelae and did not develop TTP relapse.

DISCUSSION

Caplacizumab has been tested in two clinical trials as an add-on therapy to conventional TTP treatment associating PE, steroids, and RTX (6, 7). Both trials were associated with faster thrombocytopenia correction in the caplacizumab groups. Interestingly, in the phase III trial HERCULES, no patient in the caplacizumab group experienced refractory TTP defined by the absence of platelet count doubling after 4 days of treatment, whereas three patients did in the conventional treatment group. At the difference of clinical trials where it was tested, caplacizumab was used as rescue therapy for a severe resistant form of TTP in our patient. Interestingly, platelet count increased very early after caplacizumab initiation, suggesting an immediate action of the molecule at the microcirculation level. We observed concomitantly a fast correction of the hemolytic parameters and clinical improvement of the patient. Thus, these observations clearly suggest that caplacizumab can improve microcirculation and prevent tissue damage.

Consequently, as illustrated in our case, the ability of caplacizumab to control very severe TTP forms strengthens the view that its use, along with first-line treatment, should reduce significantly life-threatening forms of the disease and the need for more aggressive salvage therapies (twice daily PE, cyclophosphamide, splenectomy). Finally, in our patient, if caplacizumab were not available to use, we would have considered cyclophosphamide administration or splenectomy. Both have been reported in the literature as salvage therapies that should be considered alone or sequentially in patients with severe and resistant TTP forms (9, 10). However, the use of these treatments relies mainly on small retrospective

studies, and their safety remains controversial, especially in regard to bleeding risk after splenectomy and infectious complications (9, 10).

ETHICS STATEMENT

The patient gave her written, informed consent for publication of the case report.

AUTHOR CONTRIBUTIONS

CM and NH: took care of the patient and wrote the first version of manuscript. P-MF and SM: took care of the patient in the ICU. J-FS: revised the manuscript. AV: did the ADAMTS13 measurement. PC: revised the manuscript and helped in treatment decision. J-FA: wrote and revised the manuscript, took care of the patient, and derived treatment decisions. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Kremer Hovinga JA, Coppo P, Lammle B, Moake JL, Miyata T, Vanhoorelbeke K. Thrombotic thrombocytopenic purpura. *Nat Rev Dis Primers.* (2017) 3:17020. doi: 10.1038/nrdp.2017.20
2. Joly BS, Coppo P, Veyradier A. Thrombotic thrombocytopenic purpura. *Blood.* (2017) 129:2836–46. doi: 10.1182/blood-2016-10-709857
3. Sayani FA, Abrams CS. How i treat refractory thrombotic thrombocytopenic purpura. *Blood.* (2015) 125:3860–7. doi: 10.1182/blood-2014-11-551580
4. Alwan F, Vendramin C, Vanhoorelbeke K, Langley K, McDonald V, Austin S, et al. Presenting adamts13 antibody and antigen levels predict prognosis in immune-mediated thrombotic thrombocytopenic purpura. *Blood.* (2017) 130:466–71. doi: 10.1182/blood-2016-12-758656
5. Veyradier A. Von willebrand factor–a new target for ttp treatment? *N Engl J Med.* (2016) 374:583–5. doi: 10.1056/NEJMe1515876
6. Peyvandi F, Scully M, Kremer Hovinga JA, Cataland S, Knobl P, Wu H, et al. Caplacizumab for acquired thrombotic thrombocytopenic purpura. *N Engl J Med.* (2016) 374:511–22. doi: 10.1056/NEJMoa1505533
7. Scully M, Cataland SR, Peyvandi F, Coppo P, Knobl P, Kremer Hovinga JA, et al. Caplacizumab treatment for acquired thrombotic thrombocytopenic purpura. *N Engl J Med.* (2019) 380:335–46. doi: 10.1056/NEJMoa1806311
8. Vigna E, Petrungaro A, Perri A, Terzi D, Recchia AG, Mendicino F, et al. Efficacy of eculizumab in severe adamts13-deficient thrombotic thrombocytopenic purpura (ttp) refractory to standard therapies. *Transfus Apher Sci.* (2018) 57:247–9. doi: 10.1016/j.transci.2018.03.005
9. Beloncle F, Buffet M, Coindre JP, Munoz-Bongrand N, Malot S, Pene F, et al. Splenectomy and/or cyclophosphamide as salvage therapies in thrombotic thrombocytopenic purpura: the French TMA reference center experience. *Transfusion.* (2012) 52:2436–44. doi: 10.1111/j.1537-2995.2012.03578.x
10. Kappers-Klunne MC, Wijermans P, Fijnheer R, Croockewit AJ, van der Holt B, de Wolf JT, et al. Splenectomy for the treatment of thrombotic thrombocytopenic purpura. *Br J Haematol.* (2005) 130:768–76. doi: 10.1111/j.1365-2141.2005.05681.x

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Outcomes of Immune Thrombotic Thrombocytopenic Purpura (iTTP) With Upfront Cyclophosphamide vs. Rituximab

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Background: Immune thrombotic thrombocytopenic purpura (iTTP) is a rare, life-threatening disorder managed with plasma exchange (PLEX) and steroids. Addition of rituximab (RTX) to initial disease treatment has been shown to lower future relapse rates. Information as to whether upfront cyclophosphamide (CTX) treatment is helpful in reducing relapse is not known.

Methods: In a retrospective cohort study, we identified all patients at our institution diagnosed with iTTP between 2010 and 2019. We analyzed outcomes of cumulative incidence of relapse (CIR) and duration of remission.

Results: Thirty Nine patients were studied. Group A ($n = 10$) included patients who received upfront PLEX and steroids alone, and Group B ($n = 28$) included those who received either upfront RTX ($n = 23$) or CTX ($n = 5$) in addition to PLEX and steroids. The 2-year CIR was 50% in Group A and 27.7% in Group B, with a median duration of remission of 43.6 months vs. 108.3 months, respectively ($p = 0.04$). Group A was associated with a HR=8.7 (95% CI: 1.27, 59.45), $p = 0.027$ for duration of remission. There was no significant difference between CTX and RTX in both outcomes of CIR and duration of remission. We observed a potential impact on remission duration based on the presenting absolute neutrophil count (HR = 0.74, 95% CI: 0.58, 0.96) and serum creatinine (HR = 1.42, 95% CI: 1.03, 1.94).

Conclusion: There was no significant difference in iTTP relapse outcomes between upfront RTX and CTX. Absolute neutrophil count and serum creatinine may have a role in predicting relapse. Larger, prospective studies are needed to evaluate these findings.

Keywords: thrombotic thrombocytopenic purpura, rituximab, cyclophosphamide, treatment, relapse

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INTRODUCTION

Auto-immune thrombotic thrombocytopenic purpura (iTTP) is a rare, life-threatening disorder caused by auto-antibodies against ADAMTS13. It is characterized by a severe thrombotic microangiopathy (TMA) that leads to organ failure and is associated with high morbidity and mortality. The mortality rate of the untreated disease is around 90%, and treatment with

corticosteroids and therapeutic plasma exchange (PLEX) reduces that rate to around 10%. (1, 2) Although this treatment induces remission, disease relapse remains a common problem. Relapse is estimated to occur in around 30–50% of patients after achieving initial remission (3, 4). Since iTTP is mediated by an antibody against ADAMTS13, additional immunosuppressive therapy given upfront has been shown to lower relapse rates (5–9).

Rituximab (RTX) is a monoclonal antibody that targets B-cells, which produce the antibody responsible for causing iTTP. It was first used in treating this disease in the relapsed or refractory setting in the early 2000s, and was successful in inducing remission (10, 11). The Phase II trials by Scully et al. and Chen et al. demonstrated safety and efficacy of using upfront RTX (8, 12). In the former trial, RTX was associated with 10% relapse rate compared to 57% in historical controls, which was a statistically significant reduction (8). It has since been used in the upfront setting in various studies and shown to lower relapse rates (5–9). While recent common practice has shifted toward adding RTX to steroids and PLEX as front-line treatment for acute initial iTTP, this practice has not been rigorously examined.

An alternative therapy, cyclophosphamide (CTX), has been used in relapsed iTTP. CTX is effectively used in the management of acquired hemophilia that is caused by auto-antibodies to factor VIII (13, 14). In the European Acquired Hemophilia Registry, steroids combined with cyclophosphamide resulted in more stable complete remission (70%) than rituximab-based regimens (59%) (14). Several small studies have utilized cyclophosphamide in the treatment of relapsed or refractory iTTP where it has been shown to be effective (15–19). However, studies evaluating CTX use in the upfront setting as a first-line treatment along with PLEX and corticosteroids are lacking. There are several advantages to the use of CTX. First, many patients develop infusion reactions to RTX or have other contraindicating comorbidities that may preclude its use upfront. Furthermore, if given concurrently with PLEX, ∼65% of RTX may be removed making the optimal dose and frequency of this drug difficult to determine (20). Alternatively, CTX is unlikely to be removed by PLEX due to its low protein-binding rate of 23%, and higher volume of distribution of 0.8 L/kg (21). Furthermore, CTX is far less costly than RTX. As such, having an alternative agent for use in the upfront setting may be beneficial. In this retrospective clinical review, we hypothesized that CTX is as effective as RTX in lowering relapse rate when used in the upfront setting for initial acute iTTP. We compared outcomes of cumulative incidence of relapse (CIR) as well as duration of remission between patients who received upfront CTX and those who received RTX, in addition to PLEX and steroids. Subsequently, in order to evaluate whether there was benefit in the use of any additional immunosuppression besides PLEX and steroids, we compared the cohort of patients who received either additional CTX or RTX with those who received PLEX and steroids alone without any additional immunosuppression. This retrospective investigation also examined several baseline characteristics on initial disease presentation and assessed whether they correlated with a higher risk of relapse.

METHODS

Patient Selection and Data Collection

In a retrospective chart review, we identified all patients diagnosed with iTTP at University Hospitals—Cleveland Medical Center between 2010 and 2019. The study was approved by the Institutional Review Board (IRB). A billing diagnosis code search for "thrombotic microangiopathy" (ICD9: 446.6, ICD10: M31.1) was conducted. We included all patients diagnosed with iTTP, defined as having an ADAMTS13 level of <10% and a positive ADAMTS13 inhibitor or antibody, with clinical evidence of TMA per provider documentation. We excluded patients with a follow-up duration of <30 days, or with TMA due to other causes such as congenital TTP, hemolytic-uremic syndrome, anti-phospholipid syndrome, HELLP syndrome, systemic lupus erythematosus, preeclampsia, accelerated hypertension, and diffuse intravascular coagulopathy. The ADAMTS13 assays were performed at the Blood Center of Wisconsin, measured by Fluorescence Resonance Energy Transfer with a synthetic substrate. Inhibitor assays were performed on those patients with low ADAMTS13 levels.

We divided our cohort into four groups (**Figure 1**). Group A included patients who received steroids and PLEX alone, without any additional upfront therapy. Group B included patients who received steroids, PLEX, in addition to either RTX or CTX. The latter group was further divided into RTX group and CTX group to compare the effects of these two agents. For our secondary objective of predictors of relapse, we utilized the collective data from the entire cohort (all groups combined).

Outcomes and Definitions

We defined the "onset of remission" as the date after which the platelet count was greater than or equal to 150,000 /μl and lactate dehydrogenase (LDH) ≤246 U/L for a minimum of 48 h. Clinical relapse was defined as recurrence of TMA in addition to ADAMTS13 level of <10%. The "duration of remission" was measured from the date of onset of remission, until the date of first clinical relapse of TTP. For patients who are alive and did not relapse, the end point was the date of last-follow-up.

Statistical Methods

The cumulative incidence of relapse (CIR) was estimated using Kaplan-Meier method (22) and its difference among treatment groups was examined by log-rank test. The effect of continuous and categorical covariates on thrombotic thrombocytopenic purpura relapse rate was estimated by univariate Cox model (23). The association of categorical variables and continuous variables was examined using chi-square test and Pearson correlation coefficient, respectively, and the difference of continuous measurements among groups was tested using *T*-test (two groups) or ANOVA (more than 2 groups). The effect of continuous and categorical covariates on time to eradication of inhibitor (from diagnosis time) was estimated by univariate and multivariable linear regression. The effect of continuous and categorical covariates on days of hospitalization duration was also estimated by univariate and multivariable linear

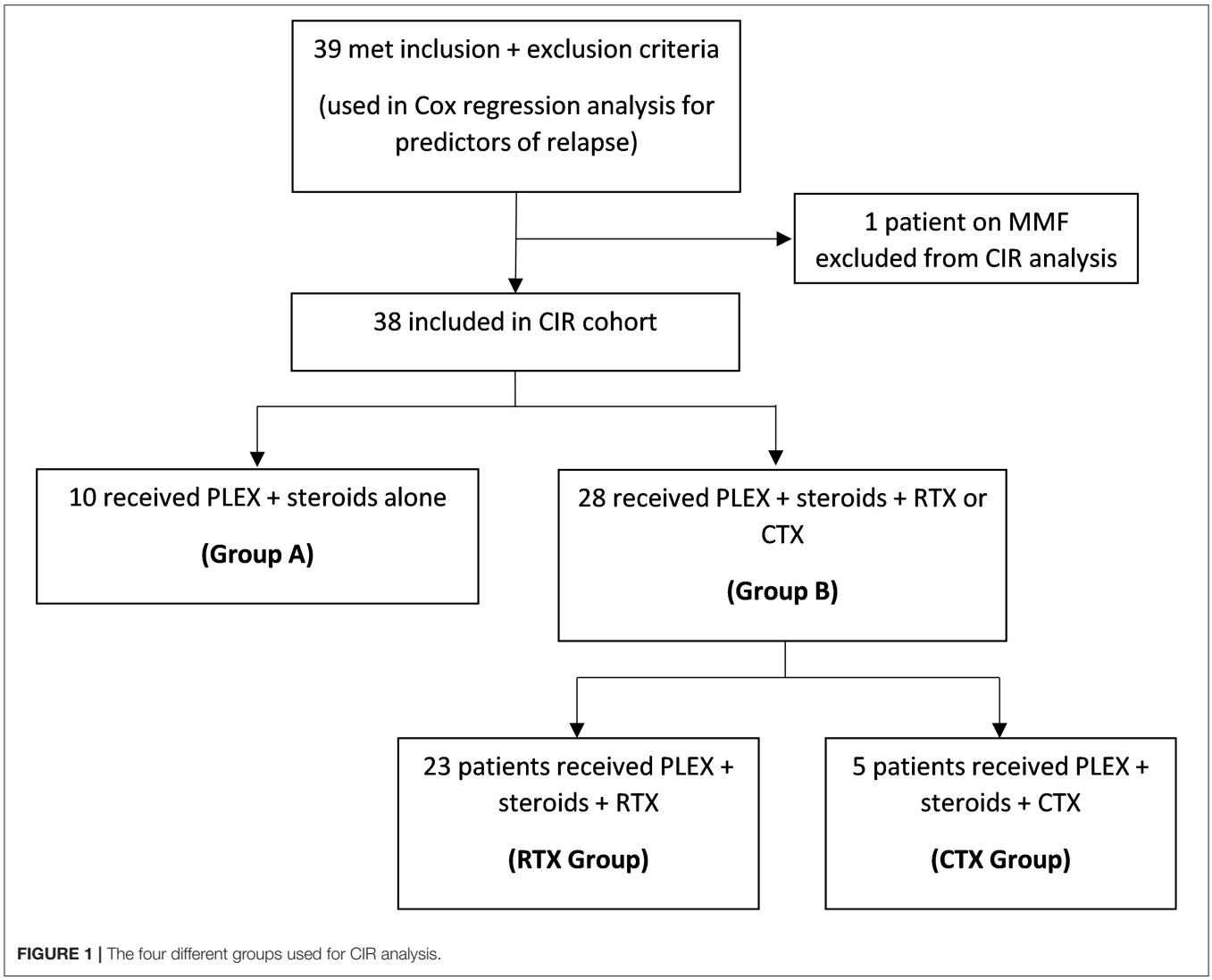

FIGURE 1 | The four different groups used for CIR analysis.

regression. All tests are two-sided and $p \leq 0.05$ was considered statistically significant.

RESULTS

A total of 39 patients met the inclusion and exclusion criteria. One patient received mycophenolate mofetil, and was not included in the primary analysis of CIR, but was included in the secondary objective of the study on laboratory predictors of relapse. Ten patients received steroids and PLEX alone (Group A), and 28 patients received steroids, PLEX, and either RTX or CTX (Group B). Of those, 23 received RTX (375 mg/m^2 weekly for four doses), and five received CTX (400 mg/m^2 every 3 weeks for six doses). The average age of diagnosis was 44 years. The average duration of follow-up was 76 months. There were no significant differences in baseline characteristics between any of the groups (**Table 1**). Upfront administration of CTX was not associated with any significant side effects in the five treated patients.

Clinical Relapse

The median duration of remission in Group A vs. Group B was 43.6 months (95% CI: 0.5, 114.3) and 108.3 months (95% CI: 36.8, N/A) respectively ($p = 0.04$). The CIR at 48 months in Group A was 50 vs. 27.7% (CTX = 33.3%, RTX = 27.2%) in Group B (**Table 2**) In the multivariable analysis, Group A was associated with a statistically significant hazard ratio of 8.7 (95% CI: 1.27, 59.45), $p = 0.027$ for time to clinical relapse, compared to Group B. There was no statistically significant difference in CIR between RTX and CTX in the Kaplan–Meier analysis (**Figure 2**, **Table 2**).

Predictors of Relapse

The Cox regression analysis of the effect of initial inhibitor level, absolute neutrophil count (ANC), nadir platelet count, and serum creatinine on the outcome of duration of remission is summarized below, and highlighted in **Table 3**. The analysis on all other variables, including age, absolute lymphocyte count, neutrophil/lymphocyte ratio, peak LDH on admission, fibrinogen, D-Dimer, presence of autoimmune disease, and

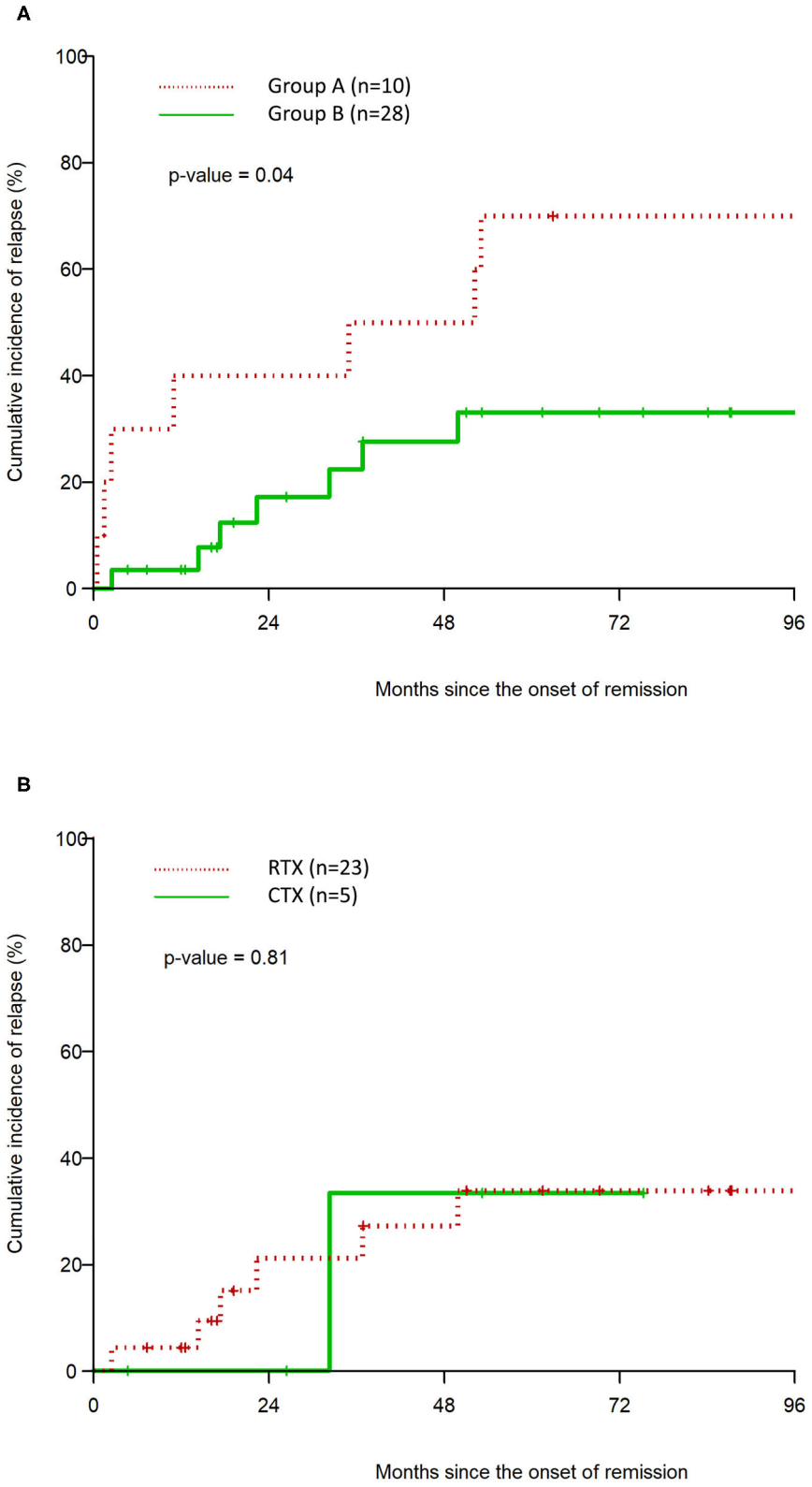

FIGURE 2 | Kaplan–Meier estimation of cumulative incidence of clinical relapse after remission for Group A vs. Group B **(A)**, and CTX vs. RTX **(B)**.

TABLE 1 | Baseline Characteristics.

Variables	CTX group ($n = 5$) mean (STD) or frequency	RTX group ($n = 23$) mean (STD) or frequency	Group A ($n = 10$) mean (STD) or frequency	*p*-value
Age (years)	33 (7.80)	44 (20.55)	43 (13.4)	0.44
Gender (F/M)	5/0	13/10	7/3	0.169
Initial inhibitor level	1.98 (2.50)	2.97 (2.72)	2.30 (2.51)	0.73
Initial ADAMTS13 antibody level	37.5 (34.65)	33.83 (29.08)	N/A	0.89
D-Dimer	767.00 (202.34)	2255.57 (1801.57)	1590.75 (600.06)	0.31
Fibrinogen	264.40 (67.42)	302.95 (90.46)	319.00 (117.66)	0.63
Nadir platelet count	8.8 (2.28)	12.5 (8.6)	12.20 (7.36)	0.64
Absolute neutrophil count	7.1 (4.72)	8.8 (4.1)	6.97 (2.14)	0.55
Absolute lymphocyte count	1.61 (1.06)	2.07 (1.05)	1.91 (1.40)	0.70
Neutrophil-to-lymphocyte ratio	9.59 (12.59)	6.23 (5.85)	5.61 (4.09)	0.61
Creatinine on admission	1.02 (0.22)	1.93 (1.98)	3.03 (1.47)	0.31
Peak LDH on admission	1,444.4 (790.47)	1,222.91 (604.31)	926.20 (374.53)	0.41
Platelet normalization (days)	11.2 (7.73)	11.32 (11.41)	11.20 (16.13)	0.99
LDH normalization (days)	11.6 (6.62)	11.33 (10.6)	6.00 (3.16)	0.58
Resolution time (days)	14.6 (8.32)	14.5 (12.07)	12.80 (15.15)	0.96
Duration of hospitalization (days)	23.6 (9.29)	26.75 (15.98)	11.2 (6.72)	0.13
Altered mental status (Yes/ No)	2/3	8/13	2/2	0.91
Autoimmune disease (Yes/No)	3/2	3/20	2/8	0.07
Malignancy (Yes/No)	3/2	4/19	1/9	0.06

TABLE 2 | Kaplan–Meier estimation of the cumulative incidence of clinical relapse (CIR, %).

Factor	Cumulative incidence of relapse (CIR) for clinical relapse				*p*-value
	24 months	48 months	72 months	120 months	
Group A ($n = 10$)	40.0	50.0	70.0	85.0	0.04
Group B ($n = 28$)	17.2	27.7	33.2	66.6	
Cyclophosphamide ($n = 5$)	0.0	33.3	33.3		0.81
Rituximab ($n = 23$)	21.1	27.2	33.8	66.9	

presence of malignancy did not show a statistically significant impact on duration of remission. Similarly, the time to initiation of CTX or RTX from the time of disease diagnosis did not demonstrate an effect on the duration of remission.

Initial Inhibitor Level

Although the initial inhibitor level showed a trend toward an increased risk of clinical relapse per unit increase the results did not meet statistical significance in the univariate analysis (HR: 1.16, 95% CI: 0.95, 1.44), $p = 0.17$. However, this effect was amplified after controlling for type of upfront therapy in the multivariable analysis (HR: 1.23, 95% CI: 0.97, 1.56), $p = 0.084$.

Absolute Neutrophil Count (ANC)

A higher ANC on presentation was associated with a statistically significantly longer duration of remission per unit increase. This effect maintained statistical significance in the multivariable analysis, with an HR of 0.74 (95% CI: 0.58, 0.96). Also, a Kaplan-Meier graph of a dichotomized separation of high or low ANC (above or below the median of 7.6×10^9/L) shows a statistically significant decrease in the risk of CIR in the high ANC group **(Figure 3A)**, with $p = 0.015$. Although the average ANC between the groups was not different, within the entire cohort there is a gradient of ANC values. Those ANC values $>7.6 \times 10^9$/L demonstrate the improved CIR finding.

Nadir Platelet Count

The nadir platelet count on presentation was not associated with a statistically significant change in time to clinical relapse, with HR of 0.98 (0.90, 1.06), *p*-value: 0.60.

Serum Creatinine

The serum creatinine level on presentation was associated with a decreased time to clinical relapse per unit increase, which was statistically significant in the multivariable analysis (HR: 1.42, 95% CI: 1.03, 1.94). A Kaplan–Meier graph of a dichotomized separation of high or low serum creatinine (above or below the median of 1.2 mg/dL) shows a statistically significant increase in the risk of CIR in the high serum creatinine group **(Figure 3B)**, with $p = 0.046$.

DISCUSSION

Our study results demonstrate that the upfront use of immunosuppressive therapy, in addition to steroids and PLEX is associated with a reduction in the cumulative incidence of relapse and prolongs the duration of remission. The median

TABLE 3 | Univariate and multivariable Cox regression analysis on time to clinical relapse.

Variable (per unit increase)	Clinical relapse HR (95% CI)	
	Univariate	Multivariable
Initial inhibitor level	1.16 (0.94, 1.44), *p*-value: 0.17	1.23 (0.97, 1.56), *p*-value: 0.084*
Nadir platelet count	0.98 (0.90, 1.06), *p*-value: 0.60	
Initial ANC	0.79 (0.65, 0.98), *p*-value: 0.029	0.74 (0.58, 0.96), *p*-value: 0.020
Initial serum creatinine	1.36 (1.02, 1.82), *p*-value: 0.035	1.42 (1.03, 1.94), *p*-value: 0.032

Controlled for type of therapy only. All the other analyses controlled for all the baseline characteristics listed in **Table 1.*

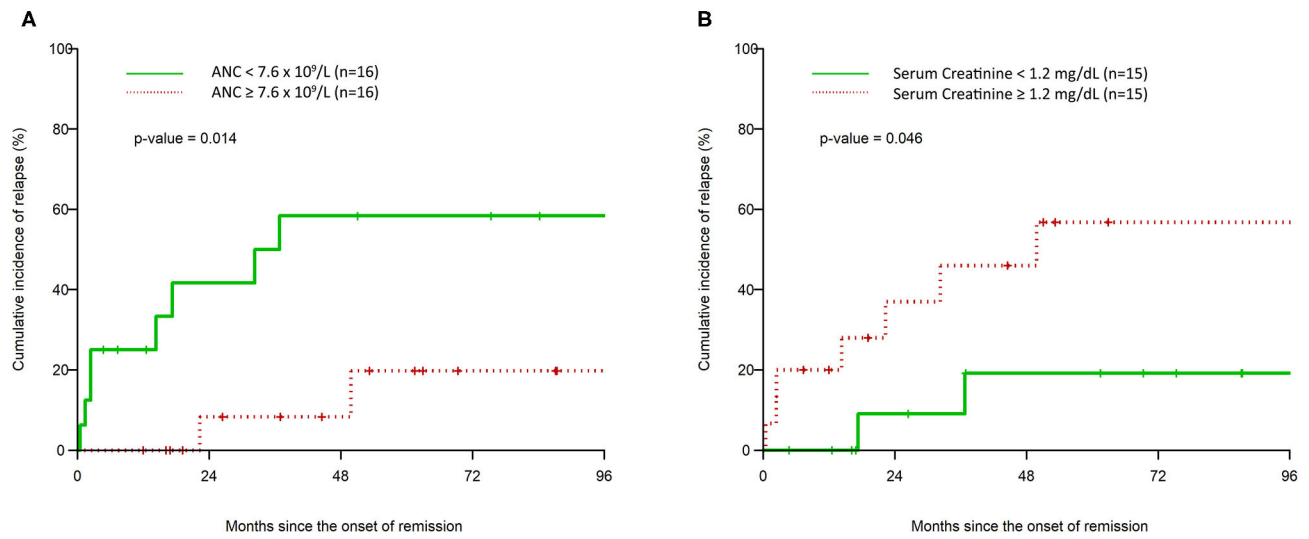

FIGURE 3 | Kaplan–Meier estimation of cumulative incidence of clinical relapse after remission based on high vs. low initial absolute neutrophil count **(A)** and high vs. low initial serum creatinine **(B)**.

duration of remission was significantly higher in Group B (108.3 months) compared to steroids and PLEX alone in Group A (43.6 months) with a HR of 8.7 (95% CI: 1.27, 59.45) after controlling for baseline characteristics in the multivariable model. Consistent with previously reported data, these findings demonstrate that the addition of immunosuppressive therapy is associated with a longer initial remission in iTTP. Our data is derived from a combined cohort of patients who received either CTX or RTX and demonstrates the benefit of any additional upfront immunosuppression besides PLEX and steroids in this disease. The sample size in the RTX and CTX groups individually was too small to demonstrate a statistically significant difference in outcomes in either group.

We then compared the outcomes between those who received RTX or CTX upfront. The use of upfront RTX has been shown in several studies to lower relapse rates (6–9). CTX, on the other hand, has not been studied in the upfront setting, as opposed to relapsed or refractory disease. While our sample size is small, the Kaplan–Meier curves showed clear overlap and no statistically significant changes in relapse outcomes in patients who received upfront CTX as compared to RTX. However, in order to achieve 80% power to detect a difference, the required total sample size is 308 patients. Nevertheless, our data demonstrate that it would be beneficial to evaluate the use of upfront CTX in larger, prospective studies. Low-dose pulse intravenous CTX used for iTTP is associated with far less adverse effects compared to higher doses of CTX, which can lead to infectious complications, bone marrow suppression, and long-term risk for malignancy (24). The risks with low-dose CTX are outweighed in the context of preventing relapse of life-threatening iTTP.

The second goal of our study was to investigate baseline characteristics that may help predict the risk of relapse. Identification of risk factors predictive of relapse would make a stronger argument for adding an upfront immunosuppressive agent, or using more aggressive therapy at diagnosis. A previous study by Tuncer et al. demonstrated that male sex, severe thrombocytopenia, and higher LDH pre-/post-treatment ratio were associated with higher risk of relapse (3). However, from all the different baseline characteristics we analyzed, we identified three different variables that may impact relapse outcomes: inhibitor level, ANC, and serum creatinine.

The inhibitor level on presentation did not show a statistically significant effect on duration of remission in the univariate analysis. However, when we controlled for the type of therapy

used (Group A vs. Group B), its effect was amplified and trended closer toward statistical significance. This suggests that with larger study samples, this effect may be more pronounced. Of note, there are limitations in the inhibitor assay that we use since it does not report levels above 8 arbitrary units. Unlike previously reported data, our study did not demonstrate a statistically significant impact of nadir platelet count on relapse risk (3). Further, we found that a higher serum creatinine was associated with a shorter duration of remission and a statistically significant separation in the Kaplan–Meier analysis for CIR. We suspect that this may reflect a more aggressive nature of the disease and its microvascular complications, which may also translate into a higher tendency for clinical relapse.

We proposed that an elevated ANC may correlate with higher risk of relapse. However, contrary to our hypothesis, we found that a higher ANC was actually associated with a longer duration of remission. The protective effect of a higher ANC maintains statistical significance in the multivariable analysis after controlling for all other characteristics and is statistically significant in the Kaplan-Meier separation. At present, the mechanistic basis of this observation is not known. There is emerging evidence in the literature that neutrophils may inhibit B-cell responses, especially in the context of autoimmune diseases (25). In murine models, data suggest a role for neutrophils in suppressing immunoglobulin and antibody production in B lymphocytes, as well as slowing disease progression of murine lupus (26–28). There is also evidence that such an effect may occur in humans as well. A study by Lelis et al. has shown that myeloid derived suppressor cells, which may function as pathologically activated neutrophils, have a role in modulating B cell responses by suppressing B-cell proliferation and antibody production (29). Since iTTP is also an autoimmune disease caused by auto-antibodies against ADAMTS13, a modulatory effect on the B cell production of antibodies probably would correlate with a more durable remission.

CONCLUSION

Our study is the first to examine outcomes in a subset of patients treated with upfront CTX therapy for initial iTTP, and suggests similar time to first relapse and 4-year CIR as compared to RTX. Although our numbers are small, our institutional experience suggests that CTX may be considered as alternative therapy in patients intolerant to RTX. Consistent with the currently published literature, PLEX and steroid therapy alone was associated with a significantly shorter duration of remission compared to additional immunosuppressive therapy. Initial inhibitor level, serum creatinine, and ANC may offer a predictive role in the risk for disease relapse. The results of these investigations indicate that there is room for more diverse approaches to the management of iTTP. Larger prospective studies are warranted to confirm these observations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MA-I and YA collected the data. MA-I, AHS, and LN wrote the manuscript. AHS and LN supervised and mentored the study. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Rock GA, Shumak KH, Buskard NA, Blanchette VS, Kelton JG, Nair RC, et al. Comparison of plasma exchange with plasma infusion in the treatment of thrombotic thrombocytopenic purpura. Canadian apheresis study group. *N Engl J Med*. (1991) 325:393–7. doi: 10.1056/NEJM199108083250604
2. Bell WR, Braine HG, Ness PM, Kickler TS. Improved survival in thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. Clinical experience in 108 patients. *N Engl J Med.* (1991) 325:398–403. doi: 10.1056/NEJM199108083250605
3. Tuncer HH, Oster RA, Huang ST, Marques MB. Predictors of response and relapse in a cohort of adults with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome: a single-institution experience. *Transfusion.* (2007) 47:107–14. doi: 10.1111/j.1537-2995.2007.01071.x
4. Vesely SK, George JN, Lammle B, Studt JD, Alberio L, El-Harake MA, et al. ADAMTS13 activity in thrombotic thrombocytopenic purpura-hemolytic uremic syndrome: relation to presenting features and clinical outcomes in a prospective cohort of 142 patients. *Blood.* (2003) 102:60–8. doi: 10.1182/blood-2003-01-0193
5. Tsai HM, Shulman K. Rituximab induces remission of cerebral ischemia caused by thrombotic thrombocytopenic purpura. *Eur J Haematol.* (2003) 70:183–5. doi: 10.1034/j.1600-0609.2003.00026.x
6. Lim W, Vesely SK, George JN. The role of rituximab in the management of patients with acquired thrombotic thrombocytopenic purpura. *Blood.* (2015) 125:1526–31. doi: 10.1182/blood-2014-10-559211
7. Westwood JP, Thomas M, Alwan F, McDonald V, Benjamin S, Lester WA, et al. Rituximab prophylaxis to prevent thrombotic thrombocytopenic purpura relapse: outcome and evaluation of dosing regimens. *Blood Adv.* (2017) 1:1159–66. doi: 10.1182/bloodadvances.2017008268

8. Scully M, McDonald V, Cavenagh J, Hunt BJ, Longair I, Cohen H, et al. A phase 2 study of the safety and efficacy of rituximab with plasma exchange in acute acquired thrombotic thrombocytopenic purpura. *Blood.* (2011) 118:1746–53. doi: 10.1182/blood-2011-03-341131
9. Page EE, Kremer Hovinga JA, Terrell DR, Vesely SK, George JN. Rituximab reduces risk for relapse in patients with thrombotic thrombocytopenic purpura. *Blood*. (2016) 127:3092–4. doi: 10.1182/blood-2016-03-703827
10. Chemnitz J, Draube A, Scheid C, Staib P, Schulz A, Diehl V, et al. Successful treatment of severe thrombotic thrombocytopenic purpura with the monoclonal antibody rituximab. *Am J Hematol.* (2002) 71:105–8. doi: 10.1002/ajh.10204
11. Zheng X, Pallera AM, Goodnough LT, Sadler JE, Blinder MA. Remission of chronic thrombotic thrombocytopenic purpura after treatment with cyclophosphamide and rituximab. *Ann Intern Med.* (2003) 138:105–8. doi: 10.7326/0003-4819-138-2-200301210-00011
12. Chen H, Fu A, Wang J, Wu T, Li Z, Tang J, et al. Rituximab as first-line treatment for acquired thrombotic thrombocytopenic purpura. J *Int Med Res*. (2017) 45:1253–60. doi: 10.1177/0300060517695646
13. Lian EC, Larcada AF, Chiu AY. Combination immunosuppressive therapy after factor VIII infusion for acquired factor VIII inhibitor. *Ann Intern Med.* (1989) 110:774–8. doi: 10.7326/0003-4819-110-10-774
14. Collins P, Baudo F, Knoebl P, Levesque H, Nemes L, Pellegrini F, et al. Immunosuppression for acquired hemophilia a: results from the european acquired haemophilia registry (EACH2). *Blood.* (2012) 120:47–55. doi: 10.1182/blood-2012-02-409185
15. Beloncle F, Buffet M, Coindre JP, Munoz-Bongrand N, Malot S, Pene F, et al. Splenectomy and/or cyclophosphamide as salvage therapies in thrombotic thrombocytopenic purpura: the French TMA reference center experience. *Transfusion.* (2012) 52:2436–44. doi: 10.1111/j.1537-2995.2012.03578.x
16. Hertzberg MS, Koutts J. Oral cyclophosphamide for refractory or relapsing thrombotic thrombocytopenic purpura (TTP). *Aust N Z J Med.* (1997) 27:439. doi: 10.1111/j.1445-5994.1997.tb02204.x
17. Allan DS, Kovacs MJ, Clark WF. Frequently relapsing thrombotic thrombocytopenic purpura treated with cytotoxic immunosuppressive therapy. *Haematologica.* (2001) 86:844–50.
18. Coppo P, Froissart A. Treatment of thrombotic thrombocytopenic purpura beyond therapeutic plasma exchange. *Hematol Am Soc Hematol Educ Program.* (2015) 2015:637–43. doi: 10.1182/asheducation-2015.1.637
19. Guzicka-Kazimierczak R, Kazimierczak A, Clark J. Promising results from cyclophosphamide based immunosuppression therapy of thrombotic thrombocytopenic purpura. *Pomeranian J Life Sci.* (2015) 61:34–40. doi: 10.21164/pomjlifesci.48
20. McDonald V, Manns K, Mackie IJ, Machin SJ, Scully MA. Rituximab pharmacokinetics during the management of acute idiopathic thrombotic thrombocytopenic purpura. *J Thromb Haemost.* (2010) 8:1201–8. doi: 10.1111/j.1538-7836.2010.03818.x
21. Samtleben W, Mistry-Burchardi N, Hartmann B, Lennertz A, Bosch T. Therapeutic plasma exchange in the intensive care setting. *Ther Apher.* (2001) 5:351–7. doi: 10.1046/j.1526-0968.2001.00383.x
22. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc.* (2012) 53:458–480.
23. Cox DR. Regression models and life-tables. *J Roy Stat Soc Ser B.* (1972) 34:187–202. doi: 10.1111/j.2517-6161.1972.tb00899.x
24. Martin-Suarez I, D'Cruz D, Mansoor M, Fernandes AP, Khamashta M, Hughes G. Immunosuppressive treatment in severe connective tissue diseases: effects of low dose intravenous cyclophosphamide. *Ann Rheum Dis.* (1997) 56:481–7. doi: 10.1136/ard.56.8.481
25. Costa S, Bevilacqua D, Cassatella MA, Scapini P. Recent advances on the crosstalk between neutrophils and B or T lymphocytes. *Immunology*. (2019) 156:23–32. doi: 10.1111/imm.13005
26. Bird AK, Chang M, Barnard J, Goldman BI, Meednu N, Rangel-Moreno J, et al. Neutrophils slow disease progression in murine lupus via modulation of autoreactive germinal centers. *J Immunol.* (2017) 199:458–66. doi: 10.4049/jimmunol.1700354
27. Jee J, Bonnegarde-Bernard A, Duverger A, Iwakura Y, Cormet-Boyaka E, Martin TL, et al. Neutrophils negatively regulate induction of mucosal IgA responses after sublingual immunization. *Mucosal Immunol.* (2015) 8:735–45. doi: 10.1038/mi.2014.105
28. Kamenyeva O, Boularan C, Kabat J, Cheung GY, Cicala C, Yeh AJ, et al. Neutrophil recruitment to lymph nodes limits local humoral response to staphylococcus aureus. *PLoS Pathog.* (2015) 11:e1004827. doi: 10.1371/journal.ppat.1004827
29. Lelis FJN, Jaufmann J, Singh A, Fromm K, Teschner AC, Poschel S, et al. Myeloid-derived suppressor cells modulate B-cell responses. *Immunol Lett.* (2017) 188:108–15. doi: 10.1016/j.imlet.2017.07.003

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Molecular Studies and an *ex vivo* Complement Assay on Endothelium Highlight the Genetic Complexity of Atypical Hemolytic Uremic Syndrome: The Case of a Pedigree With a Null CD46 Variant

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Atypical hemolytic uremic syndrome (aHUS) is an ultra-rare disease characterized by microangiopathic hemolysis, thrombocytopenia, and renal impairment and is associated with dysregulation of the alternative complement pathway on the microvascular endothelium. Outcomes have improved greatly with pharmacologic complement C5 blockade. Abnormalities in complement genes (*CFH*, *CD46*, *CFI*, *CFB*, *C3*, and *THBD*), *CFH–CFHR* genomic rearrangements, and anti-FH antibodies have been reported in 40–60% of cases. The penetrance of aHUS is incomplete in carriers of complement gene abnormalities; and multiple hits, including the *CFH–H3* and $CD46_{GGAAC}$ risk haplotypes and the *CFHR1*B* risk allele, as well as environmental factors, contribute to disease development. Here, we investigated the determinants of penetrance of aHUS associated with *CD46* genetic abnormalities. We studied 485 aHUS patients and found *CD46* rare variants (RVs) in about 10%. The c.286+2T>G RV was the most prevalent (13/485) and was associated with <30% penetrance. We conducted an in-depth study of a large pedigree including a proband who is heterozygous for the c.286+2T>G RV who experienced a severe form of aHUS and developed end-stage renal failure. The father and paternal uncle with the same variant in homozygosity and six heterozygous relatives are unaffected. Flow cytometry analysis showed about 50% reduction of CD46 expression on blood mononuclear cells from the heterozygous proband and over 90% reduction in cells from the proband's unaffected homozygous father and aunt. Further genetic studies did not reveal RVs in known aHUS-associated genes or common genetic modifiers that segregated with the disease. Importantly, a specific *ex vivo* test showed excessive complement deposition on endothelial cells exposed to sera from the proband, and also from his mother and maternal uncle, who do not carry the c.286+2T>G RV, indicating that they share a circulating defect that results in complement dysregulation on the endothelium. These results highlight the complexity of the genetics of aHUS and indicate that

CD46 deficiency may not be enough to induce aHUS. We hypothesize that the proband inherited from his mother a genetic abnormality in a complement circulating factor that has not been identified yet, which synergized with the *CD46* RV in predisposing him to the aHUS phenotype.

Keywords: atypical hemolytic uremic syndrome, complement, membrane cofactor protein, incomplete penetrance, splicing, CD46 expression, *ex-vivo* assay, rare variants

INTRODUCTION

Hemolytic uremic syndrome (HUS) is an ultra-rare disease characterized by microangiopathic hemolytic anemia, thrombocytopenia, and renal impairment (1) caused by platelet thrombi in the microcirculation of the kidney and other organs. Atypical HUS (aHUS) accounts for about 10% of all cases and has a poor prognosis compared with the most common form of HUS in children, which is caused by Shiga-like toxin producing *Escherichia coli* (*STEC*) (1). Before the introduction of C5 inhibition therapy, up to 50% of aHUS cases progressed to end-stage renal failure (ESRF) or developed irreversible brain damage, and 25% died during the acute phase of the disease (2, 3).

The term primary aHUS identifies cases characterized by dysregulation of the alternative complement pathway (2, 4). Rare recessive forms of aHUS are associated with genetically determined cobalamin C (5) or diacylglycerol kinase 3 deficiency (6, 7). Finally, aHUS may be secondary to other conditions, such as autoimmune diseases, systemic diseases, malignant hypertension, and transplantation (4, 8).

In 40–60% of patients with primary aHUS, genetic abnormalities affecting the complement regulatory proteins factor H (*CFH*), membrane cofactor protein (*MCP*), factor I (*CFI*), and thrombomodulin (*THBD*) and the components of the alternative pathway C3 convertase C3 and factor B (*CFB*) or anti-FH autoantibodies have been identified (2, 9–13). Less than 20% of cases are considered familial, that is, cases where two or more members of the same family are affected by the disease and exposure to STEC has been ruled out. All the other patients do not have a family history of the disease (sporadic aHUS), and most of them inherited the complement abnormality from an unaffected parent. Indeed, incomplete penetrance has been reported for all genes associated with aHUS. Other genetic modifiers, including risk haplotypes and polymorphisms and environmental factors have been shown to contribute to the development of disease phenotypes (14–16). A wide variety of triggers have been identified, including common viral and bacterial infections, ischemia, organ transplantation, and pregnancy.

Mutations in the gene encoding membrane cofactor protein (also known as *CD46*), first described in association with aHUS in 2003 (17, 18), account for 5 to 9% of cases with primary aHUS. MCP is a transmembrane protein made by four N-terminal short consensus repeats (SCRs), a serine/threonine-rich (ST) domain, a transmembrane domain (TM), and a cytoplasmic tail (CYT) and serves as cofactor for factor I (FI), a plasma serine protease that cleaves C3b and C4b. It is widely expressed on all nucleated cells and is particularly highly expressed in the kidney (19), where it regulates C3 activation in the glomerulus.

aHUS-associated CD46 variants usually cluster in the extracellular complement regulatory SCR domains (14, 20–22). Seventy-five percent cause a reduction in MCP expression on the cell surface (23, 24).

The penetrance of aHUS among subjects with *CD46* mutations is incomplete, and 25% of patients had combined mutations in other complement genes (12).

Here, we investigated the determinants of *CD46* mutation penetrance. We found that the splicing variant c.286+2T>G (also known as IVS2+2T>G; dbSNP: rs769742294) is the most prevalent *CD46* genetic abnormality in our cohort of patients ($n = 485$) with primary aHUS, and within families, disease penetrance in c.286+2T>G carriers was 28%. We performed an in-depth study of the large pedigree of a patient with sporadic aHUS who is heterozygous for this variant. Fremeaux-Bacchi et al. demonstrated that this splice-site variant results in abnormal splicing, causing a deletion of 48 amino acids in the SCR1 of the protein (24). Afterwards, Maga et al. reported that the c.286+2T>G results in another abnormally spliced mRNA, leading to a frameshift and the translation of a truncated protein (p.Glu97Lysfs*33) (25).

In published studies, the c.286+2T>G variant was associated with a variable phenotype: heterozygous carriers have a milder form of aHUS, while the disease is more severe and has an earlier onset in homozygous carriers (24, 26). In contrast, in our pedigree, the heterozygous proband manifested a severe form of aHUS and developed ESRF, while two adult relatives (his father and paternal uncle) with the same variant in homozygosity are unaffected. Screening for other known aHUS-associated complement genes did not reveal rare or common variants that segregated with the disease. An *ex vivo* test showed excessive complement deposition on endothelial cells exposed to sera from the proband, and also from his mother and his maternal uncle, who do not carry the c.286+2T>G variant, indicating that the proband inherited a maternal circulating defect in complement regulation. These results highlight the complexity of the genetics of aHUS and indicate that CD46 deficiency may not be not enough to cause aHUS.

MATERIALS AND METHODS

Study Subjects

Four hundred eighty-five unrelated patients with a diagnosis of primary aHUS were recruited through the International Registry of HUS/TTP, under the coordination of the Aldo and

Cele Daccò Clinical Research Center for Rare Diseases (Ranica, Bergamo, Italy).

Clinical information and demographic and laboratory data for all patients and their available relatives were collected using a case report form. Biochemical and genetic tests were performed on blood, plasma, serum, and DNA samples collected from all aHUS patients and available relatives.

All participants received detailed information on the purpose and design of the study, according to the guidelines of the Declaration of Helsinki.

aHUS was diagnosed in all cases with microangiopathic hemolytic anemia and thrombocytopenia [defined as hematocrit (Ht) <30%, hemoglobin (Hb) <10 g/dl, serum lactate dehydrogenase (LDH) of >500 IU/L, undetectable haptoglobin, fragmented erythrocytes in the peripheral blood smear, and platelet count less of than 150,000/μl] associated with acute renal failure (serum creatinine >1.3 mg/dl for adults, >0.5 mg/dl for children under 5 years old and >0.8 mg/dl for children aged 5–10, and/or urinary protein–creatinine ratio >200 mg/g; or an increase in serum creatinine or urinary protein–creatinine ratio >15% compared with baseline levels). Thrombotic thrombocytopenic purpura was ruled out because all patients exhibited ADAMTS13 activity >10% and no anti-ADAMTS13 antibodies. Primary aHUS was defined in aHUS patients when both secondary underlying conditions and infections by Stx-*E.coli* were ruled out.

Ethnically matched healthy controls ($n = 319$) were also recruited from blood donors and were screened for *CD46* variants that we found in primary aHUS patients.

All participants provided informed written consent. The study protocol was approved by the Ethics Committee of the Azienda Sanitaria Locale, Bergamo, Italy.

Genetic Screening and Biochemical Testing

Genomic DNA was extracted from peripheral blood leukocytes (Nucleon BACC2 kit, Amersham; NucleoSpin Blood kit, Macherey-Nagel). All coding exons and the intronic flanking regions of membrane cofactor protein (*CD46*), complement factor H, (*CFH*), complement factor I (*CFI*), complement factor B (*CFB*), complement C3 (*C3*), and thrombomodulin (*THBD*) genes were amplified by polymerase chain reaction (PCR), using gene-specific primers and standard conditions. Amplification products were sequenced using standard Big-Dye Terminator v.1.1 protocols on 48-capillary 3730 DNA Analyzer. Sixty patients who were recruited more recently were analyzed using a home-made next-generation sequencing (NGS) diagnostic mini panel for simultaneous sequencing of the six complement genes reported above using a combination of multiplex PCR and high-throughput sequencing (PGM Ion Torrent, Life technologies). dbSNP, 1,000 genomes, ESP6500, and ExAC databases were used to distinguish new variants from those that had already reported. Ethnically matched healthy controls (n = 319) were screened for rare *CD46* variants found in non-Stx-HUS patients.

Genetic variants with a reported minor allelic frequency (MAF) below 0.001 in 1,000 Genomes and in the Exome Aggregation Consortium (ExAC), not found in 319 healthy controls and with a Combined Annotation Dependent Depletion (CADD) phred score $\geq$ 10 were considered rare variants (RVs). *CD46* RVs were further classified in "pathogenic," "likely pathogenic," "uncertain significance," "likely benign," or "benign" using the guidelines from the American College of Medical Genetics and Genomics (ACMG) (27) and Kidney Disease: Improving Global Outcomes (KDIGO) conference (4).

Once a RV was identified, the parents and relatives of the proband were invited for genetic testing to study the transmission model.

In patients carrying the *CD46* c.286+2T>G RV and their available relatives, we genotyped by direct sequencing the *CFH* single-nucleotide polymorphisms (SNPs) (c.1-331C>T, rs3753394; c.184G>A, p.V62I, rs800292; c.1204C>T, p.H402Y, rs1061170; c.2016A>G, p.Q672Q, rs3753396; c.2237-543G>A, rs1410996; c.2808G>T, p.E936D, rs1065489) that define the disease risk haplotype CFH_{TGTGGT} (known as *CFH–H3* haplotype) and one SNP in *CD46* (rs7144, c.*897T>C) that tags the risk $CD46_{GGAAC}$ haplotype.

All *CFHR5* exons and the three nucleotide differences in exon 4 of *CFHR1* (c.469, c.475, and c.523) that distinguish the *CFHR1*A* and *CFHR1*B* alleles were also genotyped in the proband and his relatives, using direct sequencing. The three differences cause three amino acid changes in SCR3 of FHR1 that make SCR3 of FHR1 identical to SCR18 of FH (28). The SCR3 with Tyr157, Val159, and Gln175 amino acids characterizes the basic isoform of FHR1 and is identical to the SCR18 of FH, indicating that the *CFHR1*B* allele could be the result of a gene conversion between *CFH* and *CFHR1*. The *CFHR1*B* allele has been found to be associated with aHUS patients carrying the homozygous *CFHR1*B* allele.

Multiplex ligation-dependent probe amplification (SALSA MLPA P236-A3 ARMD, MCR-Holland) was used to evaluate copy number variations in *CFH*, *CFHR3*, *CFHR1*, *CFHR2*, and *CFHR5* genes.

The mRNA extracted from peripheral blood mononuclear cells (PBMCs) from the proband and his relatives in family #646 was reverse transcripted using SuperScript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA, United States). cDNA amplification and sequencing were performed using a forward primer constructed on exon 1 (signal peptide) (5′-GCTTTCCTGGGTTGCTTCT) and a reverse primer constructed on exon 3 (SCR2) (5′-CATTTGCAGGGACTGCTTG).

Complement C3 and C4 serum levels were evaluated using kinetic nephelometry (14). Plasma SC5b-9 levels were evaluated with the MicroVue SC5b-9 Plus EIA commercial kit (SC5b-9 Plus, Quidel). The presence of anti-FH antibodies was evaluated using enzyme-linked immunosorbent assay (ELISA) (13).

CD46 Expression Studies on Peripheral Blood Mononuclear Cells

PBMCs were isolated from the peripheral blood of the proband, his relatives, and healthy volunteers by performing density gradient centrifugation using Ficoll-Paque. Fresh or thawed

PBMCs were labeled with anti-human CD3 APC-Cy7 or alternatively anti-human CD3 BV510 (clone SK7) and with antibody anti-human CD46 fluorescein isothiocyanate (FITC) (clone E4.3 that recognizes SCR1 epitope) or with antibody anti-human CD46 FITC (clone MEM258 that recognizes SCR4 epitope). Each experiment was performed using PBMCs from healthy volunteers labeled for CD3 and CD46 (SCR1) or CD3 and CD46 (SCR4) or unlabeled as negative controls (29). The gating strategy is reported in **Supplementary Figure 1**.

Samples were acquired with FACSAria or LSR-Fortessa X-20 cytofluorimeter (BD) and analyzed using FlowJo software (BD).

Samples were labeled with the VIABILITY dye probe to exclude dead cells from analysis 10 min before acquisition.

FMO (Fluorescence minus one) was used to analyze samples. Samples were gated in single cells, and T live cells were then analyzed for the specific expression of CD46 [as median fluorescence intensity (MFI)] for SCR1, or for SCR4. CD46 expression was indicated as MFI percentage compared with the control.

Complement Deposition on Human Microvascular Endothelial Cells

A human microvascular endothelial cell line of dermal origin (HMEC-1) was plated on glass slides and used when confluent. Cells were activated with 10 μM of adenosine 5′-diphosphate (ADP) for 10 min and then incubated for 4 h with serum diluted 1:2 with test medium [Hanks' Balanced Salt Solution (HBSS) with 0.5% bovine serum albumin (BSA)]. At the end of the incubation step, HMEC-1 were stained with FITC-conjugated rabbit anti-human C3c-complement or rabbit anti-human complement C5b-9 followed by FITC-conjugated secondary antibody. In each experiment, a pool of sera from healthy controls was tested parallel with the patient's serum. We verified the cellular integrity after exposure to serum samples in parallel slides in which HMEC-1 were stained with May-Grunwald Giemsa (30). A confocal inverted laser microscope was used for the acquisition of the fluorescent staining on the endothelial cell surface. Fifteen fields per sample were acquired, and the area with fluorescent staining was evaluated with automatic edge detection using built-in functions in the Image J software and expressed as pixel^2 per field analyzed. The fields with the lowest and highest values were excluded from calculation. Results were expressed as percentage of staining compared with control serum pool.

Statistical Analysis

MedCalc software was used for statistical tests. Fisher's exact test was used to compare the frequency of *CD46* RVs between aHUS patients and healthy control populations. Chi-square test or Fisher's exact test was used to calculate the risk of *CFH–H3* and $CD46_{GGAAC}$ haplotypes to increase the risk of developing aHUS. The results of C5b-9 deposition on activated HMEC were expressed as mean ± SE and analyzed by ANOVA.

P-values of less than 0.05 were considered statistically significant.

RESULTS

c.286+2T>G Is the Most Frequent *CD46* Rare Variant in Atypical Hemolytic Uremic Syndrome and Is Associated With Incomplete Penetrance

Screening of complement disease-associated genes in 485 unrelated patients with primary aHUS (herein defined aHUS) identified RVs in 189 cases. Specifically, 17% of patients carry *CFH* RVs; 7% *CFH–CFHR* rearrangements; 8% RVs in *CD46*; and 4, 8, 2, and 1% in *CFI*, *C3*, *CFB*, and *THBD* genes, respectively. In addition, in 10% of patients, we identified anti-FH antibodies.

We identified 15 *CD46* RVs in 39 patients. Of these, 20% ($n = 8$) exhibited additional RVs in *CD46* ($n = 3$) and/or in other complement genes ($n = 5$) (**Table 1**). Nonsense and frameshift RVs were found in 13 aHUS patients, whereas no non-sense or frameshift RVs were found in any of the 319 healthy controls (13/485 vs. 0/319; $P = 0.002$).

c.286+2T>G is the most frequent RV that we identified in aHUS patients of our Registry and is significantly overrepresented in patients compared with controls (patients 13/485 vs: healthy controls 0/319, $P = 0.002$; ExAC controls 4/60412, $P = 1 \times 10^{-6}$) (**Table 1**). Patients carrying this RV had an age of onset ranging from 1 to 58 years. Through the genetic analysis of seven pedigrees, we found 25 subjects carrying the c.286+2T>G variant. Given that only seven individuals in the above pedigrees developed the disease, the penetrance of aHUS in c.286+2T>G carriers is 28%.

Among the seven studied pedigrees, we further focused on the large family identified as #646 as a prototype of incomplete penetrance associated with the c.286+2T>G variant (**Figure 1**). In this pedigree, the proband was born from non-consanguineous, healthy parents. After an episode of acute gastroenteritis at 29.5 years of age, the man developed hypertension, jaundice, and thrombocytopenia (platelet count 25,000/μl) with borderline serum creatinine levels (1.2 mg/dl) and normal Hb levels (15 g/dl). The episode resolved spontaneously, platelet count normalized (293,000/μl), and Hb levels and renal function were normal (16.6 g/dl and 1.1 mg/dl, respectively) (**Figure 2**). At age 32, he was hospitalized with a fever and jaundice. Laboratory results revealed a low platelet count (12,000/μl, **Figure 2**), an increased LDH level (1,619 U/L), hyperbilirubinemia, fragmented red cells (schistocytes), and slightly elevated serum creatinine levels (1.36 mg/dl). A clinical diagnosis of aHUS was made. After 10 plasma exchanges and oral therapy with high doses of steroids (1 mg/kg), the hematologic features improved but renal function worsened (creatinine levels up to 2.9 mg/dl, **Figure 2**). The patient continued steroid therapy, leading to improvement in renal function (serum creatinine: 1.5 mg/dl) and an increase in platelet numbers (203,000/μl, **Figure 2**). After 3 months, serum creatinine was 4 mg/dl (**Figure 2**), and the patient underwent a kidney biopsy, which showed thrombotic microangiopathy with ischemic nephropathy. Two months later, he had an aHUS relapse with severe thrombocytopenia (44,000/μl) severe renal failure (serum creatinine 10.6 mg/dl, **Figure 2**) and lower than normal C3

TABLE 1 | List of *CD46* rare variants (RVs; variants with minor allele frequency < 0.001 in 1000 Genomes and ExAC databases and with CADD phred score ≥10) identified in 485 atypical hemolytic uremic syndrome (aHUS) patients recruited through the International Registry of HUS/TTP.

Exon	*CD46* rare variants	Patients ($n = 485$)	Ctrs ($n = 319$)	*P*-value	dbSNP rs	HGMD	1,000 K frequency	ExAC global frequency	Pathogenic in functional studies	CADD	Variant classification
Intron 1	c.98-1G>C (p.C35X)	4	0	0.16	rs1441937053	CS064376	NA	NA	Yes[1]	24.1	P
Exon2	c.104G>A (p.C35Y)	3[a]	0	0.28	rs121909591	CM062498	NA	8.26×10^{-6}	Yes[1]	24.5	LP
Exon2	c.175C>T (p.R59X)	6[b]	0	0.09	rs121909590	CM062495	NA	NA	Yes[1]	24.5	P
Exon2	c.192_198delinsC (p.C64fs)	1	0	1.00	NA	CX064751	NA	NA	NA	22.5	LP
Intron 2	c.286+2T>G	13[c]	0	*0.002*	rs769742294	CS066620	NA	3.3×10^{-5}	Yes[2]	23.7	P
Intron 2	c.287-2A>G	3[d]	0	0.28	rs759813089	CS064377	NA	3.1×10^{-5}	Yes[1]	23.9	LP
Exon3	c.295T>C (p.C99R)	1	0	1.00	NA	CM062496	NA	NA	Yes[1]	23.8	P
Exon3	c.307C>T (p.R103W)	1	0	1.00	rs1486782648	CM050660	NA	NA	Yes[3]	10.3	US
Intron 4	c.475+1_4delGTAA	1[e]	0	1.00	NA	CD136074	NA	NA	NA	16.8	NA
Exon 5	c.565T>G (p.Y189D)	4[f]	0	0.16	rs202071781	CM103418	1.99×10^{-4}	8.2×10^{-6}	Yes[2]	23.8	LP
Exon 5	c.648G>C (p.W216C)	1	0	1.00	NA	CM103419	NA	NA	NA	24.9	NA
Exon 6	c.685C>T (p.R229X)	1	0	1.00	rs1553251787	CM1516829	NA	NA	NA	35	LP
Exon 6	c.799-800delAC (p.T267fs)	1	0	1.00	NA	CD034158	NA	NA	Yes[4]	15.03	P
Exon 6	c.815_832delinsATT+ c.841C>A (p.272-276del+D277N+P278S)	1	0	1.00	NA	CX064752	NA	NA	Yes[1]	25.4	NA
Exon 6	c.725T>G (p.F242C)	1[g]	0	1.00	NA	CM062499	NA	NA	Yes[1]	22.3	P

Nucleotide CD46 numbering was used according to GenBank sequence NM_002389.4. Statistical analysis was performed using Fisher's exact test. Statistical significance: $P < 0.05$. Italic characters indicate statistically significant values.

HGMD, Human Gene Mutation Database; 1000 K, 1000 Genome Project; ExAC, Exome Aggregation Consortium; CADD, Combined Annotation Dependent Depletion; Variant Classification reported in the Database on complement gene variant (https://www.complement-db.org/home.php) based on guidelines from ACMG and Goodship et al. (4); P, pathogenic; LP, likely pathogenic; US, uncertain significance; NA, not available.

[a] *A patient (#F106) carries two additional RVs (CD46 p.R59X; FH p.R1210C).*

[b] *Two patients have combined complement RVs: patient #1075 also carries a THBD pathogenic variant (p.A43T); patient #F106 carries two additional RVs (CD46 p.C35Y; FH p.R1210C).*

[c] *Four patients have additional complement variants: patient #S978 also carries a FI (p.I357M) and an FH (p.R1210C) RVs; patient #1503 carries an additional RV in CD46 (p.Y189D). Patient #1873 also carries an FH RV (p.N516K). Patient #1793 also carries a FI RV (p.R317Q).*

[d] *A patient (#657) also carries a FI RV (p.L484Vfs3X).*

[e] *This patient (#1430) carries an additional CD46 RV (p.Y189D).*

[f] *Two patients (#1430 and #1503) also carry additional complement RVs (#1430: c.475+1_delGTAA; #1503: c.286+2T>G).*

[g] *This patient (#F169) also carries an FH RV (p.G1194D).*

[1] *Caprioli et al. (23).*

[2] *Fremeaux-Bacchi et al. (24).*

[3] *Fang et al. (31).*

[4] *Noris et al. (17).*

and C4 levels (44 and 4 mg/dl, respectively). He was treated with plasma exchange and steroid therapy and underwent hematological remission but developed ESRF requiring chronic hemodialysis. One year later, C3 and C4 serum levels were normal, Hb levels were borderline (between 11 and 11.9 g/dl), and the platelet count was normal (**Table 2**).

At age 37, the patient underwent a cadaveric renal transplantation with prophylactic plasma exchange to prevent post-transplant recurrence (**Figure 2**). The patient received an induction treatment with low doses of thymoglobulin and basiliximab, and maintenance immunosuppression with steroids, cyclosporine, and azathioprine. Hypertension was treated with atenolol and nifedipine. On day 9 post-transplant, the platelet count and Hb level were normal, and serum creatinine was 1.6 mg/dl. At the last follow-up, 5 years after transplantation, blood pressure was normal (136/72 mmHg), serum creatinine was 1.43 mg/dl, and the platelet count and Hb levels were within normal ranges, while proteinuria was absent (0.08 g/24 h).

The proband carries the *CD46* c.286+2T>G RV in heterozygosity. The family history is negative for aHUS and other kidney diseases. Genetic analysis for the c.286+2T>G variant was extended to 16 healthy relatives. As shown in **Figure 1** (pedigree #646), the c.286+2T>G variant is of paternal origin. Surprisingly, the proband's unaffected father (III-7) and one paternal aunt (III-6) are homozygous for the c.286+2T>G RV. Furthermore, six paternal relatives are healthy carriers of the same variant in heterozygosity (**Figure 1**).

Sequencing of other complement aHUS-associated genes (*CFH*, *CFI*, *C3*, *CFB*, *THBD*, and *CFHR5*) in the proband and his parents did not reveal additional RVs. The proband has a normal copy number of *CFH–CFHR* genes. The assay for anti-FH antibodies that was done before transplantation showed negative results.

We then investigated whether genetic modifiers previously associated with aHUS, including the *CFH–H3* and the

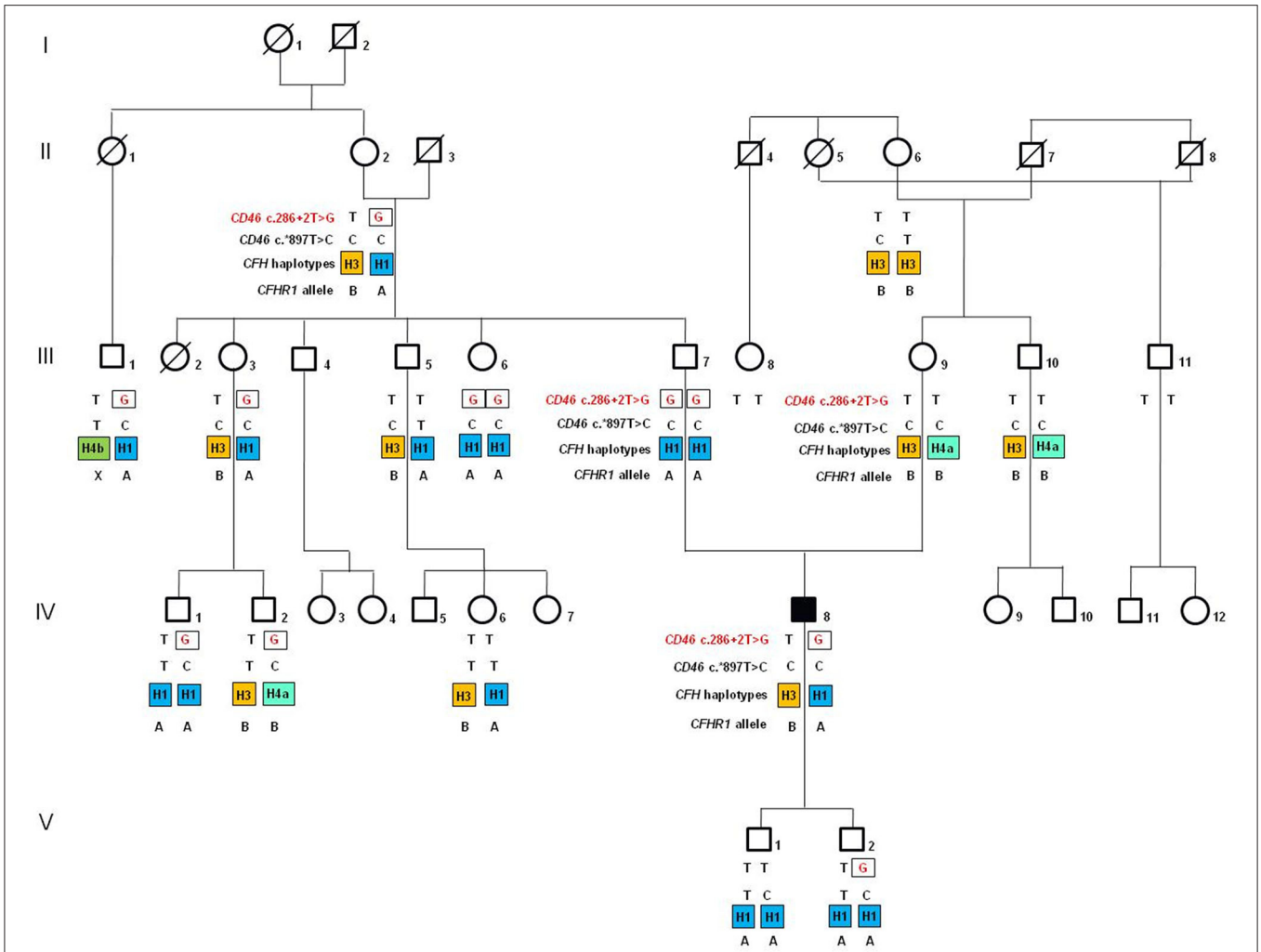

FIGURE 1 | Pedigree (#646) of the atypical hemolytic uremic syndrome (aHUS) patient (IV-8; indicated by the black square) carrying the heterozygous c.286+2T>G rare variant (rs769742294) in the gene encoding membrane cofactor protein (*CD46*). The *CD46* c.286+2G rare variant is indicated in red. The *CD46* c.*897T>C SNP (rs7144) tags the risk $CD46_{GGAAC}$ haplotype. In orange is indicated the disease risk haplotype CFH_{TGTGGT} (*CFH–H3* haplotype), while in blue and in green are indicated *CFH* haplotypes (CFH_{CGGAGG} or *CFH-H1*; CFH_{CGTAAG} or *CFH-H4a*; CFH_{TGTAAG} or *CFH-H4b*) not associated with aHUS. A indicates the *CFHR1*A* allele, which encode the acid (A) isoform of FHR1, and B indicates the *CFHR1*B* allele that is a risk factor for aHUS when present in homozygosity and encodes the basic (B) isoform of FHR1.

$CD46_{GGAAC}$ haplotypes (32–35), and the *CFHR1*B* allele (28) could account for disease penetrance in family #646. The proband is heterozygous for the *CFH* SNPs of the risk H3 haplotype (CFH_{TGTGGT}), which he inherited from his mother. In addition, he is homozygous for the c.*897T>C SNP (rs7144), which tags the $CD46_{GGAAC}$ risk haplotype and was inherited from both parents, who are homozygous for this SNP. Finally, he is heterozygous for the *CFHR1*B* allele (**Figure 1**).

Unlike the proband, his father does not carry the risk *CFH–H3* haplotype. Indeed, he is homozygous for the neutral *CFH-H1*, previously found with the same frequency in aHUS patients and in the control population (34, 36). In addition, the father does not carry the *CFHR1*B* allele. However, as shown in **Figure 1**, two unaffected adult paternal relatives who are heterozygous for c.286+2T>G RV (II-2, the paternal grandmother and III-3, a paternal aunt; 83 and 49 years old, respectively) share the same risk factors as the proband. Indeed, they have one copy of *CFH–H3*, two copies of the $CD46_{GGAAC}$, and one copy of the *CFHR1*B*. These data indicate that the presence of *CFH* and *CD46* risk haplotypes and *CFHR1*B* alleles is not enough to explain the disease in c.286+2T>G carriers.

We extended the analysis of *CFH* and *CD46* risk haplotypes to the other aHUS patients and their available unaffected relatives carrying the *CD46* c.286+2T>G RV. We found no significant association between the presence of *CFH–H3* ($P = 0.29$) or $CD46_{GGAAC}$ ($P = 0.12$) haplotypes and the disease (**Table 3**), confirming the results obtained in pedigree #646.

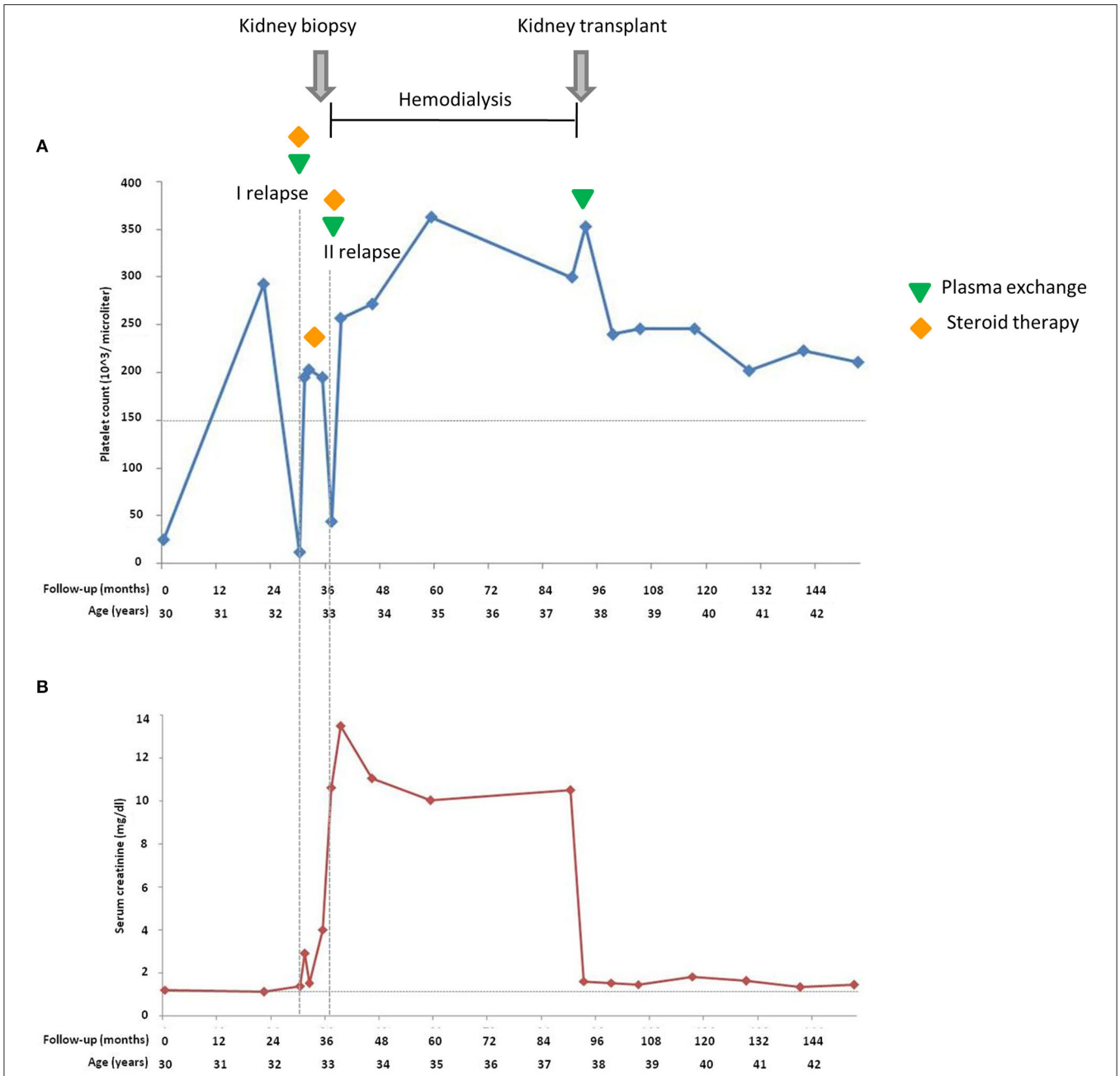

FIGURE 2 | Platelet count **(A)** and serum creatinine **(B)** of the proband during 153 months of follow-up from the atypical hemolytic uremic syndrome (aHUS) onset. *Horizontal dashed lines* in **(A)** indicate the lower limit of normal platelet count (150,000/μl); in **(B)** indicate the upper limit of the normal serum creatinine range. *Vertical dashed lines* highlight the two relapses.

In Pedigree #646, the *CD46* c.286+2T>G Rare Variant Causes the Formation of Two Abnormal mRNA Variants, Leading to Reduced Cell Surface Protein CD46 Expression

The c.286+2T>G RV is in the donor splice site of exon 2 of CD46 coding for short consensus repeat 1 (SCR1), which is one of the four extracellular complement regulatory domains of the protein. The variant has already been described as affecting exon 2 splicing (24, 25). In 2006, Fremeaux-Bacchi et al. (24) described that it results in aberrant mRNA that lacks 144 bp of exon 2 and encodes a protein that is missing 48 amino acids in the SCR1 domain in phase with the wild-type protein sequence. In 2010, Maga et al. (25) provided different results and showed that the c.286+2T>G RV leads to a frameshift at p.R96 (SCR2) of the

TABLE 2 | Clinical, complement, genetic, and CD46 expression data in the patient and his asymptomatic relatives.

				Genetic data	Clinical parameters				Complement parameters					CD46 protein expression[†]	
Disease status	**Subject**	**Gender**	**Age (years)[a]**	***CD46*: c.286+2T>G**	**Platelets ($\times 10^3/\mu l$)[b]**	**LDH (IU/L)[c]**	**Hb (g/dl)[d]**	**sCr (mg/dl)[e]**	**C3 (mg/dl)[f]**	**C4 (mg/dl)[g]**	**sC5b-9 (ng/ml)[h]**	**C3 deposits[i] (% of control)**	**C5b-9 deposits[i] (% of control)**	**With anti-SCR1 antibody (% of control)**	**With anti-SCR4 antibody (% of control)**
Affected	IV-8	M	34	TG	363	434	11.9	10.03	114	47	250.7	380	464	46	63
Unaffected	III-7	M	57	GG	204	392	14.6	0.83	nd	nd	136.2	135	104	7	15
Unaffected	III-9	F	52	TT	226	334	13.1	0.76	nd	nd	140.1	275	358	95	100
Unaffected	II-2	F	82	TG	209	454	13.4	1.13	nd	nd	nd	nd	nd	37	54
Unaffected	III-6	F	49	GG	277	376	12.4	0.56	nd	nd	nd	nd	nd	8	9
Unaffected	III-3	F	60	TG	151	401	13.4	0.81	nd	nd	nd	nd	nd	40	56
Unaffected	III-5	M	50	TT	151	437	14.1	0.81	nd	nd	nd	nd	nd	91	100
Unaffected	V-1	M	7	TT	215	nd	13.5	0.47	nd	nd	nd	nd	nd	97	nd
Unaffected	III-1	M	51	TG	245	482	16	0.76	nd	nd	211.5	nd	103	nd	nd
Unaffected	III-10	M	55	TG	nd	334	nd	1.08	nd	nd	nd	193	337	nd	nd

[a] *At this age, the proband was in hematological remission and chronic hemodialysis. Age of relatives refers to the data of the visit done at our center for genetic, clinical, and complement CD46 expression analyses. Abbreviations and limit of normal range.*

[b] *150–400 $\times$ $10^3/\mu l$.*

[c] *LDH, lactate dehydrogenase: 266–500 IU/L.*

[d] *Hb, hemoglobin: 14–18 g/dl (men), 12–16 g/dl (women).*

[e] *sCr, serum creatinine: 0.55–1.25 mg/dl.*

[f] *C3: 79–170 mg/dl.*

[g] *C4: 10–40 mg/dl.*

[h] *Normal plasma sC5b9 levels: ≤400 ng/ml.*

[i] *Expressed as percentage of control. Limit of normal range < 150%.*

[†] *CD46 protein levels in CD3+ positive peripheral blood mononuclear cells (PBMCs). Values are expressed as percentage median fluorescence intensity in control CD3+ PBMCs. Data are mean of results of 2/3 experiments.*

nd, not done.

TABLE 3 | Analysis showing that *CFH–H3* and $CD46_{GGAAC}$ haplotypes are not associated with *CD46* c.286+2T>G rare variant.

	***CFH–H3* haplotype**				***CD46* c.*897T>C**			
Status	**het**	**hom**	**No *CFH H3*-haplotype**	**Total subjects screened**	**TT**	**TC**	**CC**	**Total subjects screened**
Affected	3	3	3	9	0	1	10	11
Unaffected	7	1	5	13	0	6	7	13
			$P = 0.293$				$P = 0.124$	

*Patients and relatives carrying the CD46 c.286+2T>G rare variant were genotyped for CFH single-nucleotide polymorphisms (SNPs) and for the CD46 SNP (rs7144; c.*897T>C) tagging the* $CD46_{GGAAC}$ *haplotype.*

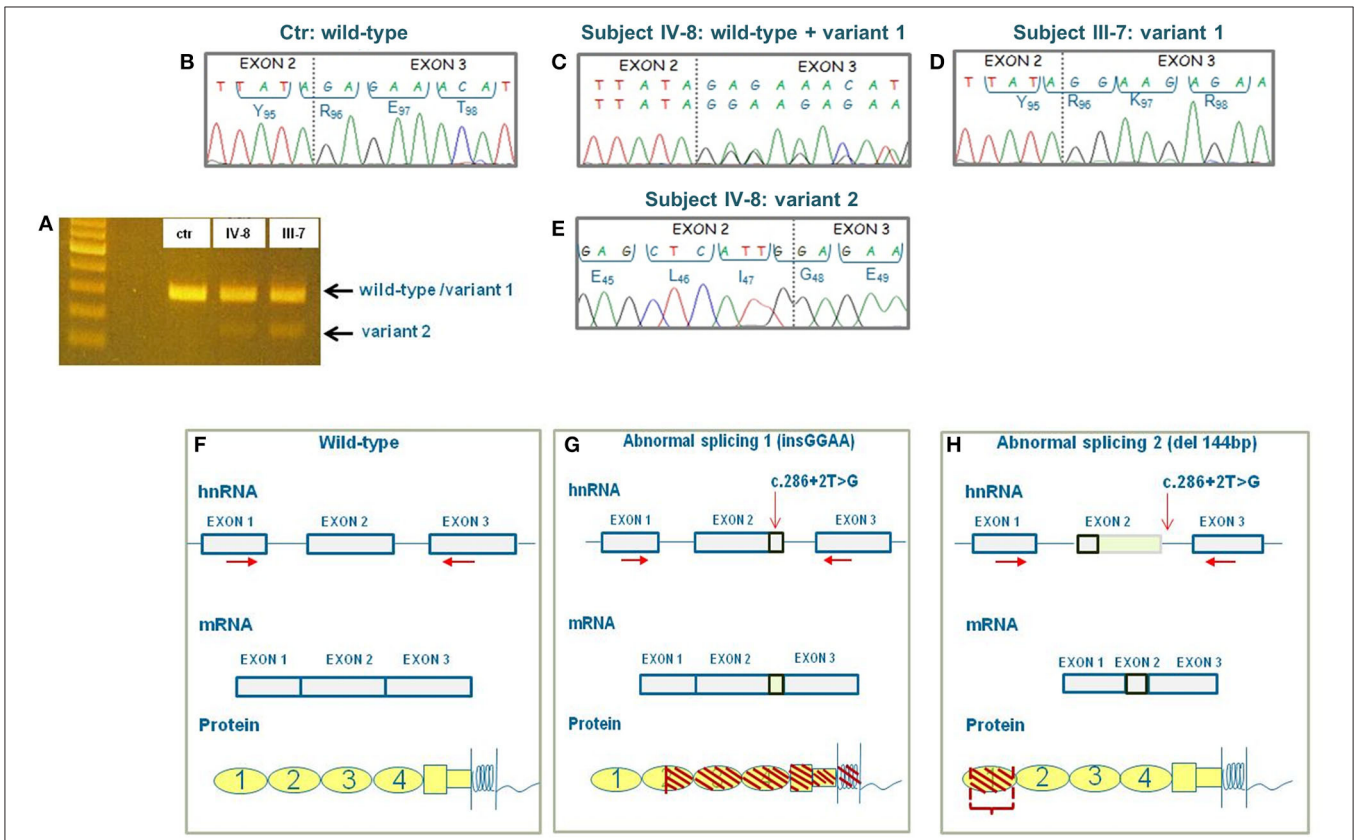

FIGURE 3 | Results of CD46 cDNA sequencing and prediction of the effect of the abnormal splicings at protein level. **(A)** Agarose gel image of cDNA showing a single band in the control (ctr) and two bands in the proband (IV-8) and in his healthy father (III-7). First lane: 100-bp ladder used as a size marker. **(B)** Electropherogram showing the wild-type cDNA sequence of the control. **(C)** Electropherogram of the upper band in the proband (subject IV-8) showing the wild-type sequence and the variant 1's sequence with 4-bp insertion. **(D)** Electropherogram of the upper band in the homozygous proband's father (subject III-7) showing the sequence of variant 1 only. **(E)** Electropherogram of the lower minor band in the proband showing the sequence of variant 2 with the deletion of 144 bp. **(F)** Wild-type CD46 heterogeneous nuclear RNA (hnRNA), messenger RNA (mRNA), and protein. **(G)** Representation of the effect of splicing variant 1 on CD46 hnRNA, mRNA and protein. The predicted protein is truncated in the SCR2 domain and includes only the first 128 amino acids. **(H)** Representation of the effect of splicing variant 2 on CD46 hnRNA, mRNA, and protein. The predicted protein lacks 48 amino acids of SCR1. Horizontal red arrows indicate the localization of primers used for cDNA sequencing. The vertical red arrow indicates the localization of c.286+2T>G variant.

protein, with the addition of several amino acids, followed by a premature truncation at position p.129X (SCR2).

To clarify the effect of the c.286+2T>G variant in pedigree #646 and to evaluate whether there was any difference in the abnormal splicing between the proband and the unaffected carriers, we extracted RNA from PBMCs from the proband and his relatives. cDNA amplification using the forward primer located on exon 1 and reverse primer located on exon 3 revealed two different bands on agarose gel electrophoresis in the proband and in all carriers (**Figure 3A**). Sequencing of the two bands revealed the wild-type sequence and two variants in the heterozygous proband, as well in the heterozygous healthy relatives. The upper band includes both the wild-type sequence and a second sequence (variant 1) with a 4-bp insertion, which

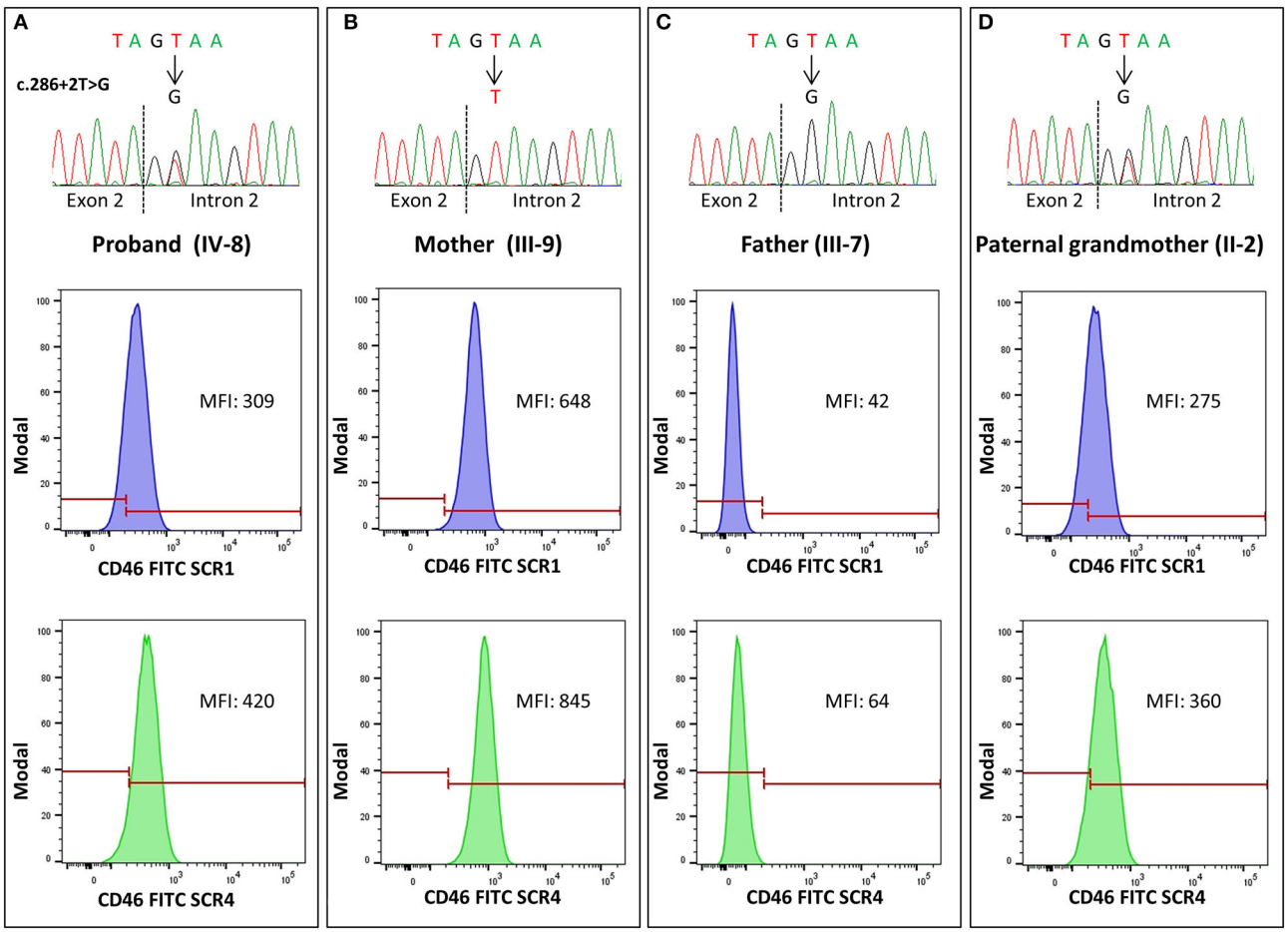

FIGURE 4 | Representative histograms of flow cytometry analysis in pedigree #646, performed with anti-SCR1 and anti-SCR4 antibodies showing levels of CD46 on CD3+ peripheral blood mononuclear cells (PBMCs), expressed as median fluorescence intensity (MFI). The heterozygous carriers of the 286+2T>G rare variant [the proband IV-8, **(A)**, and the paternal grandmother II-2, **(D)**] showed about 50% CD46 expression compared with the wild-type mother [subject III-9, **(B)**], while in the homozygous patient's father [subject III-7, **(C)**], a severe reduction of CD46 expression (<8% compared with the wild-type mother) was observed. The electropherograms of DNA sequences in the proband and his relatives are shown on the top of each panel.

corresponds to the variant described by Maga et al. (25) (control: **Figure 3B**, proband: **Figure 3C**). cDNA sequence analysis of the upper band from two homozygous relatives showed the variant 1 sequence, but not the wild-type sequence (**Figure 3D**). The sequence of the minor band (variant 2) corresponds to the splicing variant with the 144-bp deletion described by Frémeaux-Bacchi et al. (24) (**Figure 3E**). These data demonstrate that (1) the two aberrant mRNA splicing isoforms coexist both in the proband and in his unaffected carrier relatives; (2) variant 1 (with the 4-bp insertion) is the predominantly expressed variant both in heterozygous and homozygous carriers; and (3) in the homozygous unaffected carriers, no appreciable wild-type mRNA is transcribed. Thus, the characterization of abnormal RNAs and their proportion in the pedigree do not explain the incomplete penetrance of the c.286+2T>G RV in this pedigree.

The predicted effect of the two aberrant mRNA splicing isoforms on protein sequence in respect to wild-type protein is shown in **Figures 3F–H**. To investigate whether the c.286+2T>G RV affected the levels of CD46 protein on cell surfaces in the proband and in his unaffected carrier relatives differentially, we performed fluorescence-activated cell sorting (FACS) studies in peripheral blood leukocytes (**Table 2** and **Figure 4**). With the use of an anti-SCR1 antibody, the MFI on PBMCs from the proband was 46%, compared with that found on PBMCs from healthy controls (**Table 2**). Similar levels of MFI were observed on PBMCs from the heterozygous healthy carriers (37% in II-2 and 40% in III-3, **Table 2**). These results indicate that the c.286+2T>G RV severely impairs cell surface protein expression.

Impressively, the PBMCs from the two healthy homozygous relatives (the father, III-7 and the paternal aunt, III-6) exhibited <8% of normal CD46 expression (**Table 2**), demonstrating an almost complete CD46 deficiency.

Since the anti-SCR1 antibody cannot recognize the less abundant splicing variant 2 that lacks 48 amino acids in SCR1, we repeated the FACS analysis using an antibody targeting the

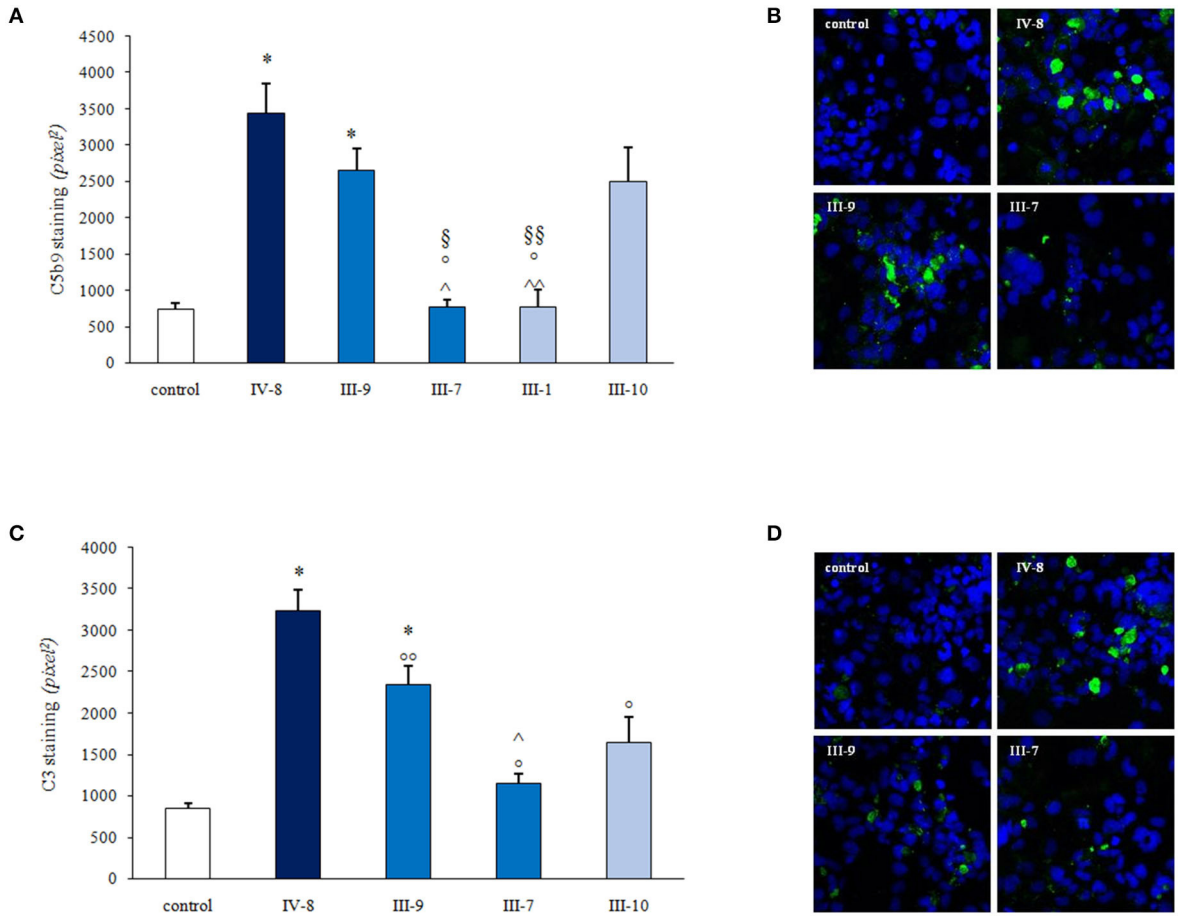

FIGURE 5 | Serum-induced C3 and C5b-9 deposition on human microvascular endothelial cell line (HMEC-1) activated with adenosine 5′-diphosphate (ADP). **(A,C)** Histograms show the quantification of C5b9 **(A)** or C3 **(C)** staining on activated HMEC-1 after 4 h of incubation with the serum from a healthy control (control), the patient (IV-8; heterozygous for *CD46* c.286+2T>G variant), patient's father (III-7; homozygous for *CD46* c.286+2T>G variant), the patient's mother (III-9; with no complement RVs), a paternal relative (III-1; heterozygous for *CD46* c.286+2T>G variant), and a maternal relative (III-10; with no complement RVs). Data are means ± SE; $^{*}P < 0.0001$ vs. control; $^{\circ}P < 0.0001$ vs. IV-8; $^{\circ\circ}P < 0.01$ vs IV-8; $^{\wedge}P < 0.0001$, $^{\wedge\wedge}P < 0.001$ vs. III-9; $^{\S}P < 0.01$, $^{\S\S}P < 0.05$ vs. III-10. Representative confocal microscopy images show strong C5b-9 **(B)** and C3 **(D)** staining (green) on activated H-MEC1 induced by serum from the patient (IV-8) and from his unaffected mother (III-9) but not by the serum from the proband's father (III-7; **B,D**). The blue color indicates the 4′-6-diamidino-2-phenylindole (DAPI) staining of cell nuclei.

SCR4 of CD46. CD46 surface expression was about 63% normal in the proband, 54–56% in the heterozygous healthy carriers, and 12% in the homozygous family members (**Table 2**).

Thus, with both antibodies, CD46 protein expression was similar and close to half-normal levels in the proband and the heterozygous carriers, whereas healthy homozygous relatives had an almost complete CD46 deficiency.

In the wild-type family members, the CD46 expression on PBMCs was similar to that observed in healthy controls with both anti-SCR1 and anti-SCR4 antibodies (**Table 2**).

Representative FACS histograms are shown in **Figure 4**.

In Pedigree #646, a Circulating Defect of Maternal Origin Causes Complement Dysregulation on Endothelial Cells

The complement system is constituted by over 50 components, and its activation is strictly regulated by circulating and membrane bound proteins to prevent injury to host cells. We hypothesized that a defect in a circulating factor resulting in complement dysregulation at the endothelial cell level could have synergized with *CD46* RV in inducing the disease in the proband. To address this issue, we used a specific *ex vivo* test in which serum from patients with aHUS, studied either during the acute phase or in remission, induces intense deposition of complement products on microvascular endothelial cells (HMEC-1) (37, 38). We exposed ADP-activated HMEC-1 to serum from the proband and from his relatives and evaluated the surface area covered by deposits of the terminal complement complex C5b-9. Serum from the proband (IV-8 in **Figure 5**), collected while he was in remission when he was on chronic hemodialysis, deposited significantly more C5b-9 on HMEC-1 than control serum (464% of C5b-9 deposits recorded with a pool of sera from healthy controls, **Figures 5A,B**). Similar results (589%) were obtained with serum collected 2 weeks after kidney transplantation.

Serum from the unaffected father (III-7), who is homozygous for the *CD46* RV, and serum from a heterozygous unaffected relative (III-1) did not increase endothelial C5b-9 deposits (**Figures 5A,B**). At variance, abnormally high C5b-9 deposits were induced on HMEC-1 by serum from the proband's mother (III-9; 358% of control sera) and from a maternal uncle (III-10; 356%) who do not carry the *CD46* RV (**Figures 5A,B**). These results indicate that the proband inherited from his mother an abnormality in a circulating factor that predisposes to complement activation on endothelial cells.

Similar results were obtained when we repeated the *ex vivo* test on activated HMEC-1 using an anti-C3c antibody to detect deposits of C3 activation products. Results showed that serum from the proband in remission induced significantly higher C3 deposits than the pool of control sera (380%) (**Figures 5C,D**). Elevated endothelial C3 deposits were also observed on cells incubated with sera from the proband's mother (III-9, 275%) and the maternal uncle (III-10, 193%) but not with the serum from the proband's father (III-7, 135%) (**Figures 5C,D**).

DISCUSSION

Through a retrospective analysis of a cohort of 485 aHUS patients, we performed genetic, molecular, and functional studies to investigate the determinants of penetrance of aHUS associated with genetic abnormalities in *CD46*. This gene encodes a transmembrane glycoprotein that is highly expressed in all tissues, on endothelial cells, and on all circulating cells with the exception of erythrocytes, which regulates both the alternative and classical complement pathways, acting as cofactor for FI to degrade C3b and C4b and to prevent C3 activation on cell surfaces (39).

In this study, *CD46* RVs were found in about 10% of aHUS patients. Consistent with data reported earlier by our group and others, aHUS-associated *CD46* RVs cluster in the extracellular domains of the protein, which are involved in cell surface complement regulation (21, 40).

We focused on the splicing variant c.286+2T>G, which is the most frequent *CD46* RV in our and other aHUS cohorts (21). The c.286+2T>G is also present in the general population, and the allele frequency of this variant in European non-Finnish population is 6.02×10^{-5}. If this allele were fully penetrant, it would have resulted in 0.012% of population developing aHUS. However, the reported prevalence of aHUS is far lower, ranging between two to ten per million population (0.0002–0.001%) (41), implying that the c.286+2T>G is not enough to develop the disease. Consistently, through the analysis of our pedigrees, we have found several unaffected carriers of the c.286+2T>G and that aHUS penetrance was lower than 30%.

As a prototype of incomplete penetrance associated with c.286+2T>G RV, we investigated in depth a large pedigree (#646) from our cohort with nine carriers, of whom only one was affected by aHUS. The proband, as well as six unaffected family members, is heterozygous for the c.286+2T>G RV, and surprisingly, the healthy proband's father and aunt were even homozygous for this variant. Bathia et al. (26) described the incomplete penetrance of the c.286+2T>G RV in a family with three homozygous siblings, two of whom developed aHUS at 5 and 8 years of age but one of whom was still asymptomatic at age 10. The authors' hypothesis that the latter may develop HUS at a later age (26) is not supported by findings in our pedigree, showing two adults who are homozygous for this RV and are asymptomatic at 66 and 58 years of age at present.

Altogether, these data indicate that genetic deficiency of CD46 due to pathogenetic RVs is not enough to induce aHUS and that other genetic risk factors are likely important for disease manifestation.

This is consistent with the present and other published (12) findings that show that around 25% of aHUS patients with RVs in *CD46* carry a second or third RV in other complement genes. Specifically, in 23% of patients with the c.286+2T>G variant, we found RVs in other known disease-associated genes. However, this is not the case for the large pedigree #646 described here, since the proband did not carry other RVs or anti-FH antibodies.

In addition to complement gene RVs, common genetic modifiers, such as the *CFH–H3* and $CD46_{GGAAC}$ haplotypes and the *CFHR1*B* allele, may influence susceptibility to aHUS (16, 32–34, 36). Indeed, the *CFH–H3* and $CD46_{GGAAC}$ haplotypes and the homozygous *CFHR1*B* allele have been reported more frequently in aHUS patients than in controls. It is significant that our group and others have shown that in some aHUS families the disease manifested only in individuals who inherited a complement RV from one parent and the *CFH* and/or *CD46* risk haplotype from the other parent (32, 34, 42). However, the above findings are not universally true, since in other families aHUS penetrance was still incomplete in carriers of complement RVs and both *CFH–H3* and $CD46_{GGAAC}$ risk haplotypes, suggesting that the synergic effect of these haplotypes may depend on the specific concurrent RV (12).

Consistently, here, we have found that the proband of the large pedigree with the 286+2T>G RV carries the *CFH–H3* risk haplotype, the homozygous $CD46_{GGAAC}$ haplotype, and one copy of the *CFHR1*B* allele, but this combination does not explain penetrance, since the same risk factors are present in unaffected relatives who are carriers of the c.286+2T>G RV. The finding that in the other pedigrees with the c.286+2T>G RV the *CFH–H3* and $CD46_{GGAAC}$ did not segregate with aHUS confirms that the above haplotypes do not have a significant effect on the risk of aHUS in *CD46* c.286+2T>G carriers.

c.286+2T>G is a splice variant and as such can affect mRNA processing and generate aberrant proteins with impaired structural and/or functional properties. However, different data on its effect have been reported. In one study, this RV was shown to cause an in-frame deletion generating a shorter CD46 protein that lacks part of SCR1 (24) and likely maintains a partial regulatory function. At variance, in another report, c.286+2T>G was shown to have a greater impact, resulting in the premature truncation of CD46 at SCR2 with the loss of all the other complement regulatory SCRs and of the transmembrane and intracellular domains (25). Thus, we wondered whether differences in the mRNA and protein products of the abnormal

splicing between the proband and the unaffected carriers could explain incomplete aHUS penetrance in our pedigree. However, this was not the case, since we found that the proband and all healthy carrier relatives express mRNAs encoding both the shorter and truncated CD46 variants and that the latter was prominent in all carriers. The finding that the proband and the heterozygous c.286+2T>G carriers had approximately half-normal CD46 levels on blood cells and homozygous carriers had an almost total CD46 deficiency confirmed at the protein level that the truncated variant was the main product of the c.286+2T>G RV. Severe CD46 protein deficiency was also reported in a patient with aHUS and his healthy sister, both of whom were homozygous for another RV, *CD46* c.286+1T>G, affecting the same splice site (43). Together, these findings indicate that CD46 deficiency is not enough to induce the aHUS phenotype.

As multiple complement regulatory proteins, including the transmembrane decay-accelerating factor (DAF), protectin (CD59), and plasma factor H are expressed or bind to endothelial cell surfaces, the endothelium can regulate complement even when gene abnormalities cause impaired activity of one of them (44). This is supported by studies in blood outgrowth endothelial cells showing that the inhibition of CD46 with a specific antibody followed by exposure to normal human serum as a source of complement did not cause C3 deposition on cell surfaces (45).

In search of additional abnormalities in the proband of our pedigree that could predispose him to aHUS, we used an *ex vivo* assay of serum-induced complement activation on a cultured endothelial cell line (HMEC-1) [34], based on evidence that in aHUS the microvascular endothelium is the main target of complement dysregulation (2). In previous studies, the assay with ADP-activated HMEC-1 allowed us to specifically pick up genetic complement abnormalities that affect circulating regulators both in aHUS patients and in their unaffected relatives (37).

The finding here that serum from the proband, and also sera from the mother and a maternal uncle, caused extensive C3 and C5b-9 deposits on activated HMEC-1 indicates that the proband inherited from his mother a genetic abnormality that causes complement activation on endothelial cells.

At variance, the unaffected paternal relatives carrying the c.286+2T>G *CD46* RV had normal serum-induced complement deposition on endothelial cells, which is an expected finding because CD46 is a surface complement regulatory protein and the endothelial cell line used in the assay expresses normal CD46. Thus, the proband has two main genetic predisposing factors, the c.286+2T>G *CD46* RV inherited from his father and a defect of maternal lineage that remains unknown and which, together with the *CFH–H3* and $CD46_{GGAAC}$ risk haplotypes, have synergized and induced aHUS. The finding that both the paternal relatives carrying the c.286+2T>G *CD46* variant and the maternal relatives with an abnormal complement deposition test are unaffected indicates that either genetic defect alone were not enough to induce aHUS.

Whole exome sequencing studies in this pedigree are required to identify the genetic defect inherited from the patient's mother, using segregation with the phenotype "elevated complement deposits" as criterion for selection of candidate variants.

Terminal complement activation associated with aHUS has been shown to activate platelets and neutrophils and to induce the formation of neutrophil-platelet aggregates (46, 47). Further *ex vivo* studies with platelets and blood leukocytes from the proband and his relatives could also contribute to clarify the complex pathogenetic mechanisms leading to aHUS in this pedigree.

aHUS patients with *CD46* RVs usually have a good prognosis. Although recurrences are frequent, spontaneous remissions are common and the long-term outcome appears to be good, with about 90% of patients maintaining normal renal function. The proband described here, however, had a severe disease course and developed ESRF, despite intensive plasma-exchange therapy. This phenotype closely resembles that of patients with combined RVs in *CD46* and other complement genes (14), thus supporting the hypothesis that the proband inherited an additional genetic defect from his mother. The observation that the proband did not experience HUS recurrence in the kidney graft and exhibits good a graft function at the 5-year follow-up after transplantation can be explained by the transplant correcting the *CD46* genetic defect, since endothelial cells within the kidney allograft express normal CD46. This finding also supports our interpretation that both the paternal CD46 variant and the unidentified maternal defect synergized to induce aHUS in this pedigree.

In conclusion, we demonstrate that *CD46* c.286+2T>G is a variant with a severe functional effect and also confirm that CD46 deficiency alone is not sufficient to develop aHUS, even though it is a strong risk factor.

The results from this study are consistent with earlier reports in the literature (33) that have documented the complexity of genetic abnormalities associated with aHUS, which range from highly penetrant RVs and genomic rearrangements that affect *CFH* to RVs that cause aHUS only in the presence of other RVs and/or risk haplotypes. Combining genetic studies with the *ex vivo* test of complement activation on endothelium may contribute to explaining the determinants of penetrance in this complex disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Azienda Sanitaria Locale, Bergamo, Italy. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RP, MN, and GR designed research, interpreted data, and wrote the paper. RP, PI, MT, SG, EV, MA, CM, MG, and PC performed the research and analyzed the data.

EB provided detailed clinical information of patients. AB analyzed the data and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.579418/full#supplementary-material

Supplementary Figure 1 | Gating strategy used to evaluate CD46 surface expression on lymphocytes by flow cytometry. Fresh or thawed PBMCs were labeled with anti-human CD3 antibody and with antibody anti-human CD46 FITC recognizing SCR1 epitope **(A)** or with antibody anti-human CD46 FITC recognizing SCR4 epitope **(B)**.

REFERENCES

1. Noris M, Remuzzi G. Hemolytic uremic syndrome. *J Am Soc Nephrol.* (2005) 16:1035–50. doi: 10.1681/ASN.2004100861
2. Noris M, Remuzzi G. Atypical hemolytic-uremic syndrome. *N Engl J Med.* (2009) 361:1676–87. doi: 10.1056/NEJMra0902814
3. Kaplan BS, Meyers KE, Schulman SL. The pathogenesis and treatment of hemolytic uremic syndrome. *J Am Soc Nephrol.* (1998) 9:1126–33.
4. Goodship TH, Cook HT, Fakhouri F, Fervenza FC, Fremeaux-Bacchi V, Kavanagh D, et al. Atypical hemolytic uremic syndrome and C3 glomerulopathy: conclusions from a "Kidney Disease: Improving Global Outcomes" (KDIGO) controversies conference. *Kidney Int.* (2017) 91:539–51. doi: 10.1016/j.kint.2016.10.005
5. Huemer M, Scholl-Burgi S, Hadaya K, Kern I, Beer R, Seppi K, et al. Three new cases of late-onset cblC defect and review of the literature illustrating when to consider inborn errors of metabolism beyond infancy. *Orphanet J Rare Dis.* (2014) 9:161. doi: 10.1186/s13023-014-0161-1
6. Lemaire M, Fremeaux-Bacchi V, Schaefer F, Choi M, Tang WH, Le Quintrec M, et al. Recessive mutations in DGKE cause atypical hemolytic-uremic syndrome. *Nat Genet.* (2013) 45:531–6. doi: 10.1038/ng.2590
7. Mele C, Lemaire M, Iatropoulos P, Piras R, Bresin E, Bettoni S, et al. Characterization of a new DGKE intronic mutation in genetically unsolved cases of familial atypical hemolytic uremic syndrome. *Clin J Am Soc Nephrol.* (2015) 10:1011–9. doi: 10.2215/CJN.08520814
8. George JN, Nester CM. Syndromes of thrombotic microangiopathy. *N Engl J Med.* (2014) 371:654–66. doi: 10.1056/NEJMra1312353
9. Bu F, Borsa N, Gianluigi A, Smith RJ. Familial atypical hemolytic uremic syndrome: a review of its genetic and clinical aspects. *Clin Dev Immunol.* (2012) 2012:370426. doi: 10.1155/2012/370426
10. Warwicker P, Goodship TH, Donne RL, Pirson Y, Nicholls A, Ward RM, et al. Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int.* (1998) 53:836–44. doi: 10.1111/j.1523-1755.1998.00824.x
11. Fakhouri F, Zuber J, Fremeaux-Bacchi V, Loirat C. Haemolytic uraemic syndrome. *Lancet.* (2017) 390:681–96. doi: 10.1016/S0140-6736(17)30062-4
12. Bresin E, Rurali E, Caprioli J, Sanchez-Corral P, Fremeaux-Bacchi V, Rodriguez de Cordoba S, et al. Combined complement gene mutations in atypical hemolytic uremic syndrome influence clinical phenotype. *J Am Soc Nephrol.* (2013) 24:475–86. doi: 10.1681/ASN.2012090884
13. Valoti E, Alberti M, Iatropoulos P, Piras R, Mele C, Breno M, et al. Rare functional variants in complement genes and anti-FH autoantibodies-Associated aHUS. *Front Immunol.* (2019) 10:853. doi: 10.3389/fimmu.2019.00853
14. Noris M, Caprioli J, Bresin E, Mossali C, Pianetti G, Gamba S, et al. Relative role of genetic complement abnormalities in sporadic and familial aHUS and their impact on clinical phenotype. *Clin J Am Soc Nephrol.* (2010) 5:1844–59. doi: 10.2215/CJN.02210310
15. Rodriguez de Cordoba S, Hidalgo MS, Pinto S, Tortajada A. Genetics of atypical hemolytic uremic syndrome (aHUS). *Semin Thromb Hemost.* (2014) 40:422–30. doi: 10.1055/s-0034-1375296
16. Arjona E, Huerta A, Goicoechea de Jorge E, Rodriguez de Cordoba S. The familial risk of developing atypical hemolytic uremic syndrome. *Blood.* (2020) 136:1558–61. doi: 10.1182/blood.2020006931
17. Noris M, Brioschi S, Caprioli J, Todeschini M, Bresin E, Porrati F, et al. Familial haemolytic uraemic syndrome and an MCP mutation. *Lancet.* (2003) 362:1542–7. doi: 10.1016/S0140-6736(03)14742-3
18. Richards A, Kathryn Liszewski M, Kavanagh D, Fang CJ, Moulton E, Fremeaux-Bacchi V, et al. Implications of the initial mutations in membrane cofactor protein (MCP; CD46) leading to atypical hemolytic uremic syndrome. *Mol Immunol.* (2007) 44:111–22. doi: 10.1016/j.molimm.2006.07.004
19. Nangaku M. Complement regulatory proteins in glomerular diseases. *Kidney Int.* (1998) 54:1419–28. doi: 10.1046/j.1523-1755.1998.00130.x
20. Schaefer F, Ardissino G, Ariceta G, Fakhouri F, Scully M, Isbel N, et al. Clinical and genetic predictors of atypical hemolytic uremic syndrome phenotype and outcome. *Kidney Int.* (2018) 94:408–18. doi: 10.1016/j.kint.2018.02.029
21. Osborne AJ, Breno M, Borsa NG, Bu F, Fremeaux-Bacchi V, Gale DP, et al. Statistical validation of rare complement variants provides insights into the molecular basis of atypical hemolytic uremic syndrome and C3 glomerulopathy. *J Immunol.* (2018) 200:2464–78. doi: 10.4049/jimmunol.1701695
22. Bu F, Maga T, Meyer NC, Wang K, Thomas CP, Nester CM, et al. Comprehensive genetic analysis of complement and coagulation genes in atypical hemolytic uremic syndrome. *J Am Soc Nephrol.* (2014) 25:55–64. doi: 10.1681/ASN.2013050453
23. Caprioli J, Noris M, Brioschi S, Pianetti G, Castelletti F, Bettinaglio P, et al. Genetics of HUS: the impact of MCP, CFH, and IF mutations on clinical presentation, response to treatment, and outcome. *Blood.* (2006) 108:1267–79. doi: 10.1182/blood-2005-10-007252

24. Fremeaux-Bacchi V, Moulton EA, Kavanagh D, Dragon-Durey MA, Blouin J, Caudy A, et al. Genetic and functional analyses of membrane cofactor protein (CD46) mutations in atypical hemolytic uremic syndrome. *J Am Soc Nephrol.* (2006) 17:2017–25. doi: 10.1681/ASN.2005101051
25. Maga TK, Nishimura CJ, Weaver AE, Frees KL, Smith RJ. Mutations in alternative pathway complement proteins in American patients with atypical hemolytic uremic syndrome. *Hum Mutat.* (2010) 31:E1445–60. doi: 10.1002/humu.21256
26. Bhatia D, Khandelwal P, Sinha A, Hari P, Cheong HI, Bagga A. Incomplete penetrance of CD46 mutation causing familial atypical hemolytic uremic syndrome. *Pediatr Nephrol.* (2015) 30:2215–20. doi: 10.1007/s00467-015-3189-0
27. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of medical genetics and genomics and the association for molecular pathology. *Genet Med.* (2015) 17:405–24. doi: 10.1038/gim.2015.30
28. Abarrategui-Garrido C, Martinez-Barricarte R, Lopez-Trascasa M, de Cordoba SR, Sanchez-Corral P. Characterization of complement factor H-related (CFHR) proteins in plasma reveals novel genetic variations of CFHR1 associated with atypical hemolytic uremic syndrome. *Blood.* (2009) 114:4261–71. doi: 10.1182/blood-2009-05-223834
29. Hsu EC, Dorig RE, Sarangi F, Marcil A, Iorio C, Richardson CD. Artificial mutations and natural variations in the CD46 molecules from human and monkey cells define regions important for measles virus binding. *J Virol.* (1997) 71:6144–54. doi: 10.1128/JVI.71.8.6144-6154.1997
30. Morigi M, Galbusera M, Binda E, Imberti B, Gastoldi S, Remuzzi A, et al. Verotoxin-1-induced up-regulation of adhesive molecules renders microvascular endothelial cells thrombogenic at high shear stress. *Blood.* (2001) 98:1828–35. doi: 10.1182/blood.V98.6.1828
31. Fang CJ, Fremeaux-Bacchi V, Liszewski MK, Pianetti G, Noris M, Goodship TH, et al. Membrane cofactor protein mutations in atypical hemolytic uremic syndrome (aHUS), fatal Stx-HUS, C3 glomerulonephritis, and the HELLP syndrome. *Blood.* (2008) 111:624–32. doi: 10.1182/blood-2007-04-084533
32. Caprioli J, Castelletti F, Bucchioni S, Bettinaglio P, Bresin E, Pianetti G, et al. Complement factor H mutations and gene polymorphisms in haemolytic uraemic syndrome: the C-257T, the A2089G and the G2881T polymorphisms are strongly associated with the disease. *Hum Mol Genet.* (2003) 12:3385–95. doi: 10.1093/hmg/ddg363
33. Esparza-Gordillo J, Goicoechea de Jorge E, Buil A, Carreras Berges L, Lopez-Trascasa M, Sanchez-Corral P, et al. Predisposition to atypical hemolytic uremic syndrome involves the concurrence of different susceptibility alleles in the regulators of complement activation gene cluster in 1q32. *Hum Mol Genet.* (2005) 14:703–12. doi: 10.1093/hmg/ddi066
34. Pickering MC, de Jorge EG, Martinez-Barricarte R, Recalde S, Garcia-Layana A, Rose KL, et al. Spontaneous hemolytic uremic syndrome triggered by complement factor H lacking surface recognition domains. *J Exp Med.* (2007) 204:1249–56. doi: 10.1084/jem.20070301
35. Fremeaux-Bacchi V, Kemp EJ, Goodship JA, Dragon-Durey MA, Strain L, Loirat C, et al. The development of atypical haemolytic-uraemic syndrome is influenced by susceptibility factors in factor H and membrane cofactor protein: evidence from two independent cohorts. *J Med Genet.* (2005) 42:852–6. doi: 10.1136/jmg.2005.030783
36. de Cordoba SR, de Jorge EG. Translational mini-review series on complement factor H: genetics and disease associations of human complement factor H. *Clin Exp Immunol.* (2008) 151:1–13. doi: 10.1111/j.1365-2249.2007.03552.x
37. Noris M, Galbusera M, Gastoldi S, Macor P, Banterla F, Bresin E, et al. Dynamics of complement activation in aHUS and how to monitor eculizumab therapy. *Blood.* (2014) 124:1715–26. doi: 10.1182/blood-2014-02-558296
38. Galbusera M, Noris M, Gastoldi S, Bresin E, Mele C, Breno M, et al. An *ex vivo* test of complement activation on endothelium for individualized eculizumab therapy in hemolytic uremic syndrome. *Am J Kidney Dis.* (2019) 74:56–72. doi: 10.1053/j.ajkd.2018.11.012
39. Liszewski MK, Leung M, Cui W, Subramanian VB, Parkinson J, Barlow PN, et al. Dissecting sites important for complement regulatory activity in membrane cofactor protein (MCP; CD46). *J Biol Chem.* (2000) 275:37692–701. doi: 10.1074/jbc.M004650200
40. Liszewski MK, Atkinson JP. Complement regulator CD46: genetic variants and disease associations. *Hum Genomics.* (2015) 9:7. doi: 10.1186/s40246-015-0029-z
41. Yan K, Desai K, Gullapalli L, Druyts E, Balijepalli C. Epidemiology of atypical hemolytic uremic syndrome: a systematic literature review. *Clin Epidemiol.* (2020) 12:295–305. doi: 10.2147/CLEP.S245642
42. Ermini L, Goodship TH, Strain L, Weale ME, Sacks SH, Cordell HJ, et al. Common genetic variants in complement genes other than CFH, CD46 and the CFHRs are not associated with aHUS. *Mol Immunol.* (2012) 49:640–8. doi: 10.1016/j.molimm.2011.11.003
43. Couzi L, Contin-Bordes C, Marliot F, Sarrat A, Grimal P, Moreau JF, et al. Inherited deficiency of membrane cofactor protein expression and varying manifestations of recurrent atypical hemolytic uremic syndrome in a sibling pair. *Am J Kidney Dis.* (2008) 52:e5–9. doi: 10.1053/j.ajkd.2008.02.359
44. Jokiranta TS, Zipfel PF, Fremeaux-Bacchi V, Taylor CM, Goodship TJ, Noris M. Where next with atypical hemolytic uremic syndrome? *Mol Immunol.* (2007) 44:3889–900. doi: 10.1016/j.molimm.2007.06.003
45. Noone DG, Riedl M, Pluthero FG, Bowman ML, Liszewski MK, Lu L, et al. Von Willebrand factor regulates complement on endothelial cells. *Kidney Int.* (2016) 90:123–34. doi: 10.1016/j.kint.2016.03.023
46. Noris M, Mescia F, Remuzzi G. STEC-HUS, atypical HUS and TTP are all diseases of complement activation. *Nat Rev Nephrol.* (2012) 8:622–33. doi: 10.1038/nrneph.2012.195
47. Riedl M, Noone DG, Khan MA, Pluthero FG, Kahr WHA, Palaniyar N, et al. Complement activation induces neutrophil adhesion and neutrophil-platelet aggregate formation on vascular endothelial cells. *Kidney Int Rep.* (2017) 2:66–75. doi: 10.1016/j.ekir.2016.08.015

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Immature Platelet Dynamics in Immune-Mediated Thrombocytopenic States

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A major challenge encountered by clinicians is differentiating presentations characterized by significant thrombocytopenia due to overlapping clinical symptoms and signs in the setting of ambiguous laboratory results. Immature platelets represent the youngest platelets that can be measured in peripheral blood by current hematology analyzers. These young platelets are larger, with higher RNA content recently released from the bone marrow. Thrombocytopenic presentations caused directly or indirectly by immune responses can lead to compensatory bone marrow responses seeking to normalize the platelet count; thus obtaining absolute immature platelet counts may be informative while triaging patients. Over the last decade, their use has expanded beyond being an early biomarker of bone marrow reconstitution post-hematopoietic stem cell transplantation to being used to establish bone marrow responses to infection and thrombocytopenias due to immune etiologies. Its accessibility as part of more detailed platelet indices obtained with routine laboratories makes it a promising option to understand the bone marrow's real-time response to disease states characterized by thrombocytopenia. This review will look at the immature platelet count as a biomarker, while presenting current attempts trying to understand how it could be used in thrombocytopenias occurring secondary to a given immune etiology.

Keywords: absolute immature platelet count, thrombotic thrombocytopenic purpura, immune thrombocytopenia, immature platelet fraction, immature platelets

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INTRODUCTION

Immunological processes that affect platelet production and/or platelet counts represent clinical challenges not only to diagnose but also to treat. Indeed, over the years a better understanding of some of these immune processes causing thrombocytopenia has led to more timely and targeted treatment approaches resulting in better outcomes. However, difficulties still remain when overlapping clinical pictures make a definitive diagnosis challenging. This can be exemplified by microangiopathic hemolytic anemia presentations where a given etiology may not be immediately apparent (1), and even in some instances when malignant states could be misdiagnosed as immune thrombocytopenia (ITP) (2). It is with this in mind that a growing body of literature describing potential alternative biomarkers lending support to a given diagnosis that results in timelier and etiology-specific interventions can be found. One of these markers, readily obtainable in modern automated hematology analyzers with fluorescence capability, is the immature platelet fraction

(%-IPF) and specifically the absolute immature platelet count (A-IPC). Regardless of how the acronyms are presented in the literature, such as designation of either immature or reticulated platelets, it has become more evident that this is an important variable when discerning processes leading to a thrombocytopenic state which has long been overlooked in clinical practice (3–8).

Immature platelets have been shown to be much larger, with higher RNA content, and more biochemically active than their mature counterparts (9). They can be affected by chemotherapy treatments and irradiation, and when immune reactions target platelets they can be elevated well above reference ranges (9, 10). They can be accurately measured in blood samples even 24 h after they have been collected (11), likely due to their increased longevity compared to mature platelets (9). Additionally, consumptive thrombocytopenic processes and those characterized by platelet hypoproduction can be readily identified looking at immature platelets (12). These counts can potentially point out if the thrombocytopenia-inducing etiology is either centrally (at bone marrow) or peripherally driven (5, 13). Likewise, immature platelets appear not to be affected by gender (14) or age since their production is preserved even in older individuals with lower platelet counts (15).

Nevertheless, tests that have the potential to be used clinically need to undergo scrutiny that at times may come into a collision course with the constrains of the technology behind them. The number of reports describing the utility of immature platelet counts has been mostly positive favoring it as a gauge to zero-in on a narrower group of potential etiologies. Yet, technological differences exemplified by changes in dyes with improved specificity for platelet elements, changes in gating, size correction, testing platform used, and wavelengths influence the analytical limits of this test (16–18); while those factors that are pre-clinical in nature such as timing of measurement with respect to specimen collection, degree of platelet activation, anticoagulant used, and quality of the sample (degree of hemolysis) may begin to explain the finite discrepancies that appear in the literature (16, 19–22).

Measurement of immature platelet counts requires establishing better defined reference intervals (16, 17). Importantly, reports describing that A-IPC changes precede those of their mature counterparts at times by 2–3 days may be an important observation when treating patients with thrombocytopenia (23–25). This is also the case in patients recovering from chemotherapy or stem cell transplantation in which immature platelets are first to return indicating that engraftment has occurred (23, 26, 27). This is undoubtedly of benefit to predict patients engrafting or for those recovering from their immune-consumptive processes. This may also help even those patients whose bone marrow fails to produce platelets in sufficient numbers to compensate for an existing thrombocytopenic state (3). Finally, research into their biology is likely to become easier thanks to the development of newer assays that immunostain, sort, and isolate immature platelets from the peripheral circulation (28, 29). Thus, this is the focus of this review to present the most current information of potential uses of immature platelets counts in clinical practice.

IMMATURE PLATELETS IN ITP

When the first data of changes in baseline %-IPF in the setting of ITP was reported, it raised the possibility that a real-time response by the bone marrow in this disease was possible to measure (30). Pathologically both higher platelet destruction that results in decreased platelet counts and potentially impaired thrombopoiesis influence risk of bleeding (31). Thus it should be of interest that reports appear to suggest that bone marrow attempts to compensate for platelet destruction by markedly increasing %-IPF to cope with the consumptive/destructive process (2, 8, 21, 30, 32–34); that these increases appear higher in those with chronic ITP (35); and that these dynamics may help risk stratify patients at risk of bleeding since they appear to have a higher preponderance of immature platelets (32, 36, 37). Notably, the magnitude of these compensatory increases in patients with ITP may be conveyed in a more consistent manner by looking at A-IPC at presentation (38).

However, few reports have found limited utility in using immature platelets to differentiate ITP where one hematology analyzer found some value to measuring it while the other had discrepant results (39). Regrettably, this study lacks A-IPC data analysis and determination if immature platelet counts were comparable with stratification according to platelet count (39). Despite this, recent data indicates that as ITP patients respond to treatment (40), specific changes in A-IPCs identify those with the disease (8). Based on these results, potentially helpful clinical scoring models that include immature platelet counts and favor ITP as a diagnosis have been proposed (2, 33), that take into consideration the high positive predictive value of these A-IPC changes (41). What adds to their potential usefulness is that in ITP, the higher immature platelet counts seen as patients recover from the disease (high turnover/destruction) precede by >3 days changes in mature platelet counts (25), similar to what has been described in other thrombocytopenic presentations. Thus, a model can be derived taking into account the published literature which favors a preserved and at times enhanced immature platelet/ mature platelet feedback (**Figure 1A**). In this model, as platelets get consumed or destroyed in the periphery, the bone marrow concomitantly increases its immature platelet output to compensate for platelet losses. Once platelet counts improve in response to disease remission and/or as a result of therapy, and platelet counts improve there is a corresponding decrease in immature platelet output as they return to baseline (10).

IMMATURE PLATELETS IN THROMBOTIC THROMBOCYTOPENIC PURPURA (TTP)

In the TTP literature, without a doubt the discovery that deficiency either innate or immune-mediated (acquired) of the metalloprotease ADAMTS13 is at the center of its pathology has changed the way in which patients are diagnosed and triaged (42, 43). This test, however, is sent-out to reference laboratories by most institutions often leading to initiation of therapeutic plasma exchange (TPE) prior to testing results becoming available. This is an important point to consider, since

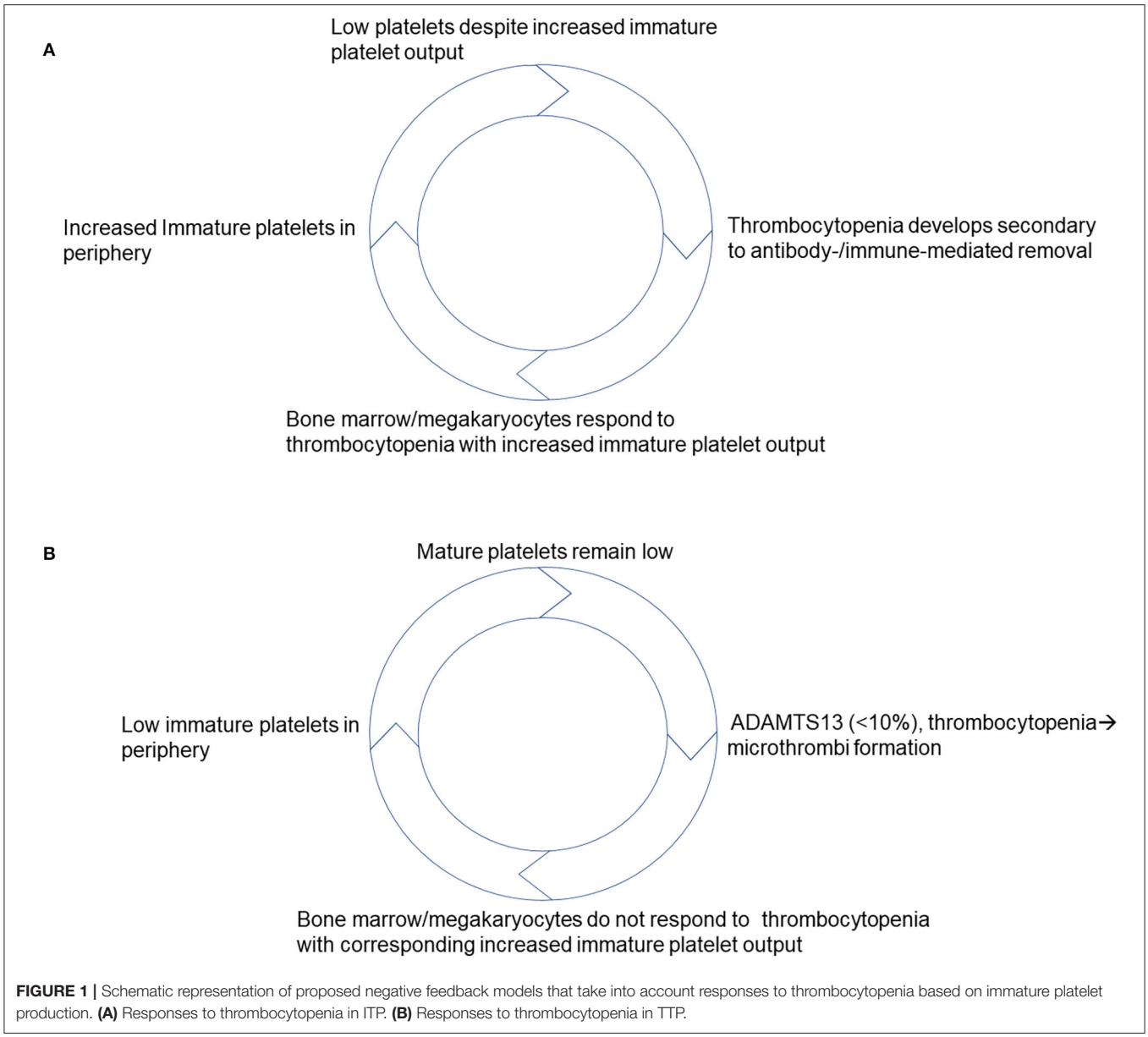

FIGURE 1 | Schematic representation of proposed negative feedback models that take into account responses to thrombocytopenia based on immature platelet production. **(A)** Responses to thrombocytopenia in ITP. **(B)** Responses to thrombocytopenia in TTP.

TPE is associated with higher adverse reactions when plasma is used as replacement fluid as required in TTP patients, making a timely accurate diagnosis paramount (44, 45). As a result, alternatives that increase the index of suspicion for TTP while not delaying prompt initiation of therapy could prove useful in such clinical settings. Early reports indicate that the immature platelet counts of TTP patients are much lower than those of ITP patients (30). Furthermore, recent data has shown that TTP patients (ADAMTS13 <10%) have A-IPCs at presentation that are markedly decreased compared to healthy controls that differentiates this group from other thrombocytopenic patients without the enzyme deficiency (24, 46, 47), and in some, such as refractory TTP cases, it may facilitate adjustments in therapy (48, 49). In TTP patients, improvement in A-IPC precedes that of mature platelet counts by about 2 days following initiation of TPE; and counts return to baseline once mature platelet counts stabilize at a normal level (24, 46, 50).

A-IPCs have been shown to predict response to TPE in patients with high ADAMTS13 inhibitors who may require longer treatment protocols to restore platelet counts (50). However, it remains to be determined if they would be helpful to assess responses of those TTP patients who are likely to require more TPE based on their ABO blood group (51). Patients with TTP as indicated by their immature platelet counts at presentation, appear to have a suppressed negative feedback i.e., rapidly relieved by TPE initiation (24, 46, 50). Based on this, we can propose a model in which an impaired immature platelet/ mature platelet negative feedback characterizes new onset TTP. This proposed model suggests that the bone marrow appears not to respond to the existing

thrombocytopenia with a corresponding increase in immature platelets until TPE is initiated (**Figure 1B**). Nonetheless, post TPE initiation A-IPC increases, preceding the mature platelet count changes restoring the negative feedback. Once mature platelet counts reach a normal level, A-IPC begins to decline back to baseline indicating that patients are on their way to recovery. This apparent suppressed A-IPC response implies that the feedback mechanisms at play between mature and immature platelets are disrupted in new onset TTP. This should be the focus of future research looking at the mechanisms mediating this observation to establish how disease precipitates in TTP patients.

IMMATURE PLATELETS DURING INFECTIOUS PROCESSES

Infectious processes may lead to decreases in mature platelets as the body fends off the infectious agent(s). Notably, even though thrombocytopenia can be seen in infections, A-IPCs are generally maintained so that at least platelet production attempts to keep up with the higher consumption (52); yet such A-IPC increases appear to correlate with higher mortality risk and disease severity in septic patients (53). These increases in immature platelets have been reported to occur earlier in patients prior to sepsis onset (54), which may be predictive of subsequent decreases in mature platelet counts once infection sets in Muronoi et al. (55). In this regard %-IPF has been reported as highly sensitive in identifying patients with sepsis regardless of extent of infection or severity (56, 57). This may relate to the significant immune hyperreactivity observed under states of severe infection leading to a disseminated platelet consumption requiring a higher immature platelet output. However, these increases may not be applicable to neonates where suppressed A-IPC characterizes those patients who did not survive disseminated infections (58). On the other hand, older children with dengue fever who recovered from the infection had increased immature platelet outputs up to 3 days prior to recovering their platelet count (59). Therefore, in an infectious presentation, the negative feedback between immature platelets and mature platelets appears generally preserved in older children and adults.

IMMATURE PLATELET COUNTS IN INFLAMMATORY SETTINGS

Inflammation-inducing disease processes that lead to impaired thrombopoiesis can be triaged looking at immature platelets as shown in patients with impaired liver function/cirrhosis (60). Similarly, states in which inflammation leads to platelet count changes can be ascertained looking at immature platelets. For example, higher counts of immature platelets in circulation may predict those patients at risk of subsequent inflammation post-cardiac surgery (61). Even 7 days after these surgical procedures a correlation between pro-inflammatory interleukin (IL)-6 and immature platelet counts has been reported, where the former is associated with the inflammation encountered by these patients (62). Potentially, these increases in immature platelets may be directly driven by IL-6 since this cytokine leads to thrombocytosis and platelet activation in intestinal inflammatory settings (63). However, the risk may be related to cardiovascular disease itself since human immunodeficiency virus (HIV) patients on antiretroviral therapy with cardiovascular disease have a higher number of immature platelets compared to HIV patients on therapy without cardiovascular disease (64).

Inflammation has also been shown associated with hypertension and this may lead the cardiovascular disease sequelae among other complications (65). For example, patients with malignant hypertension who present with thrombocytopenia have significantly higher immature platelet counts that are distinct from other microangiopathic hemolytic anemia processes including TTP (47, 66). Likely, sheer forces associated with hypertension lead to vascular damage and platelet consumption that result in a higher immature platelet output. Similarly, just as in other inflammatory processes it appears that the A-IPC allows for a better distinction between preeclampsia and those patients with hemolysis, elevated liver enzymes, and low platelet count syndrome (66). Along these lines, smoking causes vascular stenosis that lead to hypertensive complications and patients who are smokers have higher proportions of immature platelets (67). Paradoxically, some reports indicate that low grade inflammation may not provide enough of a stimulus to drive immature platelet production (68). Additional research is required to further characterize differences among these presentations.

IMMATURE PLATELETS AND DRUG-INDUCED PRESENTATIONS

Immature platelets have been used to establish when a given drug has no effect over thrombopoiesis (69). Antibody-mediated reactions to complexes that include platelet factor four are mediated by use of heparin. Since platelets are affected by the presence of antibody to the PF4-heparin complexes, this can result in changes to immature platelet output. Mild increases in %-IPF have been reported in samples tested during heparin-induced thrombocytopenia (HIT) investigations (70). However, recently it was shown that patients who test positive (HIT^+) for the presence of anti-PF4-heparin antibodies have A-IPC similar to the reference range unlike patients who test negative whose immature platelets are well-below this range (71). This implies that HIT^+ patients may have immature platelet responses that attempt to maintain the platelet count though not necessarily leading to an increase in net immature platelet production. Future investigation should expand upon these observations.

CONCLUDING REMARKS

Expansion over the last decade of the literature showing potential uses of immature platelet measurement in a variety of thrombocytopenic clinical settings (23, 38, 52, 71, 72),

represents a promising development that has evidently resulted in heightened interest on their use.

Yet, it must be acknowledged that these promising reports, favor the establishment of well-controlled clinical trials that look at how immature platelet counts could affect disease management without compromising therapy timing (73). Likewise, as mentioned earlier, there are still remaining limitations since different analyzers will require establishment of reference ranges prior to potential application, and as technology advances with newer analyzers becoming available with higher specificity and sensitivity these ranges will undoubtedly require revision. Despite these apparent shortcomings, counts below, at, or above a reference range may prove clinically informative when discerning the etiology behind a thrombocytopenic presentation.

In summary, immature platelets are equivalent to reticulocyte counts in the setting of anemia and thus provide valuable information to the clinician when treating thrombocytopenic patients. These immature platelet counts provide the nearest to real-time information of bone marrow response to the etiology causing the thrombocytopenia, with the added benefit that once therapy is initiated it can guide a clinician to determine when therapy is causing a net shift in the production of these young platelets.

AUTHOR CONTRIBUTIONS

HR and RM contributed to drafting, editing, and finalizing manuscript to its current form. Both authors contributed to the article and approved the submitted version.

REFERENCES

1. Bittencourt CE, Ha JP, Maitta RW. Re-examination of 30-day survival and relapse rates in patients with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. *PLoS ONE.* (2015) 10:e0127744. doi: 10.1371/journal.pone.0127744
2. Jeon MJ, Yu ES, Kang KW, Lee BH, Park Y, Lee SR, et al. Immature platelet fraction based diagnostic predictive scoring model for immune thrombocytopenia. *Korean J Intern Med.* (2020) 35:970–8. doi: 10.3904/kjim.2019.093
3. Li J, Li Y, Ouyang J, Zhang F, Liang C, Ye Z, et al. Immature platelet fraction related parameters in the differential diagnosis of thrombocytopenia. *Platelets.* (2019) 31:771–6. doi: 10.1080/09537104.2019.1678118
4. Benlachgar N, Doghmi K, Masrar A, Mahtat EM, Harmouche H, Tazi Mezalek Z. Immature platelets: a review of the available evidence. *Thromb Res.* (2020) 195:43–50. doi: 10.1016/j.thromres.2020.06.048
5. Van De Wyngaert Z, Fournier E, Bera E, Carrette M, Soenen V, Gauthier J, et al. Immature platelet fraction (IPF): a reliable tool to predict peripheral thrombocytopenia. *Curr Res Transl Med.* (2020) 68:37–42. doi: 10.1016/j.retram.2019.04.002
6. Pons I, Monteagudo M, Lucchetti G, Munoz L, Perea G, Colomina I, et al. Correlation between immature platelet fraction and reticulated platelets. Usefulness in the etiology diagnosis of thrombocytopenia. *Eur J Haematol.* (2010) 85:158–63. doi: 10.1111/j.1600-0609.2010.01468.x
7. Dusse LM, Freitas LG. Clinical applicability of reticulated platelets. *Clin Chim Acta.* (2015) 439:143–7. doi: 10.1016/j.cca.2014.10.024
8. Ali I, Graham C, Dempsey-Hibbert NC. Immature platelet fraction as a useful marker in the etiological determination of thrombocytopenia. *Exp Hematol.* (2019) 78:56–61. doi: 10.1016/j.exphem.2019.09.001
9. Hong H, Xiao W, Maitta RW. Steady increment of immature platelet fraction is suppressed by irradiation in single-donor platelet components during storage. *PLoS ONE.* (2014) 9:e85465. doi: 10.1371/journal.pone.0085465
10. Abe Y, Wada H, Tomatsu H, Sakaguchi A, Nishioka J, Yabu Y, et al. A simple technique to determine thrombopoiesis level using immature platelet fraction (IPF). *Thromb Res.* (2006) 118:463–9. doi: 10.1016/j.thromres.2005.09.007
11. Ruisi MM, Psaila B, Ward MJ, Villarica G, Bussel JB. Stability of measurement of the immature platelet fraction. *Am J Hematol.* (2010) 85:622–4. doi: 10.1002/ajh.21748
12. Barsam SJ, Psaila B, Forestier M, Page LK, Sloane PA, Geyer JT, et al. Platelet production and platelet destruction: assessing mechanisms of treatment effect in immune thrombocytopenia. *Blood.* (2011) 117:5723–32. doi: 10.1182/blood-2010-11-321398
13. Blandinieres A, Arlet JB, Gaussem P, Pouchot J, Darnige L. Usefulness of immature platelet fraction measurement for diagnosis and monitoring of iron deficiency associated thrombocytopenia: about two cases. *Ann Biol Clin.* (2020) 78:433–7. doi: 10.1684/abc.2020.1564
14. Negro F, Verdoia M, Tonon F, Nardin M, Kedhi E, De Luca G, et al. Impact of gender on immature platelet count and its relationship with coronary artery disease. *J Thromb Thrombolysis.* (2020) 49:511–21. doi: 10.1007/s11239-020-02080-0
15. Verdoia M, Nardin M, Negro F, Tonon F, Gioscia R, Rolla R, et al. Impact of aging on immature platelet count and its relationship with coronary artery disease. *Platelets.* (2020) 31:1060–8. doi: 10.1080/09537104.2020.1714572
16. Buttarello M, Mezzapelle G, Freguglia F, Plebani M. Reticulated platelets and immature platelet fraction: clinical applications and method limitations. *Int J Lab Hematol.* (2020) 42:363–70. doi: 10.1111/ijlh.13177
17. Wu MF, Hsieh HH, Huang WC, Lu KM, Lin JH, Lin YC, et al. Effects of analyzer detection method, ethnicity, and reference individuals on reference interval of immature platelet fraction. *Clin Lab.* (2018) 64:699–708. doi: 10.7754/Clin.Lab.2018.171025
18. Hoffmann JJ. Reticulated platelets: analytical aspects and clinical utility. *Clin Chem Lab Med.* (2014) 52:1107–17. doi: 10.1515/cclm-2014-0165
19. Wada A, Takagi Y, Kono M, Morikawa T. Accuracy of a new platelet count system (PLT-F) depends on the staining property of its reagents. *PLoS ONE.* (2015) 10:e0141311. doi: 10.1371/journal.pone.0141311
20. Hoffmann JJ, van den Broek NM, Curvers J. Reference intervals of reticulated platelets and other platelet parameters and their associations. *Arch Pathol Lab Med.* (2013) 137:1635–40. doi: 10.5858/arpa.2012-0624-OA
21. Schmoeller D, Picarelli MM, Paz Munhoz T, Poli de Figueiredo CE, Staub HL. Mean platelet volume and immature platelet fraction in autoimmune disorders. *Front Med.* (2017) 4:146. doi: 10.3389/fmed.2017.00146
22. Ibrahim H, Nadipalli S, Usmani S, DeLao T, Green L, Kleiman NS. Detection and quantification of circulating immature platelets: agreement between flow cytometric and automated detection. *J Thromb Thrombolysis.* (2016) 42:77–83. doi: 10.1007/s11239-016-1338-3
23. Have LW, Hasle H, Vestergaard EM, Kjaersgaard M. Absolute immature platelet count may predict imminent platelet recovery in thrombocytopenic children following chemotherapy. *Pediatr Blood Cancer.* (2013) 60:1198–203. doi: 10.1002/pbc.24484
24. Hong H, Xiao W, Stempak LM, Sandhaus LM, Maitta RW. Absolute immature platelet count dynamics in diagnosing and monitoring the clinical course of thrombotic thrombocytopenic purpura. *Transfusion.* (2015) 55:756–65. doi: 10.1111/trf.12912
25. Jeon K, Kim M, Lee J, Lee JS, Kim HS, Kang HJ, et al. Immature platelet fraction: a useful marker for identifying the cause of thrombocytopenia and predicting platelet recovery. *Medicine.* (2020) 99:e19096. doi: 10.1097/MD.0000000000019096
26. Yamaoka G, Kubota Y, Nomura T, Inage T, Arai T, Kitanaka A, et al. The immature platelet fraction is a useful marker for predicting the timing of platelet recovery in patients with cancer after chemotherapy and hematopoietic stem cell transplantation. *Int J Lab Hematol.* (2010) 32(6 Pt 1):e208–16. doi: 10.1111/j.1751-553X.2010.01232.x

27. Zucker ML, Murphy CA, Rachel JM, Martinez GA, Abhyankar S, McGuirk JP, et al. Immature platelet fraction as a predictor of platelet recovery following hematopoietic progenitor cell transplantation. *Lab Hematol.* (2006) 12:125–30. doi: 10.1532/LH96.06012
28. Bernlochner I, Klug M, Larasati D, Von Scheidt M, Santovito D, Hristov M, et al. Sorting and magnetic-based isolation of reticulated platelets from peripheral blood. *Platelets*. (2020) 1–7. doi: 10.1080/09537104.2020.1724923
29. Hille L, Cederqvist M, Hromek J, Stratz C, Trenk D, Nuhrenberg TG. Evaluation of an alternative staining method using SYTO 13 to determine reticulated platelets. *Thromb Haemost.* (2019) 119:779–85. doi: 10.1055/s-0039-1681101
30. Briggs C, Kunka S, Hart D, Oguni S, Machin SJ. Assessment of an immature platelet fraction (IPF) in peripheral thrombocytopenia. *Br J Haematol.* (2004) 126:93–9. doi: 10.1111/j.1365-2141.2004.04987.x
31. Rodeghiero F, Stasi R, Gernsheimer T, Michel M, Provan D, Arnold DM, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. *Blood.* (2009) 113:2386–93. doi: 10.1182/blood-2008-07-162503
32. Frelinger AL 3rd, Grace RF, Gerrits AJ, Berny-Lang MA, Brown T, Carmichael SL, et al. Platelet function tests, independent of platelet count, are associated with bleeding severity in ITP. *Blood.* (2015) 126:873–9. doi: 10.1182/blood-2015-02-628461
33. Kurata Y, Hayashi S, Kiyoi T, Kosugi S, Kashiwagi H, Honda S, et al. Diagnostic value of tests for reticulated platelets, plasma glycocalicin, and thrombopoietin levels for discriminating between hyperdestructive and hypoplastic thrombocytopenia. *Am J Clin Pathol.* (2001) 115:656–64. doi: 10.1309/RAW2-0LQW-8YTX-941V
34. Ferreira FLB, Colella MP, Medina SS, Costa-Lima C, Fiusa MML, Costa LNG, et al. Evaluation of the immature platelet fraction contribute to the differential diagnosis of hereditary, immune and other acquired thrombocytopenias. *Sci Rep.* (2017) 7:3355. doi: 10.1038/s41598-017-03668-y
35. Adly AA, Ragab IA, Ismail EA, Farahat MM. Evaluation of the immature platelet fraction in the diagnosis and prognosis of childhood immune thrombocytopenia. *Platelets.* (2015) 26:645–50. doi: 10.3109/09537104.2014.969220
36. McDonnell A, Bride KL, Lim D, Paessler M, Witmer CM, Lambert MP. Utility of the immature platelet fraction in pediatric immune thrombocytopenia: differentiating from bone marrow failure and predicting bleeding risk. *Pediatr Blood Cancer.* (2018) 65:e26812. doi: 10.1002/pbc.26812
37. Frelinger AL 3rd, Grace RF, Gerrits AJ, Carmichael SL, Forde EE, Michelson AD. Platelet function in ITP, independent of platelet count, is consistent over time and is associated with both current and subsequent bleeding severity. *Thromb Haemost.* (2018) 118:143–51. doi: 10.1160/TH17-06-0387
38. Greene LA, Chen S, Seery C, Imahiyerobo AM, Bussel JB. Beyond the platelet count: immature platelet fraction and thromboelastometry correlate with bleeding in patients with immune thrombocytopenia. *Br J Haematol.* (2014) 166:592–600. doi: 10.1111/bjh.12929
39. Cybulska A, Meintker L, Ringwald J, Krause SW. Measurements of immature platelets with haematology analysers are of limited value to separate immune thrombocytopenia from bone marrow failure. *Br J Haematol.* (2017) 177:612–9. doi: 10.1111/bjh.14628
40. Takami A, Mizuno S, Nakamura A, Kanasugi J, Yamamoto H, Vu Quang L, et al. Pretreatment immature platelet fraction as a surrogate of reticulated platelets predicts the response to corticosteroids in adults with immune thrombocytopenia. *Acta Haematol.* (2020) 1–5. doi: 10.1159/000510460
41. Sachs UJ. Diagnosing immune thrombocytopenia. *Hamostaseologie.* (2019) 39:250–8. doi: 10.1055/s-0039-1678739
42. Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, et al. von willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med.* (1998) 339:1578–84. doi: 10.1056/NEJM199811263392202
43. Tsai HM, Lian EC. Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med.* (1998) 339:1585–94. doi: 10.1056/NEJM199811263392203
44. Kundrapu S, Datla S, Griffin V, Maitta RW. Adverse events during apheresis: a 10-year experience at a tertiary academic medical center. *J Clin Apher.* (2019) 34:528–36. doi: 10.1002/jca.21706
45. Maitta RW. Current state of apheresis technology and its applications. *Transfus Apher Sci.* (2018) 57:606–13. doi: 10.1016/j.transci.2018.09.009
46. Stefaniuk CM, Reeves HM, Maitta RW. Dynamic changes in absolute immature platelet count suggest the presence of a coexisting immune process in the setting of thrombotic thrombocytopenic purpura. *Transfusion.* (2017) 57:913–8. doi: 10.1111/trf.13974
47. Zheng Y, Hong H, Reeves HM, Maitta RW. Absolute immature platelet count helps differentiate thrombotic thrombocytopenic purpura from hypertension-induced thrombotic microangiopathy. *Transfus Apher Sci.* (2014) 51:54–7. doi: 10.1016/j.transci.2014.07.004
48. Kier YE, Stempak LM, Maitta RW. Immature platelet fraction can help adjust therapy in refractory thrombotic microangiopathic hemolytic anemia cases. *Transfus Apher Sci.* (2013) 49:644–6. doi: 10.1016/j.transci.2013.07.005
49. Kundrapu S, Reeves HM, Maitta RW. Absolute immature platelet counts suggest platelet production suppression during complicated relapsing thrombotic thrombocytopenic purpura. *Acta Haematol.* (2020). doi: 10.1159/000510913. [Epub ahead of print].
50. Gokozan HN, Reeves HM, Maitta RW. Absolute immature platelet count dynamics of thrombotic thrombocytopenic purpura patients with high ADAMTS13 inhibitor. *Thromb Res.* (2019) 179:128–31. doi: 10.1016/j.thromres.2019.05.011
51. Behtaj M, Zhu ML, Bittencourt CE, Ha JP, Maitta RW. Non-O blood group thrombotic thrombocytopenic purpura patients take longer to recover as measured by number of therapeutic plasma exchanges needed for platelet recovery. *Thromb Res.* (2020) 185:78–84. doi: 10.1016/j.thromres.2019.11.022
52. Koyama K, Katayama S, Muronoi T, Tonai K, Goto Y, Koinuma T, et al. Time course of immature platelet count and its relation to thrombocytopenia and mortality in patients with sepsis. *PLoS ONE.* (2018) 13:e0192064. doi: 10.1371/journal.pone.0192064
53. Thorup CV, Christensen S, Hvas AM. Immature platelets as a predictor of disease severity and mortality in sepsis and septic shock: a systematic review. *Semin Thromb Hemost.* (2020) 46:320–7. doi: 10.1055/s-0039-3400256
54. Buoro S, Manenti B, Seghezzi M, Dominoni P, Barbui T, Ghirardi A, et al. Innovative haematological parameters for early diagnosis of sepsis in adult patients admitted in intensive care unit. *J Clin Pathol.* (2018) 71:330–5. doi: 10.1136/jclinpath-2017-204643
55. Muronoi T, Koyama K, Nunomiya S, Lefor AK, Wada M, Koinuma T, et al. Immature platelet fraction predicts coagulopathy-related platelet consumption and mortality in patients with sepsis. *Thromb Res.* (2016) 144:169–75. doi: 10.1016/j.thromres.2016.06.002
56. Park SH, Ha SO, Cho YU, Park CJ, Jang S, Hong SB. Immature platelet fraction in septic patients: clinical relevance of immature platelet fraction is limited to the sensitive and accurate discrimination of septic patients from non-septic patients, not to the discrimination of sepsis severity. *Ann Lab Med.* (2016) 36:1–8. doi: 10.3343/alm.2016.36.1.1
57. Enz Hubert RM, Rodrigues MV, Andreguetto BD, Santos TM, de Fatima Pereira Gilberti M, de Castro V, et al. Association of the immature platelet fraction with sepsis diagnosis and severity. *Sci Rep.* (2015) 5:8019. doi: 10.1038/srep08019
58. Cremer M, Weimann A, Szekessy D, Hammer H, Buhrer C, Dame C. Low immature platelet fraction suggests decreased megakaryopoiesis in neonates with sepsis or necrotizing enterocolitis. *J Perinatol.* (2013) 33:622–6. doi: 10.1038/jp.2013.21
59. Chuansumrit A, Apiwattanakul N, Sirachainan N, Paisooksantivatana K, Athipongarporn A, Tangbubpha N, et al. The use of immature platelet fraction to predict time to platelet recovery in patients with dengue infection. *Paediatr Int Child Health.* (2020) 40:124–8. doi: 10.1080/20469047.2019.1697574
60. Nomura T, Kubota Y, Kitanaka A, Kurokouchi K, Inage T, Saigo K, et al. Immature platelet fraction measurement in patients with chronic liver disease: a convenient marker for evaluating cirrhotic change. *Int J Lab Hematol.* (2010) 32:299–306. doi: 10.1111/j.1751-553X.2009.01184.x
61. Imperiali CE, Lopez-Delgado JC, Dastis-Arias M, Sanchez-Navarro L. Evaluation of the delta of immature platelet fraction as a predictive biomarker of inflammatory response after cardiac surgery. *J Clin Pathol.* (2019) 73:335–40. doi: 10.1136/jclinpath-2019-206068
62. Cavalca V, Rocca B, Veglia F, Petrucci G, Porro B, Myasoedova V, et al. On-pump cardiac surgery enhances platelet renewal and impairs aspirin

pharmacodynamics: effects of improved dosing regimens. *Clin Pharmacol Ther.* (2017) 102:849–58. doi: 10.1002/cpt.702
63. Senchenkova EY, Komoto S, Russell J, Almeida-Paula LD, Yan LS, Zhang S, et al. Interleukin-6 mediates the platelet abnormalities and thrombogenesis associated with experimental colitis. *Am J Pathol.* (2013) 183:173–81. doi: 10.1016/j.ajpath.2013.03.014
64. Goedel A, Muller S, Schwerdtfeger C, Zink A, Noe S, Bongiovanni D, et al. Influence of antiretroviral therapy and cardiovascular disease on the immature platelet fraction in patients living with HIV. *Platelets.* (2019) 31:756–62. doi: 10.1080/09537104.2019.1678114
65. Dale BL, Madhur MS. Linking inflammation and hypertension via LNK/SH2B3. *Curr Opin Nephrol Hypertens.* (2016) 25:87–93. doi: 10.1097/MNH.0000000000000196
66. Bernstein U, Kaiser T, Stepan H, Jank A. The immature platelet fraction in hypertensive disease during pregnancy. *Arch Gynecol Obstet.* (2019) 299:1537–43. doi: 10.1007/s00404-019-05102-2
67. Nardin M, Verdoia M, Negro F, Rolla R, Tonon F, De Luca G. Impact of active smoking on the immature platelet fraction and its relationship with the extent of coronary artery disease. *Eur J Clin Invest.* (2020) 50:e13181. doi: 10.1111/eci.13181
68. Larsen SB, Grove EL, Hvas AM, Kristensen SD. Platelet turnover in stable coronary artery disease - influence of thrombopoietin and low-grade inflammation. *PLoS ONE.* (2014) 9:e85566. doi: 10.1371/journal.pone.0085566
69. Kurahashi H, Takami A, Murotani K, Numoto S, Okumura A. Decreased platelet count in children with epilepsy treated with valproateand its relationship to the immature platelet fraction. *Int J Hematol.* (2018) 107:105–11. doi: 10.1007/s12185-017-2323-0
70. Meintker L, Haimerl M, Ringwald J, Krause SW. Measurement of immature platelets with abbott CD-sapphire and sysmex XE-5000 in haematology and oncology patients. *Clin Chem Lab Med.* (2013) 51:2125–31. doi: 10.1515/cclm-2013-0252
71. Chen W, Ha JP, Hong H, Maitta RW. Absolute immature platelet counts in the setting of suspected heparin-induced thrombocytopenia may predict anti-PF4-heparin immunoassay testing results. *Transfus Apher Sci.* (2018) 57:507–11. doi: 10.1016/j.transci.2018.04.001
72. Johnson S, Baker B. A CBC algorithm combined with immature platelet fraction is able to identify JAK2 V617F mutation-positive polycythaemia vera patients. *Int J Lab Hematol.* (2019) 41:271–6. doi: 10.1111/ijlh.12967
73. George MR, Creer MH. Advanced platelet parameters: underutilized tools in the diagnosis and management of thrombopoietic states? *Transfusion.* (2017) 57:874–5. doi: 10.1111/trf.14044

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frontiers in Medicine

BRIEF RESEARCH REPORT
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Diagnosis of Hereditary TTP Caused by Homozygosity for a Rare Complex ADAMTS13 Allele After Salmonella Infection in a 43-Year-Old Asylum Seeker

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A 43-year-old Armenian patient was diagnosed with salmonella infection and thrombotic microangiopathy (TMA). The clinical course was benign with resolution of all laboratory alterations after antibiotic treatment. Constantly deficient ADAMTS13 activity without ADAMTS13 inhibitors and evidence of homozygosity for a rare complex ADAMTS13 allele led to the diagnosis of congenital thrombotic thrombocytopenic purpura (cTTP). Half-life of ADAMTS13 after plasma infusion was calculated (27,6h) and double blinded plasma infusion as well as ergometric exercise with and without prior plasma infusion undertaken to investigate suspected smoldering TTP activity.

Keywords: cTTP, Upshaw Schulman syndrome, hereditary TTP, ADAMTS13, TMA

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Wendt R, Kalbitz S, Otto F, Falter T, Beige J, Rossmann H and Lämmle B (2021) Diagnosis of Hereditary TTP Caused by Homozygosity for a Rare Complex ADAMTS13 Allele After Salmonella Infection in a 43-Year-Old Asylum Seeker. Front. Med. 8:639441. doi: 10.3389/fmed.2021.639441

INTRODUCTION

Congenital or hereditary thrombotic thrombocytopenic purpura (cTTP) or Upshaw Schulman syndrome (USS, OMIM #274150) is a very rare disorder characterized by severe ADAMTS13 deficiency. In the vast majority TTP is caused by autoantibodies toward ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13) with consecutive ADAMTS13 deficiency. In cTTP, ADAMTS13 deficiency is due to mutations in the *ADAMTS13* gene. It is inherited as an autosomal recessive trait and accounts for <10% of all TTP cases. ADAMTS13 protease deficiency leads to persistence of ultra large von Willebrand factor multimers and eventually to acute thrombotic microangiopathy (TMA). Most cTTP cases are diagnosed in childhood or early adolescence after a first acute TTP episode. Here, we present an unusual case of late-onset cTTP occurring after salmonella infection at the age of 43 years caused by homozygosity for a rare, complex ADAMTS13 allele.

CASE PRESENTATION

A 43-year-old person of Armenian ancestry presented to our hospital due to fever and severe diarrhea. This patient was an asylum-seeking refugee in Germany, who just recently had a barbecue. Salmonella enteritidis enterocolitis was diagnosed with associated acute kidney injury AKIN I. There was severe thrombocytopenia (14 GPT/l) and signs of hemolysis (elevated lactate dehydrogenase level, low haptoglobin) (**Figure 1**). The initial differential diagnosis of the treating colleagues at the Infectiology department was Shiga toxin-producing E. coli induced hemolytic-uremic syndrome (STEC-HUS) due to the presentation with diarrhea. Based on the result of a stool culture revealing Salmonella enterica the attending physician suspected a Salmonella-induced secondary HUS. The patient received supportive treatment (volume replacement with balanced electrolyte solutions) and antibiotic treatment for salmonella (azithromycin). Platelet count normalized within 6 days, renal function completely normalized, and all other laboratory abnormalities were also resolved (**Figure 1**). Due to diagnosis of latent tuberculosis (based on positive QuantiFERON-test without evidence of active tuberculosis) the patient was treated with isoniazid for nine months.

The patient received neither plasma products nor plasma exchange at any time point during hospitalization.

The diagnosis of TTP was made after consulting with the nephrologist and after normalization of laboratory findings and resolution of the clinical symptoms. There was no measurable ADAMTS13 activity or ADAMTS13 antigen during the acute phase of the disease and no evidence of anti-ADAMTS13 IgG antibodies. ADAMTS13 deficiency was shown to be persistent during the follow-up (**Table 1**). Because of suspected cTTP, a genetic analysis of *ADAMTS13* gene was performed.

RESULTS

Family History

The parents of our patient had died at the age of 78 years (mother, unknown cause) and 75 years (father, "stroke"). The patient has 2 brothers (52 and 56 years old) and five sisters (46, 51, 55, 57, and 62 years of age), who are refugees (currently in Russia) and unfortunately not accessible for us despite all efforts. Another brother had died at the age of 22 years from suspected stroke. Two sisters are known to have heart problems (no objective data available). According to our patient there was no other sibling with any severe disorder.

Laboratory Values During the Disease Course and Diagnosis of cTTP

The patient has been subjected to a series of tests in order to clarify his symptoms and to optimize his clinical management.

The course of the most significant laboratory analyses are depicted in **Table 1**. No plasma products were given and no plasmapheresis was performed. The only treatment was oral antibiotic therapy of salmonella infection and intravenous volume replacement.

Repeated analyses of ADAMTS13 activity and inhibitor or antibodies, respectively, showed no measurable protease activity and no evidence for ADAMTS13 antibodies at any time.

Genetic Analysis

Sanger sequencing of the *ADAMTS13* (OMIM: *604134; RefSeq: NM_139025.4) exons and splice sites was performed on a CEQ 8000 Genetic Analysis System (Sciex, Darmstadt, Germany). In exon 19 the variant c.2351G>A, p.Arg784Gln (dbSNP rs377187626), in exon 22 the variant c.2746C>T, p.Arg916Cys (dbSNP rs374444423) were detected, both in

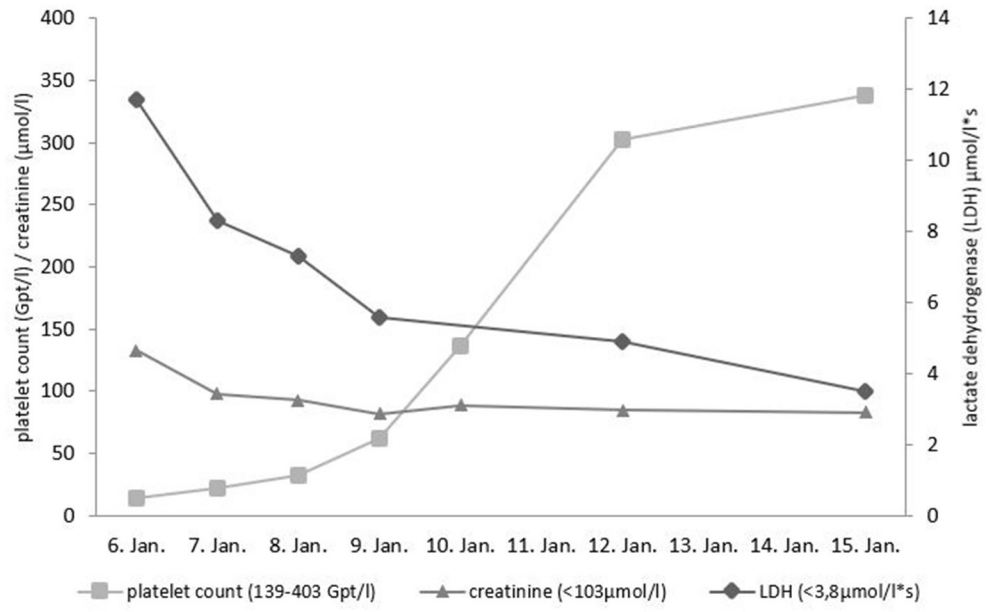

FIGURE 1 | Course of platelet count, lactate dehydrogenase (LDH) and creatinine during initial presentation. Reference ranges given in parentheses.

TABLE 1 | ADAMTS13 activity and inhibitor at presentation and during follow-up.

	09.01.2018	15.03.2018	15.10.2018	01.11.2018
ADAMTS13 activity, % (40–130)	<3.0*	<3.0*	<1.0**	<1.0**
ADAMTS13 concentration, μg/ml (0.31−0.82)	<0.01*			
ADAMTS13 inhibitor, Bethesda Units (<0.5)			0**	0**
ADAMTS13 IgG antibodies, U/ml (<12)	1.1*			

*Values measured at Institute of Transfusion Medicine and Clinical Hemostasiology, St. Georg Hospital Leipzig, Germany (Technozym® ADAMTS13 ELISA Kit and TECHNOZYM ADAMTS13 INH, measuring anti-ADAMTS13 antibodies by ELISA) **ADAMTS13 activity and functional inhibitor measured by in house assay using the FRETS-VWF73 substrate (1, 2) at the Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center Mainz, Germany.

homozygosity. A large deletion of the *ADAMTS13* gene was excluded by NGS (Next Generation Sequencing): Target enrichment was achieved by in solution custom probe hybridization (IDT, Leuven, Belgium). Sequencing was performed by a MiSeq instrument (Illumina, Berlin, Germany) confirming homozygosity for the complex allele c.[2351G>A;2746C>T] p.[Arg784Gln;Arg916Cys].

The patient's son was found to be heterozygous for c.[2351G>A;2746C>T] p.[Arg784Gln;Arg916Cys] and had an ADAMTS13 activity of 48.6%.

Plasma Infusion Trial

To further confirm cTTP we calculated plasma half-life of ADAMTS13 after donor plasma infusion (**Figure 2**). The single time infusion volume was 9 ml/kg bodyweight (BW) fresh frozen plasma (FFP) with a pooled ADAMTS13 activity of 85%. T_0 refers to the time at which the plasma volume was fully infused (2 h after start of the infusion). ADAMTS13 activity at T_0 was 15.7%. Based on the predicted plasma volume of 3.72 liters, we calculated a maximum ADAMTS13 activity of 18.6%. This value corresponds well to the extrapolated protease activity of 17.1% at 1 h after plasma infusion. Additional blood samples for ADAMTS13 activity were drawn at 2, 24, 48, 72, and 144 h after end of the infusion. A fast initial decline in ADAMTS13 activity was observed between 0 and 2 h, which afterwards changed into a first order elimination kinetics. Therefore, we calculated the ADAMTS13 activity half-life as 27.6 h (95% confidence interval: 19.9–45.12 h) according to a 2-phase elimination kinetics (3).

Investigation of Smoldering Clinical TTP Manifestations

Maximal Bicycle Exercise Testing With and Without Prior Plasma Infusion

Usual bicycle exercise testing (ergometry) was performed with blood samples taken at the following timepoints: start of ergometry, 5 min after start, at the end of exercise and 60 min after start of exercise (**Figure 3**). The first ergometry testing was performed without prior plasma infusion. Exercise was started at 50 watts and increased every 2 min by 25 watts. After almost 11 min and at 150 watts, the ergometric exercise was stopped due to patient exhaustion. 48 h later a second ergometry was performed after prior infusion of 525 ml fresh frozen plasma. Laboratory assays at the different time points of exercise testing are shown in **Supplementary Table 1**.

Double Blinded Infusion Trial (Crystalloids Vs. Fresh Frozen Plasma)

On the day of our blinded infusion trial, the patient repeatedly reported thoracic (6/10), abdominal (5/10) und back pain (8/10), quantified on a 10-point visual analog pain scale (VAPS). We blinded the patient, the treating physicians and the nurses by using aluminum foils for the infusion lines and infusion fluids (**Supplementary Figure 1**). Infusion time was set to 2 h. We started with two parallel infusions of 500 ml each of balanced electrolyte fluids (E153), followed by two parallel infusions of 250 ml each of fresh frozen plasma.

After the infusion of the balanced electrolyte solution, thoracic pain (starting from 6/10 on the VAPS) and abdominal pain (starting from 5/10 on the VAPS) completely vanished (both 1/10 VAPS), but back pain persisted at 8/10 on the VAPS (**Supplementary Table 2**). After plasma infusion the back pain was almost completely gone (2/10 VAPS).

DISCUSSION

Congenital TTP (or Upshaw-Schulman syndrome) is a very rare, autosomal recessive disease, which is caused by homozygous or compound heterozygous *ADAMTS13* mutations (4–14). The diagnosis is confirmed by evidence of a persistent severe ADAMTS13 deficiency (<10%), absence of an ADAMTS13 inhibitor and presence of biallelic mutations of *ADAMTS13*. There seems to be no clear genotype-phenotype relationship (4). Subjects, e.g., parents, heterozygous for an *ADAMTS13* mutation are unaffected, also under conditions that can precipitate TTP episodes. The son of our patient was heterozygous for the patient's mutation and showed ADAMTS13 activity of about 50%, which seems sufficient to prevent acute TTP episodes.

The patient is homozygous for the complex allele c.[2351G>A;2746C>T] p.[Arg784Gln;Arg916Cys]. Both variants, Arg784Gln in Exon 19 (dbSNP: rs377187626) and Arg916Cys in Exon 22 (dbSNP: rs374444423) are very rare (minor allele frequency of both <0.00008 [dbSNP]).

Though analysis by bioinformatic tools is inconsistent (Mutation Taster: polymorphism; PolyPhen2: probably damaging; SIFT: tolerated; PROVEAN: deleterious), a biological consequence of c.2746C>T, p.Arg916Cys is plausible as it creates an unpaired cysteine. More importantly, it was reported in compound heterozygosity with a further *ADAMTS13* mutation in several Upshaw-Schulman syndrome (USS) patients (15–17). Furthermore, a poster presented by Russo et al. (18), reported an USS patient with homozygosity for the same complex allele as found in the patient described here.

In summary, we consider the complex allele Arg784Gln; Arg916Cys to be the cause of our patient's disease, but it remains unclear whether Arg784Gln, which is classified as benign by the

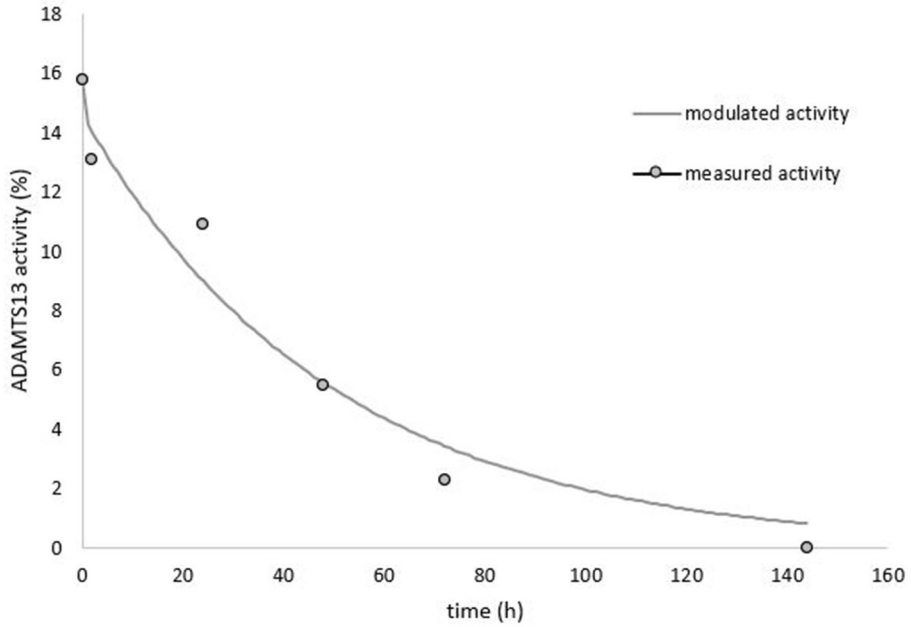

FIGURE 2 | ADAMTS13 activity measured over time after 9 ml/kg plasma infusion.

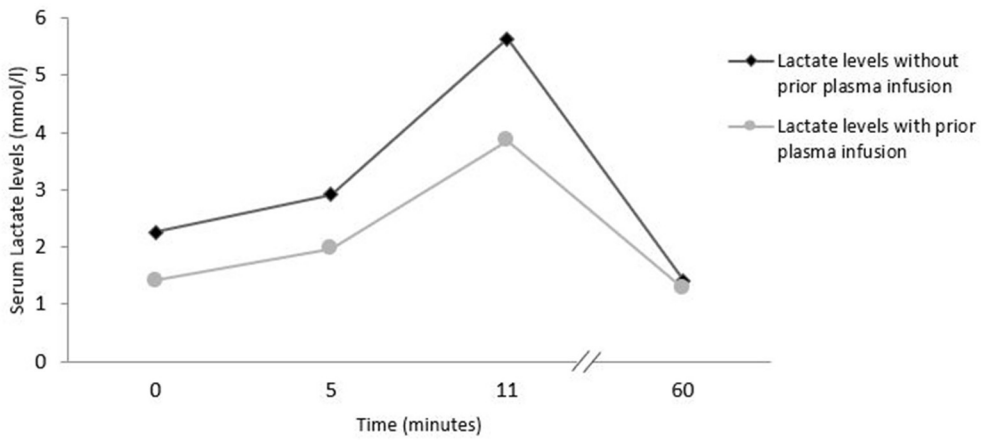

FIGURE 3 | Exercise induced lactate levels in bicycle exercise testing with and without prior plasma infusion.

applied bioinformatic tools (Mutation Taster, PolyPhen2, SIFT, PROVEAN), contributes to the allele's pathogenicity.

Since no residual plasma ADAMTS13 activity was detected, we conclude that Arg784Gln;Arg916Cys causes a severe defect of the affected *ADAMTS13* allele, suggesting that either no protein is expressed, protein maturation is severely impaired, or the protein is not secreted.

Plasma Half-Life of ADAMTS13

In patients with autoimmune TTP (iTTP) there is usually no measurable increase in ADAMTS13 activity after plasma infusion. Therefore, the increase of ADAMTS13 activity in our patient after plasma infusion confirms our diagnosis of cTTP. The ADAMTS13 activity half-life of 27.6 h in our patient is somewhat shorter than most previous reported elimination half-life values.

The expected maximal concentration of ADAMTS13 activity at the beginning of the infusion [calculated via equation described by Furlan et al. (19)] was 18.6%. The fast decline of ADAMTS13 activity at the beginning of the curve points to a multiple-compartment kinetics. Few studies have documented the same effect after plasmatherapy, but this can mostly be attributed to the lack of data points early after infusion. The half-life ($t_{1/2}$) of ADAMTS13 reported in the scarce literature is significantly longer [between 2.1 days [50.4 h] up to 7,9 days [189.5 h] (19–21)].

The plasma half-life of ADAMTS13 in another case of cTTP was close to our findings with an elimination half-life of 1.54 days (37 h) (22). We suppose that part of the infused ADAMTS13 will be trapped by binding to endothelial cell-attached VWF multimers. There seems to be no significant difference between ADAMTS13 half-life of cTTP patients with prior plasma exchange vs. patients just receiving plasma infusions (21).

The shorter ADAMTS13 half-life in our case may also be related to the multi-compartment elimination, as previous treatments with plasma infusions or plasmapheresis over an extended time period could saturate all compartments of ADAMTS13 and cause higher ADAMTS13 half-life measurements.

In the study of the pharmacokinetics of recombinant ADAMTS13 (rhADAMTS13) the $t_{1/2}$ was 2,47 d (59.2 h) with a reported initial half-life of 17 h (23). In one patient the half-life was as low as 29.5 h, comparable to the result in our patient.

There is a rather large variation of reported half-lives due to a paucity of systematic testing.

Late Onset cTTP

Several factors (most of them not understood) influence the severity of disease and first occurrence of an acute TTP episode. An important factor is residual ADAMTS13 activity (24). But results of the large International Hereditary TTP Registry with 123 enrolled cTTP patients showed that residual ADAMTS13 activity is not the only determinant of the age at first disease manifestation (4). Median age at overt disease onset was 4.52 years and at clinical diagnosis 16.7 years. Another important factor is exposition to strong triggers of disease (infections, surgery, pregnancy, drugs, etc.).

The influence of AB0 blood group type on plasma levels of von Willebrand factor (VWF) is well-documented in several studies (25–27). A large study with healthy individuals nicely showed that plasma VWF levels were lowest in blood group 0 persons and highest in blood group AB subjects (28). Among patients from the Oklahoma TTP registry with acquired TTP blood group 0 was more frequent than expected and was even discussed to be a potential risk factor (29). While blood group might play a role in iTTP, there are no data in USS patients. Whether blood group 0 may protect cTTP patients from early disease onset is unknown.

The first disease onset and clinical diagnosis in our patient was at the age of 43 years. Our patient was carrier of two homozygous *ADAMTS13* variants and had no residual plasma ADAMTS13 activity, his blood group was 0.

There was no anamnestic finding that could retrospectively be attributed to an earlier TTP episode. Because of the complete resolution of the TTP flare by just treating the triggering situation in this case, we cannot exclude earlier unknown disease manifestations which may have resolved by treating the underlying triggering condition without diagnosis of TTP.

Smoldering TTP Activity

In the literature there are hints to ongoing disease activity despite lack of classical signs and symptoms of active disease (4, 5, 30, 31). Symptoms reported were headaches, loss of concentration or abdominal discomfort (5), which were reversed by prophylactic plasma infusions.

Our patient consistently reported thoracic and abdominal pain. There were no pathological changes in ECG, transthoracic echocardiography, abdominal sonography or repeated troponin values, lipase, lactate dehydrogenase and other parameters. There were no laboratory signs of TTP flare. The most interesting observation was a complete resolution of all pain burden within 2 h of plasma infusion. The positive effect lasted for about 4 days, as reported by the patient without any knowledge nor information about half-life of ADAMTS13 or advanced knowledge of the disease. We could reproduce this effect of complete pain resolution on two further occasions. With this information we searched for signs of "smoldering TTP activity," but we could not find any laboratory value which was consistently or temporarily altered (troponin, lipase, LDH, haptoglobin, CK, platelets, lactate, coagulation parameters including D-dimers). We tried to provoke signs of smoldering clinical TTP with clinical provocation tests that could possibly cause peripheral ischemia. We did maximum ergometric cycling exercise with and without prior prophylactic plasma infusion, but–except for a lower lactate at starting point–we were unable to find any difference in any parameter evaluated: no significant differences in dynamics of troponin, lipase, LDH, haptoglobin, CK, platelets and lactate values (**Figure 3**, **Supplementary Table 1**) between the two ergometry tests.

In the further course the patient reported spontaneous disappearance of the above-named symptoms without any intervention. When the symptoms returned, we decided to do the double-blinded infusion trial with balanced crystalloid fluids vs. fresh frozen plasma infusions with an impressive effect of just crystalloid fluids on thoracic and abdominal pain. The fresh frozen plasma was then effective in reducing back pain. It is difficult to interpret these findings correctly and we rather doubt having proof of smoldering TTP activity reacting exclusively to replenishment of ADAMTS13 via plasma infusion (5, 6, 31). There seems to be a significant placebo effect, especially in this patient with a huge language barrier and fear of deportation to his home country after possible failure of asylum procedures and expiration of residency permit.

Triggers of Acute Episodes of cTTP

The salmonella infection was clearly the trigger for the acute TTP flare in our case.

It is nevertheless remarkable that the acute TTP episode was completely resolved just by treating the trigger situation (antibiotics against salmonella, supportive treatment) without any plasma infusion. Platelet count normalized on day 6 and lactate dehydrogenase on day 9 after presenting at our hospital (day 4 resp. day 7 after starting antibiotic therapy against salmonellosis). The inflammatory trigger (salmonella infection) probably caused an upregulation of endothelial VWF secretion and increase of VWF plasma levels, which caused acute flare of the disease in analogy to triggering acute TTP by shiga toxin in an ADAMTS13 deficient mouse model (32).

Until recombinant ADAMTS13 (23) becomes available, treatment is either on demand with plasma infusions during acute flares of TTP (10 to 20 ml/kg BW of fresh frozen plasma) or prophylactic with 10–15 ml/kg BW every 2–3 weeks. Data from the International hereditary TTP registry show that 71%

of the 117 registered cTTP patients with available treatment data receive prophylactic plasma infusions and just 29% on demand treatment (4). We decided for "on demand" treatment strategy and careful surveillance in our patient. In the 3 years of follow-up since diagnosis, there has been no recurrence of TTP episodes. Echocardiography, myocardial scintigraphy, and cerebral MRI were performed without pathological findings.

Although our understanding of cTTP has increased enormously over the last two decades, there is still a lack of knowledge about long-term sequelae and possible silent smoldering end organ damage, which makes lifelong careful follow-up and regular assessments imperative.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

RW: diagnosing the patient, conducting clinical tests, writing, and revision of the manuscript. BL: senior advisor in all laboratory, genetical and clinical analyses, and manuscript revision. HR: laboratory analyses of ADAMTS13 and inhibitors, genetic analysis and interpretation, and manuscript revisions. JB: clinical testing and follow up and manuscript revision. TF: manuscript revision. FO: pharmacokinetic analyses and revision of manuscript. SK: caring for the patient and revision of manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.639441/full#supplementary-material

Supplementary Figure 1 | Blinded assessment of symptoms using aluminum foil-covered infusion fluids and lines.

Supplementary Table 1 | Laboratory values during bicycle exercise testing with and without prior plasma infusion. Values of ergometry without prior plasma infusion are in black, values of ergometry with prior plasma infusion are in gray. Lactate (<2.4 mmol/l), D-dimer (<500 μg/l), troponin (<0.005 μg/l), platelets count (139 – 403 Gpt/l), lipase (<1.0 μkat/l), lactate dehydrogenase (2.2–3.8 μmol/l*s), creatine kinase (CK) (<3.2 μmol/l*s), haptoglobin (hapto) (0.2–2.04 g/l). D-dimers in the "with prior plasma-infusion" setting were elevated most likely due to clinically apparent thrombophlebitis.

Supplementary Table 2 | 10-point visual analog pain scale (VAPS) at baseline and after infusion of crystalloids and FFP.

REFERENCES

1. Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *Brit J Haematol*. (2005) 129:93–100. doi: 10.1111/j.1365-2141.2005.05420.x
2. Kremer Hovinga JA, Mottini M, Lämmle B. Measurement of ADAMTS-13 activity in plasma by the FRETS-VWF73 assay: comparison with other assay methods. *J Thromb Haemost*. (2006) 4:1146–8. doi: 10.1111/j.1538-7836.2006.01904.x
3. Shargel L, Wu-Pong S, Yu ABC. *Applied Biopharmaceutics & Pharmacokinetics*. New York, NY: Appleton & Lange Reviews/McGraw-Hill, Medical Pub Division (2005).
4. van Dorland HA, Taleghani MM, Sakai K, Friedman KD, George JN, Hrachovinova I, et al. The international hereditary thrombotic thrombocytopenic purpura registry: key findings at enrolment until 2017. *Haematologica*. (2019) 104: 2107–15 doi: 10.3324/haematol.2019.216796
5. Alwan F, Vendramin C, Liesner R, Clark A, Lester W, Dutt T, et al. Characterization and treatment of congenital thrombotic thrombocytopenic purpura. *Blood*. (2019) 133:1644–51. doi: 10.1182/blood-2018-11-884700
6. Borogovac A, George JN. Stroke and myocardial infarction in hereditary thrombotic thrombocytopenic purpura: similarities to sickle cell anemia. *Blood Adv*. (2019) 3:3973–6. doi: 10.1182/bloodadvances.2019000959
7. Furlan M, Lämmle B. Aetiology and pathogenesis of thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome: the role of von Willebrand factor-cleaving protease. *Best Pract Res Cl Ha*. (2001) 14:437–54. doi: 10.1053/beha.2001.0142
8. Levy GG, Nichols WC, Lian EC, Foroud T, McClintick JN, McGee BM, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature*. (2001) 413:488–94. doi: 10.1038/35097008
9. Kokame K, Matsumoto M, Soejima K, Yagi H, Ishizashi H, Funato M, et al. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. *Proc National Acad Sci USA*. (2002) 99:11902–7. doi: 10.1073/pnas.172277399
10. Matsumoto M, Kokame K, Soejima K, Miura M, Hayashi S, Fujii Y, et al. Molecular characterization of ADAMTS13 gene mutations in Japanese patients with Upshaw-Schulman syndrome. *Blood*. (2004) 103:1305–10. doi: 10.1182/blood-2003-06-1796
11. Lotta LA, Garagiola I, Palla R, Cairo A, Peyvandi F. ADAMTS13 mutations and polymorphisms in congenital thrombotic thrombocytopenic purpura. *Hum Mutat*. (2010) 31:11–9. doi: 10.1002/humu.21143
12. Fujimura Y, Matsumoto M, Isonishi A, Yagi H, Kokame K, Soejima K, et al. Natural history of upshaw–schulman syndrome based on ADAMTS13 gene analysis in Japan. *J Thromb Haemost*. (2011) 9:283–301. doi: 10.1111/j.1538-7836.2011.04341.x
13. Schneppenheim R, Hovinga JA, Becker T, Becker T, Budde U, Karpman D, et al. A common origin of the 4143insA ADAMTS13 mutation. *Thromb Haemostasis*. (2006) 96:3–6. doi: 10.1160/TH05-12-0817
14. Moatti-Cohen M, Garrec C, Wolf M, Boisseau P, Galicier L, Azoulay E, et al. Unexpected frequency of upshaw-schulman syndrome in pregnancy-onset thrombotic thrombocytopenic purpura. *Blood*. (2012) 119:5888–97. doi: 10.1182/blood-2012-02-408914
15. Deal T, Hovinga JAK, Marques M, Adamski J. Novel ADAMTS13 mutations in an obstetric patient with upshaw-schulman syndrome. *J Clin Apheresis*. (2013) 28:311–6. doi: 10.1002/jca.21251
16. Mariotte E, Azoulay E, Galicier L, Rondeau E, Zouiti F, Boisseau P, et al. Epidemiology and pathophysiology of adulthood-onset thrombotic microangiopathy with severe ADAMTS13 deficiency (thrombotic thrombocytopenic purpura): a cross-sectional analysis of the French national registry for thrombotic microangiopathy. *Lancet Haematol*. (2016) 3:e237–45. doi: 10.1016/S2352-3026(16)30018-7

17. Fattah H, Kumar D, George JN, Massey HD, King AL, Friedman KD, et al. Successful kidney transplantation in a patient with congenital thrombotic thrombocytopenic purpura (Upshaw-Schulman syndrome). *Transfusion*. (2017) 57:3058–62. doi: 10.1111/trf.14326
18. Russo L, Marchetti M, Testa M. Impact of severe ADAMTS-13 deficiency in patients with thrombotic thrombocytopenic purpura (TTP) on recurrences. *Haematologica*. (2015). 100 (Suppl. 3):1–239.
19. Furlan M, Robles R, Morselli B, Sandoz P, Lämmle B. Recovery and half-life of von Willebrand factor-cleaving protease after plasma therapy in patients with thrombotic thrombocytopenic purpura. *Thromb Haemost*. (1999) 81:8–13. doi: 10.1055/s-0037-1614408
20. Taylor A, Vendramin C, Oosterholt S, Pasqua OD, Scully M. Pharmacokinetics of plasma infusion in congenital thrombotic thrombocytopenic purpura. *J Thromb Haemost*. (2019) 17:88–98. doi: 10.1111/jth.14345
21. Kovarova P, Hrdlickova R, Blahutova S, Cermakova Z. ADAMTS13 kinetics after therapeutic plasma exchange and plasma infusion in patients with Upshaw-Schulman syndrome. *J Clin Apheresis*. (2019) 34:13–20. doi: 10.1002/jca.21664
22. Meyer S, Jeddi R, Meddeb B, Gouider E, Lämmle B, Hovinga JK. A first case of congenital TTP on the African continent due to a new homozygous mutation in the catalytic domain of ADAMTS13. *Ann Hematol*. (2008) 87:663. doi: 10.1007/s00277-008-0496-6
23. Scully M, Knöbl P, Kentouche K, Rice L, Windyga J, Schneppenheim R, et al. Recombinant ADAMTS-13: first-in-human pharmacokinetics and safety in congenital thrombotic thrombocytopenic purpura. *Blood*. (2017) 130:2055–63. doi: 10.1182/blood-2017-06-788026
24. Lotta LA, Wu HM, Mackie IJ, Noris M, Veyradier A, Scully MA, et al. Residual plasmatic activity of ADAMTS13 is correlated with phenotype severity in congenital thrombotic thrombocytopenic purpura. *Blood*. (2012) 120:440–8. doi: 10.1182/blood-2012-01-403113
25. Moeller A, Weippert-Kretschmer M, Prinz H, Kretschmer V. Influence of ABO blood groups on primary hemostasis. *Transfusion*. (2001) 41:56–60. doi: 10.1046/j.1537-2995.2001.41010056.x
26. Mohanty D, Ghosh K, Marwaha N, Kaur S, Chauhan AP, Das KC. Major blood group antigens–a determinant of factor VIII levels in blood? *Thromb Haemost*. (1984) 51:414. doi: 10.1055/s-0038-1661121
27. McCallum CJ, Peake IR, Newcombe RG, Bloom AL. Factor VIII levels and blood group antigens. *Thromb Haemost*. (1983) 50:757. doi: 10.1055/s-0038-1665305
28. Gill JC, Endres-Brooks J, Bauer PJ, Jr WJM, Montgomery RR. The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood*. (1987) 69:1691–5. doi: 10.1182/blood.V69.6.1691.bloodjournal6961691
29. Terrell DR, Motto DG, Hovinga JAK, Lämmle B, George JN, Vesely SK. Blood group O and black race are independent risk factors for thrombotic thrombocytopenic purpura associated with severe ADAMTS13 deficiency. *Transfusion*. (2011) 51:2237–43. doi: 10.1111/j.1537-2995.2011.03125.x
30. Tanabe S, Fujimura Y, Lämmle B, Kimura T, Isonishi A, Sakai K, et al. Stealth thrombosis of brain and kidney in a girl with Upshaw–Schulman syndrome not receiving prophylactic plasma infusions. *Int J Hematol*. (2020) 112:603–4. doi: 10.1007/s12185-020-02997-5
31. Kremer Hovinga JA, George JN. Hereditary thrombotic thrombocytopenic purpura. *New Engl J Med*. (2019) 381:1653–62. doi: 10.1056/NEJMra1813013
32. Motto DG, Chauhan AK, Zhu G, Homeister J, Lamb CB, Desch KC, et al. Shigatoxin triggers thrombotic thrombocytopenic purpura in genetically susceptible ADAMTS13-deficient mice. *J Clin* Invest. (2005) 115:2752–61. doi: 10.1172/JCI26007

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Retrospective and Systematic Analysis of Causes and Outcomes of Thrombotic Microangiopathies in Routine Clinical Practice: An 11-Year Study

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Background: Thrombotic microangiopathies (TMAs) are highly suspected in patients showing mechanical hemolytic anemia, thrombocytopenia, and haptoglobin consumption. Primary [thrombotic thrombocytopenic purpura (TTP) and atypical hemolytic uremic syndrome] and secondary TMA are considered. Even if ADAMTS13 measurements and alternative complement pathway explorations have greatly improved the ability to identify primary TMA, their diagnosis remains difficult, and their frequency relative to that of secondary TMA is undetermined. The objectives of the present study were, therefore, to describe the etiologies, management, and the outcomes of patients presenting with TMA in real-life clinical practice.

Methods: We conducted a retrospective study between 01/01/2008 and 31/12/2018 that included all consecutive patients presenting with biological TMA syndrome at admission or developing during hospitalization. Patients were identified from the laboratory databases, and their medical files were reviewed to confirm TMA diagnosis, to determine etiology, and to analyze their therapeutic management and outcomes.

Results: During this period, 239 patients with a full TMA biological syndrome were identified, and the TMA diagnosis was finally confirmed in 216 (90.4%) after the cases were reviewed. Primary TMAs (thrombotic thrombocytopenic purpura or atypical hemolytic uremic syndrome) were diagnosed in 20 of 216 patients (9.3%). Typical HUS was diagnosed in eight patients (3.7%), and the most frequent secondary TMAs were HELLP syndrome (79/216, 36.6%) and active malignancies (30/219, 13.9%). ADAMTS13 measurements and alternative complement pathway analyses were performed in a minority of patients. Multiple factors identified as TMA triggers were present in most patients, in 55% of patients with primary TMA, vs. 44.7% of patients with secondary TMA ($p = 0.377$). Death occurred in 57 patients (23.4%) during follow-up, and

dialysis was required in 51 patients (23.6%). Active malignancies [odds ratio (OR) 13.7], transplantation (OR 4.43), male sex (OR 2.89), and older age (OR 1.07) were significantly associated with death.

Conclusion: Secondary TMAs represent many TMA causes in patients presenting a full TMA biological syndrome during routine clinical practice. Multiple factors favoring TMA are present in about half of primary or secondary TMA. ADAMTS13 and complement pathway were poorly explored in our cohort. The risk of death is particularly high in patients with malignancies as compared with patients with other TMA.

Keywords: Thrombotic microangiopathies, etiology, primary, secondary, thrombotic thrombocytopenic purpura, hemolytic uremic syndrome, real-life

INTRODUCTION

Thrombotic microangiopathies (TMAs) are defined by the presence of thrombi in small arterioles and capillaries (1). Thrombosis affects microcirculation and leads to tissue ischemia and organ failure (2). Although the confirmation of TMA diagnosis relies on histological features, a biopsy of an affected organ is rarely performed. Not only do TMAs have, in most cases, a characteristic biological presentation, but also biopsy is often contraindicated, given the bleeding risk. Mechanical hemolytic anemia, schistocytosis, and thrombocytopenia are strongly suggestive of TMA. As in other hemolytic anemia syndromes, haptoglobin consumption, elevated LDH, and elevated free bilirubin levels are also detected.

The understanding of biological mechanisms implicated in TMA development has greatly improved over the past three decades, allowing for the individualization of entities with specific pathophysiology (3, 4). In parallel with the pathophysiological understanding of TMA, the classification of TMA has been enriched with new entities and has further enabled the identification of several favoring or precipitating factors (1, 5). The latter can act as triggers of TMA development in patients with conditions that make them pre-disposed to primary TMA, i.e., genetic defects, or can induce TMA by themselves. TMAs are usually classified into two subsets, primary and secondary. Primary TMAs include thrombotic thrombocytopenic purpura (TTP) and atypical hemolytic uremic syndrome (aHUS). TTP is associated with low or undetectable ADAMTS13 activity (usually <10%), which may be related to a rare genetic defect in the ADAMTS13 gene (Upshaw–Schulman syndrome) or is, in most cases, an autoimmune disease associated with auto-antibodies neutralizing the enzymatic functions of ADAMTS13 (6). aHUS is related to several inherited or acquired abnormalities affecting complement alternative pathway (cAP) (7) that result in its permanent activation. Primary TMA can manifest at all ages and frequently develops after the occurrence of triggers that induce endothelial injury. Secondary TMAs, however, include numerous conditions or diseases that have been associated with TMA development. In secondary TMA, genetic defects and autoimmune abnormalities are rarely detected, and the microvascular endothelial injuries are driven by other factors altogether. The pathophysiology of secondary forms is, therefore, less well-defined, and as a result, diagnosis is rendered much more difficult. In the light of this, several classifications have been proposed to guide clinicians to the right diagnosis. Among secondary TMAs, typical HUS (tHUS) induced by the endothelial toxicity of Shiga toxins from *Escherichia coli* is the most well-defined secondary TMA. However, numerous other conditions such as a solid organ or bone marrow transplantation, drugs, pregnancy (HELLP syndrome), malignant hypertension, and malignancies can be associated with TMA development (1, 8–11). In such cases, the mechanisms of TMA are largely unknown and frequently multifactorial, and the therapeutic management of these patients is not well-codified.

The treatment of primary TMA has greatly evolved in the last few years using specific drugs such as rituximab in TTP and eculizumab in aHUS (12–15). In these conditions, mortality and organ lesions (mainly brain and kidney injuries) occur early, within the first days or weeks. A patient's prognosis is very dependent upon any delays to commencing with possible specific treatment. It is, therefore, crucial to identify these patients among the flood of those with a secondary cause.

The relative frequency of primary to secondary TMA has been poorly analyzed in the literature (16). Moreover, the management of patients with secondary TMA and their prognosis in comparison with patients with primary TMA are not well-known.

In the present study, we identified all consecutive patients with biological features of TMA (full biological TMA syndrome) in a period spanning 11 years. The objective of the study was to analyze the relative frequency of primary and secondary TMA, their presentation, and their therapeutic management. We also analyzed the prognosis of patients according to the causes of their TMA.

METHODS

Selection of Patients

Patients admitted to the University Hospital of Angers between 01/01/2008 and 31/12/2018 and presenting with a full biological TMA syndrome were included in the study. The concomitant association of anemia defined full biological TMA syndrome (<12 g/dl in females and 13 g/dl in males), with thrombopenia ≤150 G/L, schistocytosis ≥0.5%, and a decreased haptoglobin

level ≤0.4 g/L. These patients were identified from the database of our hematological laboratory. Two datasets listing patients with (1) thrombocytopenia and schistocytosis screening and (2) haptoglobin measurements during the study were extracted and merged. Adult patients (>18 years old) with concomitant thrombocytopenia, schistocytosis, and a decreased haptoglobin level were included in a systematic review of medical records after anemia had been confirmed. The study protocol complied with the standards of the Ethics Committee of the Angers University Hospital (no. 2019/12).

Identification and Classification of Thrombotic Microangiopathic Patients

Medical records of patients identified with a full TMA biological syndrome were analyzed by four physicians (NH, CM, BB, and JFA) trained in nephrology, hematology, and critical care medicine, to confirm or rule out the diagnosis. In patients with a TMA diagnosis, its cause was determined after a hierarchical analysis according to current classifications. In a first step, patients with ADAMTS13 < 10% were classified as having TTP. Next, patients with Shiga toxin-positive bacteria (detected in stool culture or polymerase chain reaction) were classified as having tHUS. The other following TMA causes were systematically considered: patients with HELLP syndrome, drugs known to induce TMA, graft vs. host disease (GvHD)-associated TMA, cancers, autoimmune diseases, malignant hypertension, and infections (excluding those related to Shiga toxin-producing bacteria). Patients with TMA and acute renal failure but without evidence of other secondary TMA causes and/or with cAP abnormalities known to be associated with TMA were classified as having aHUS. Patients without evidence of any of the causes of secondary TMA listed earlier, with rare secondary causes of TMA and without evidence of cAP abnormalities, were classified in "other secondary TMA causes." Patients with multiple TMA potential causes, for whom clinical file review could not determine a predominant mechanism, were also classified in "other secondary TMA causes" (**Supplementary Table 1**).

In some patients with primary or secondary TMA, several TMA causes could be present. In patients with primary TMA, other possible causes were considered as favoring factors. Patients with secondary TMA were classified within the most probable diagnosis after medical file review, and other associated conditions were considered as favoring factors. Factors considered to favor TMA were concomitant infection, pregnancy, past or present history of malignancy, drugs known to induce TMA, transplantation, autoimmune diseases, and B12 deficiency.

Therapeutic Management and Outcomes

Plasma exchange and/or plasma infusion, eculizumab and rituximab use, and the requirement for transfusion or dialysis were identified in medical charts and collected. Outcomes, including death or need for renal replacement therapy, were also collected.

Statistical Analysis

Continuous variables are presented as median (minimum–maximum). Categorical variables are presented as the absolute value and percentage. Differences between groups were analyzed using the χ^2 test (or Fisher exact test if necessary) for categorical variables and the Mann–Whitney U test for continuous variables. Logistic univariate analysis was used to examine factors associated with the outcomes. P-value < 0.05 were considered significant. Statistical analysis was performed using SPSS software® 23.0 and Graphpad Prism®.

RESULTS

Baseline Characteristics

During the period mentioned earlier, we identified 427 patients with thrombocytopenia and schistocytosis ≥0.5% and 4,664 patients with haptoglobin ≤0.4 g/L. After cross-referencing the datasets, we could identify 239 patients with a full biological TMA syndrome. After the review of medical charts, 216 patients were finally diagnosed with TMA, whereas 23 had no evidence of TMA (**Figure 1**). The diagnosis retained in these 23 patients after reviewing their medical files are given in **Supplementary Table 2**. Patients with TMA were initially admitted to 17 different medical and surgical departments (**Supplementary Figure 1**). Among the 216 patients, 81 (37.5%) were admitted to a critical care unit at least partially during their hospitalization.

TMA patients were predominantly females (74.1%), with a median age of 45.3 ± 18.5 years. Hypertension and chronic kidney disease (estimated glomerular filtration rate < 60 ml/min/1.73 m²) were found in 54 (25%) and 21 (9.7%) patients, respectively. Primary TMA (TTP or aHUS) was diagnosed in 20 patients (9.3%), with mostly acquired TTP (11 patients, 5.1%), whereas secondary TMA represented 90.7% of all TMA cases. Among secondary TMA, HELLP syndrome and active cancer-associated TMA were the most frequent diagnosis, in 36.6 and 13.9%, respectively. In the case of the latter, solid cancer featured in 19 patients and acute haemopathy in 11 patients. Other secondary TMA causes were less frequently observed, with tHUS representing 3.7% (**Table 1** and **Supplementary Figure 2**). A subgroup of 30 patients was classified as "other TMA." Some of these patients had a rare TMA cause or had no identified cause of TMA or multiple causes of TMA, not allowing their classification within other groups. The characteristics of patients classified in this subgroup of TMA are given in **Supplementary Table 3**. Surprisingly, TMA diagnosis was not mentioned within the medical file at the end of the initial admission for 53 of 216 (24.4%) patients. These patients were classified as having mainly secondary TMA after file review (cancer, $n = 18$; aGVHD, $n = 12$; undetermined TMA cause, $n = 11$). ADAMTS13 determination was performed in only 25% of patients, and, therefore, the diagnosis of TTP may have been missed in some patients. C3 and C4 complement fraction concentration and alternative complement pathway were studied in 33.8 and 19.4% of the patients of the cohort, respectively (**Supplementary Table 4**).

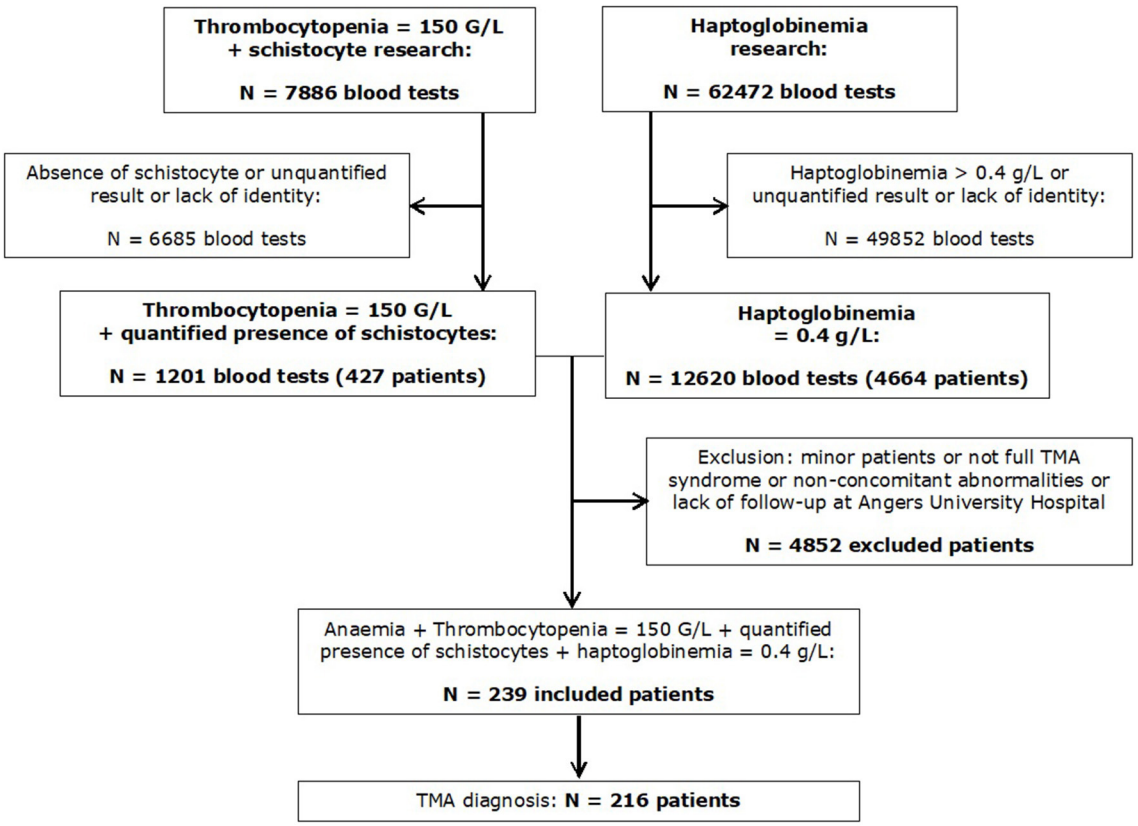

FIGURE 1 | Flowchart of the study.

Clinical and Biological Presentation According to Thrombotic Microangiopathy Cause

Table 1 reports the clinical and biological presentation according to the TMA etiology. Patients with aHUS tended to be younger compared with patients with TTP. In secondary TMA, patients with HELLP syndrome were younger than those with malignancies. Neurological signs were present at admission with variable frequencies according to TMA causes. They were present in most patients with primary TMA, either TTP or aHUS. Neurological symptoms exhibited greater variety in secondary TMA, most frequently observed in patients with malignant hypertension and malignancies. Acute kidney injury was present in all patients with aHUS and tHUS, in most patients with TTP, and with a variable frequency in other secondary TMA causes. Serum creatinine was greatly higher in patients with aHUS and malignant hypertension, as compared with other causes of TMA. The level of proteinuria showed a similar trend. However, although 75% of TTP patients developed acute kidney injury, their median serum creatinine level at admission was 94 μmol/L and therefore in the same range as HELLP syndrome patients. Diarrhea was present in all patients with tHUS and in most patients with aGvHD but was very inconstantly observed in other conditions. Anemia was present in all patients but with variable severity. Thrombocytopenia was particularly profound in patients with TTP.

Analysis of Thrombotic Microangiopathy-Favoring Factors

Except for patients with HELLP syndrome and active autoimmune diseases, the presence of an additional factor known to favor TMA was frequent (**Table 2**). There was no difference in the frequency of patients with at least one additional factor between patients with primary TMA and patients with secondary TMA (55.0 vs. 44.7%, $p = 0.377$). The most frequent additional factor was a history of malignancy and concomitant administration of a drug known to favor TMA, mostly gemcitabine or calcineurin inhibitor. Patients with aGvHD and drug-associated TMA tended to have more associated factors than patients with primary TMA and with other secondary causes (**Table 2**).

Management of Thrombotic Microangiopathy

Red blood cell transfusion was used in 43.5% of patients and platelet transfusion in 22.7% of patients. Plasma exchange or plasma infusion was initiated in 28.2% of patients. Rituximab and eculizumab were administered in 7.9 and 3.7% of patients,

TABLE 1 | Clinical and biological presentation of TMA according to etiology.

Characteristics	**Primary TMA**		**Secondary TMA**								
***N* (%)**	***n* = 20 (9.3)**		***n* = 196 (90.7)**								
	TTP	**aHUS**	**tHUS**	**HELLP syndrome**	**Malignant HBP**	**Malignancies**	**Drugs**	**Infections**	**AID**	**aGVHD**	**Others**
	***n* = 12 (5.6)**	***n* = 8 (3.7)**	***n* = 8 (3.7)**	***n* = 79 (36.6)**	***n* = 12 (5.6)**	***n* = 30 (13.9)**	***n* = 11 (5.1)**	***n* = 5 (2.3)**	***n* = 5 (2.3)**	***n* = 16 (7.4)**	***n* = 30 (13.9)**
Baseline characteristics											
Age (years)	59.2 (31–83)	36.9 (24–79)	63.9 (34–83)	29.8 (20–45)	35.6 (22–75)	63.6 (21–86)	59.1 (27–74)	63.8 (49–83)	72.3 (45–85)	53.4 (24–70)	39.5 (23–93)
Females (%)	7 (58.3)	6 (75.0)	6 (75.0)	79 (100)	5 (41.7)	20 (66.6)	5 (45.4)	3 (60.0)	2 (40.0)	8 (50.0)	17 (56.7)
Neurological signs	8 (66.6)	6 (75.0)	7 (87.5)	25 (31.6)	7 (58.3)	17 (56.7)	2 (18.2)	2 (40.0)	0 (0)	5 (31.2)	8 (26.7)
Diarrhea	3 (25.0)	0 (0)	8 (100)	0 (0)	0 (0)	0 (0)	1 (9.1)	0 (0)	0 (0)	9 (56.2)	4 (13.3)
AKI	9 (75.0)	8 (100)	8 (100)	26 (32.9)	10 (83.3)	15 (50.0)	7 (63.6)	5 (100)	5 (100)	8 (50.0)	14 (46.7)
Biological presentation											
Hemoglobin, g/Dl	7.3 (5.5–9.7)	7.5 (4.5–10.9)	9.5 (5.8–11.5)	9.8 (5.6–13)	7.2 (5.6–10.4)	7.6 (5.2–10.7)	6.9 (5.7–11.3)	6.1 (3.4–13.0)	8.0 (6.4–10.6)	8.5 (6.4–10.5)	9.6 (4.6–13.0)
Platelet count, G/L	10 (5–75)	68 (24–106)	42 (23–111)	38 (9–145)	97 (39–108)	48 (4–131)	53 (8–143)	41 (9–72)	94 (59–124)	15 (10–41)	78 (7–136)
LDH, UI/L	847 (618–1,852)	1,569 (278–4,200)	1,365 (412–3,709)	1,982 (276–6,760)	935 (297–1,955)	1,700 (288–6,546)	558 (221–1,648)	526 (478–4880)	992 (510–2318)	489 (150–14,540)	412 (240–5,900)
Schizocytes, *n* (%)											
0.5–1%	2 (16.7)	2 (25.0)	0 (0)	31 (39.2)	5 (41.7)	4 (13.3)	5 (45.5)	0 (0)	1 (20.0)	7 (43.8)	15 (50.0)
1–3%	1 (8.4)	3 (37.5)	4 (50.0)	40 (50.7)	4 (33.3)	11 (36.7)	3 (27.3)	3 (60.0)	3 (60.0)	5 (31.3)	8 (26.7)
3–5%	2 (16.7)	1 (12.5)	2 (25.0)	5 (6.3)	1 (8.3)	7 (23.3)	3 (27.3)	0 (0)	1 (20.0)	2 (12.5)	2 (6.7)
5–10%	5 (41.6)	2 (25.0)	2 (25.0)	2 (2.5)	1 (8.3)	7 (23.3)	1 (9.1)	2 (40.0)	0 (0)	2 (12.5)	2 (6.7)
>10%	2 (16.7)	0(0)	0 (0)	1 (1.3)	1 (8.3)	1 (3.3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3.3)
Elevated free bilirubin, *n* (%)	7 (58.3)	3 (37.5)	6 (75.0)	47 (59.5)	3 (25.0)	19 (63.3)	5 (45.5)	4 (80.0)	0 (0)	10 (62.5)	14 (46.7)
Elevated LE, *n* (%)	5 (41.7)	1 (12.5)	6 (75.0)	79 (100)	3 (25.0)	8 (26.7)	4 (36.4)	5 (100)	5 (100)	9 (56.3)	12 (40.0)
Fibrinogen, g/L	4.2 (1.5–7.8)	4.8 (4.3–9.9)	3.4 (2.6–8.3)	3.9 (0.3–8.4)	3.8 (2.5–5.9)	2.4 (0.3–7.5)	3.0 (2.8–4.7)	2.9 (1.7–5.0)	4.0 (2.4–6.7)	3.1 (1.8–11.5)	3.4 (2.3–8.8)
Prothrombin time (%)	78.0 (76–83)	55.5 (24–80)	90.0 (66–113)	97.0 (77–109)	96.0 (61–117)	73.0 (32–106)	88.0 (57–103)	56.0 (21–86)	60.5 (59–62)	89.0 (19–112)	79.5 (19–106)
CRP, mg/L	6 (3–105)	27 (4–65)	19.5 (3–45)	28 (4–111)	4 (3–52)	50 (4–347)	29 (3–98)	9 (3–322)	122.5 (76–169)	29.5 (3–488)	31.6 (3–230)
Serum creatinine, μmol/L	94 (62–1,131)	795 (300–1,631)	268 (97–844)	81.5 (33–746)	1,087 (120–1,491)	245 (30–700)	258 (63–499)	244 (138–350)	287 (106–505)	100 (50–343)	383 (64–962)
Proteinuria, g/L	1.0 (0.2–5.2)	4.4 (0.4–9.8)	1.8 (1.5–4.5)	7.6 (0.0–31.5)	2.7 (0.6–8)	1.1 (0.0–24.0)	1.1 (0.27.1)	0.2 (0.1–1.1)	0.4 (0.2–0.5)	0.6 (0.4–3.5)	1.8 (0.1–24)
Albuminemia, g/L	36 (14–49)	28 (21–42)	32 (23–34)	22 (18–36)	37 (21–45)	26 (17–43)	30 (24–43)	32 (31–33)	29 (21–40)	29 (19–36)	26 (20–38)

TABLE 2 | TMA-favoring factors according to TMA cause.

Characteristics	Primary		Secondary								
	TTP *n* = 12	aHUS *n* = 8	tHUS *n* = 8	HELLP syndrome *n* = 79	Malignant HBP *n* = 12	Malignancies *n* = 30	Drugs *n* = 11	Infections *n* = 5	AID *n* = 5	aGVHD *n* = 16	Others *n* = 30
Infections	**0**	**0**	**8**	**0**	**0**	**8**	**0**	**5**	**0**	**12**	**1**
Shiga toxin *E. coli*	0	0	8	0	0	0	0	0	0	0	0
Bacteria	0	0	0	0	0	2	0	3	0	4	0
Virus	0	0	0	0	0	2	0	1	0	10	0
Fungus	0	0	0	0	0	2	0	1	0	5	1
Undetermined	0	0	0	0	0	2	0	1	0	0	0
Pregnancy	**1**	**2**	**0**	**79**	**0**	**0**	**0**	**0**	**0**	**0**	**11**
History of Malignancy	**2**	**1**	**3**	**1**	**2**	**26**	**9**	**3**	**0**	**13**	**5**
Solid cancer	0	1	3	0	2	18	6	2	0	1	2
Hematological malignancy	0	0	0	0	0	9	3	1	0	13	3
Actual Malignancy	**0**	**2**	**0**	**0**	**0**	**30**	**0**	**0**	**0**	**0**	**0**
Solid cancer	0	1	0	0	0	19	0	0	0	0	0
Hematological malignancy	0	1	0	0	0	11	0	0	0	0	0
Drugs	**5**	**1**	**0**	**0**	**0**	**7**	**11**	**0**	**0**	**12**	**1**
Transplantation	**3**	**1**	**0**	**0**	**0**	**2**	**3**	**1**	**0**	**16**	**7**
Solid organ transplantation	2	1	0	0	0	0	1	0	0	0	4
Stem cell transplantation	1	0	0	0	0	2	2	1	0	16	3
Autoimmune disease	**0**	**0**	**0**	0	**1**	**0**	**0**	**0**	**5**	**0**	**0**
B12 deficiency	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**3**
No identified factor	0	0	0	0	0	0	0	0	0	0	**7**
Mean number of other factors per patient*	**0.92**	**0.87**	**0.37**	**0.01**	**0.25**	**1.43**	**1.09**	**0.8**	**0**	**2.93**	**-**

**When the factor was considered as the main etiology of TMA, it was not counted as a favoring factor.*

respectively. As expected, plasma exchange or plasma infusion was used in most patients with primary TMA. Rituximab was administered in 50% of patients with TTP and at a lower frequency in other TMA groups. Eculizumab was used in eight patients, five of them with aHUS or tHUS (**Table 3**).

Outcomes and Factors Associated With Prognosis

Fifty-one (23.6%) patients required dialysis during initial admission, and 57 (23.4%) died during follow-up at a median time of 42 days (1–1,796) from initial admission. Using univariate analysis, older age [odds ratio (OR) 1.07], male sex (OR 2.89), active malignancies (OR 13.7), and transplantation (OR 4.43) were associated with an increased risk of death. Primary TMA (OR 3.00), aHUS, and tHUS (OR 4.84) were associated with dialysis initiation during the first admission, whereas male sex (OR 0.49) and HELLP syndrome (OR 0.17) were associated with a decreased risk of requiring dialysis (**Table 4**).

DISCUSSION

The present study gives an overview of TMA etiology and management in routine clinical practice. The major finding of this work is that secondary TMAs are largely much more frequent than primary TMAs (TTP and aHUS). Importantly, using our biologically based selection and systematic medical file review, we were able to conclude TMA diagnosis in ∼25% of patients for whom the diagnosis was not clearly mentioned within the medical file. These patients had secondary TMA related to various causes. This observation probably reflects the complexity of TMA diagnosis, especially when multiple factors are involved. Data from TMA registries show a higher proportion of primary TMA. As an example, in the Oklahoma TTP-HUS registry, TTP represented 15% of TMA between 1999 and 2007 (17) and 43% in the UK TTP registry between 2009 and 2013 (18). These discrepancies are probably explained because entries into these registers are linked to plasma exchange requirement and by ADAMTS13 measurement, respectively. Thus, they include selected populations and do not, in fact, reflect the frequency of TMA, nor their distribution in "real-life" conditions. Rather, in the present study, we undertook an unbiased analysis of all consecutive patients with full TMA biological syndrome. Interestingly, a very recent study with a methodology like that used in the present study showed similar frequencies of primary and secondary TMA (16): TTP and aHUS represented 5.9% of TMA, slightly below the 9.3% observed in our study.

It is important to note that ADAMTS13 measurement and cAP analysis were performed in only a minority of patients in our cohort. Therefore, some patients may have been misclassified as having a secondary TMA. This would be in line with recent research showing abnormalities in alternative complement pathway proteins, notably patients with clinical features of malignant hypertension (19, 20) or with TMA associated with drugs, such as calcineurin inhibitors used in transplant patients (21). More recently, patients with HELLP syndrome have also been shown to present a higher incidence of germline alternative

TABLE 3 | Therapeutic management of TMA patients.

Treatment, *n* (%)		Primary TMA		Secondary TMA							
	All	TTP	aHUS	tHUS	HELLP syndrome	Malignant HBP	Malignancies	Drugs	Infections	AID	aGVHD	Others
	n = 216	*n* = 12	*n* = 8	*n* = 8	*n* = 79	*n* = 12	*n* = 30	*n* = 11	*n* = 5	*n* = 5	*n* = 16	*n* = 30
Plasma exchange	40 (18.5)	9 (75.0)	6 (75.0)	6 (75.0)	0 (0)	1 (8.3)	5 (16.6)	3 (27.3)	2 (40.0)	3 (60.0)	1 (6.25)	4 (13.3)
Plasma infusion	30 (13.9)	2 (16.6)	2 (25.0)	2 (25.0)	10 (12.7)	0 (0)	3 (10.0)	3 (27.3)	0 (0)	0 (0)	2 (12.5)	6 (20.0)
PE or PI	61 (28.2)	9 (75.0)	6 (75.0)	6 (75.0)	10 (12.7)	1 (8.3)	6 (20.0)	6 (54.5)	2 (40.0)	3 (60.0)	3 (18.8)	9 (30.0)
Eculizumab	8 (3.7)	1 (8.3)	3 (37.5)	2 (20.0)	0 (0)	0 (0)	1 (3.3)	1 (9.1)	0 (0)	0 (0)	0 (0)	0 (0)
Rituximab	17 (7.9)	6 (50.0)	1 (12.5)	0 (0)	0 (0)	0 (0)	3 (10.0)	0 (0)	0 (0)	2 (40.0)	4 (25.0)	1 (3.3)
Red blood cell transfusion	94 (43.5)	8 (66.7)	4 (50.0)	4 (50.0)	14 (17.7)	6 (50.0)	19 (63.3)	5 (45.4)	2 (40.0)	4 (80.0)	15 (93.8)	13 (43.3)
Platelet transfusion	49 (22.7)	3 (25.0)	1 (12.5)	1 (12.5)	7 (8.9)	1 (8.3)	12 (40.0)	5 (45.4)	0 (0)	0 (0)	14 (87.5)	5 (16.6)
Dialysis (during first admission)	51 (23.6)	3 (25.0)	6 (75.0)	3 (37.5)	6 (7.59)	10 (83.3)	5 (16.7)	3 (27.3)	3 (60.0)	2 (40.0)	2 (12.5)	8 (26.7)

TABLE 4 | Univariate analysis of factors associated with outcomes.

	*N**	Death (n = 57)		*N**	RRT (n = 51)	
		OR (95% CI)	*P*		OR (95% CI)	*P*
Patient characteristics						
Age**	-	1.07 (1.05–1.09)	**<0.001**	-	1.02 (0.98–1.02)	0.823
Male (yes)	32	2.89 (1.50–5.55)	**0.001**	19	0.49 (0.25–0.96)	**0.037**
TMA etiology						
Primary TMA (vs. secondary)	7	1.57 (0.59–4.16)	0.362	9	3.00 (1.17–7.71)	**0.023**
TTP (yes)	5	2.09 (0.63–6.86)	0.225	3	1.08 (0.28–4.16)	0.907
aHUS or tHUS (vs. others)	3	0.62 (0.17–2.27)	0.475	9	4.84 (1.70–13.7)	**0.003**
HELLP syndrome (vs. others)	0	-		6	0.17 (0.07–0.42)	**<0.001**
Active malignancies (vs. others)	24	13.7 (5.67–33.3)	**<0.001**	7	0.89 (0.36–2.20)	0.802
Transplantation (vs. others)	18	4.43 (2.05–9.58)	**<0.001**	10	1.51 (0.66–3.42)	0.328
aGvHD (vs. others)	10	5.42 (1.87–15.7)	**0.002**	2	0.44 (0.01–2.00)	0.289
Need for RRT (vs. no RRT)	51	1.22 (0.61–2.45)	0.576	-	-	-

**Number of events in subgroup.*
***per year.*

complement pathway gene mutations (22). The explanation for such a low rate of patients with specific investigations in our study is likely to be multifaceted, reflecting an unawareness of these relatively new data, the rarity of TMA, and the variable TMA experience of the clinician.

We observed the presence of favorable/precipitating factors that can act as "triggers" in ∼50% of patients. Interestingly, their frequency showed no significant difference between patients with primary and secondary TMAs. Therefore, this observation supports the concept of the "multi-hit" theory of TMA (23). TMA has been shown to occur in ∼10% of patients with aGvHD (24). It is interesting to note that it was the condition with the higher number of concomitant triggers in our study.

Finally, primary TMAs, for which treatment has been better codified in past years, represent a minority of TMA cases. HELLP syndrome and active malignancies were the most highly represented causes of secondary TMA in our cohort. The repartition between secondary causes in our study is closely aligned with those observed in the study of Bayer et al. (16). However, we observed far fewer patients with infection-associated TMA (2.3%). These discrepancies are probably linked to different methods for selecting patients and for classifying them between the two studies. First, in Bayer et al.'s study, screening constituted a combination of automatized medical file analysis and the inclusion of patients with partial biological TMA syndrome. The differences in patient selection probably explain why Bayer et al. included many more patients. Second, in our study, we considered infectious events as triggers rather than as causative agents *per se*.

In line with previous data, the mortality rate was high in our study, and death occurred early after TMA diagnosis. In the univariate analysis, active malignancies, transplantation, and aGvHD were significantly associated with a higher risk of death. These observations are in line with the clinical experience we have of TMA and with the few available reports on these topics (24–26). Notably, the differentiation between chemotherapy-induced TMA and cancer-associated TMA represents a challenge, especially when medical files are reviewed retrospectively. Moreover, these patients do not usually undergo extensive biological explorations given their very limited prognosis; this in turn also limits what can be achieved through reviewing files. We may, therefore, have misclassified some of them.

In conclusion, our study highlights that more than 90% of patients with full TMA biological syndrome have secondary TMAs, which are associated with very high mortality. We also point out that it is not uncommon for the diagnosis of TMA not to be mentioned, especially in patients diagnosed with secondary TMA. Therefore, these results suggest that more attention and clinical research should focus on secondary TMA to understand better the mechanisms implicated in it, especially with systematic cAP and ADAMTS13 exploration to rule out primary TMA, improve their classification and codify their treatment.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because The datasets generated for this study are available on request to the corresponding author. Requests to access the datasets should be directed to jfaugusto@chu-angers.fr.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Angers University Hospital (n°2019/12). Written informed

consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

NH, CM, and J-FA contributed to the conception and design of the study. J-FA supervised the project. NH and CM organized the database and wrote the first draft of the manuscript. NH, CM, J-FA and BB performed the statistical analysis. J-FA and BB provided critical revision of the manuscript. All authors participated in patients care. All authors read and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.566678/full#supplementary-material

Supplementary Figure 1 | Site of initial hospital admission.

Supplementary Figure 2 | Number of patients according to TMA etiology.

Supplementary Table 1 | Diagnosis criteria for classifying the causes of TMA.

Supplementary Table 2 | Patients with full TMA biological syndrome without TMA.

Supplementary Table 3 | Characteristics of patients with "other" TMA causes.

Supplementary Table 4 | ADAMTS13, complement and autoimmune evaluation.

REFERENCES

1. George JN, Nester CM. Syndromes of thrombotic microangiopathy. *N Engl J Med*. (2014) 371:654–66. doi: 10.1056/NEJMra1312353
2. Hofer J, Rosales A, Fischer C, Giner T. Extra-renal manifestations of complement-mediated thrombotic microangiopathies. *Front Pediatr*. (2014) 2:97. doi: 10.3389/fped.2014.00097
3. Fakhouri F, Fremeaux-Bacchi V. Does hemolytic uremic syndrome differ from thrombotic thrombocytopenic purpura? *Nat Clin Pract Nephrol*. (2007) 3:679–87. doi: 10.1038/ncpneph0670
4. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. *Nat Rev Immunol*. (2009) 9:729–40. doi: 10.1038/nri2620
5. Masias C, Vasu S, Cataland SR. None of the above: thrombotic microangiopathy beyond TTP and HUS. *Blood*. (2017) 129:2857–63. doi: 10.1182/blood-2016-11-743104
6. Kremer Hovinga JA, Coppo P, Lammle B, Moake JL, Miyata T, Vanhoorelbeke K. Thrombotic thrombocytopenic purpura. *Nat Rev Dis Primers*. (2017) 3:17020. doi: 10.1038/nrdp.2017.20
7. Jokiranta TS. HUS and atypical HUS. *Blood*. (2017) 129:2847–56. doi: 10.1182/blood-2016-11-709865
8. Gupta M, Feinberg BB, Burwick RM. Thrombotic microangiopathies of pregnancy: differential diagnosis. *Pregnancy Hypertens*. (2018) 12:29–34. doi: 10.1016/j.preghy.2018.02.007
9. Al-Nouri ZL, Reese JA, Terrell DR, Vesely SK, George JN. Drug-induced thrombotic microangiopathy: a systematic review of published reports. *Blood*. (2015) 125:616–8. doi: 10.1182/blood-2014-11-611335
10. Noris M, Remuzzi G. Thrombotic microangiopathy after kidney transplantation. *Am J Transplant*. (2010) 10:1517–23. doi: 10.1111/j.1600-6143.2010.03156.x
11. Obut F, Kasinath V, Abdi R. Post-bone marrow transplant thrombotic microangiopathy. *Bone Marrow Transplant*. (2016) 51:891–7. doi: 10.1038/bmt.2016.61
12. Scully M, Goodship T. How I treat thrombotic thrombocytopenic purpura and atypical haemolytic uraemic syndrome. *Br J Haematol*. (2014) 164:759–66. doi: 10.1111/bjh.12718
13. Chander DP, Loch MM, Cataland SR, George JN. Caplacizumab Therapy without plasma exchange for acquired thrombotic thrombocytopenic purpura. *N Engl J Med*. (2019) 381:92–4. doi: 10.1056/NEJMc1905426
14. Schmidtko J, Peine S, El-Housseini Y, Pascual M, Meier P. Treatment of atypical hemolytic uremic syndrome and thrombotic microangiopathies: a focus on eculizumab. *Am J Kidney Dis*. (2013) 61:289–99. doi: 10.1053/j.ajkd.2012.07.028
15. Legendre CM, Licht C, Muus P, Greenbaum LA, Babu S, Bedrosian C, et al. Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome. *N Engl J Med*. (2013) 368:2169–81. doi: 10.1056/NEJMoa1208981
16. Bayer G, von Tokarski F, Thoreau B, Bauvois A, Barbet C, Cloarec S, et al. Etiology and outcomes of thrombotic microangiopathies. *Clin J Am Soc Nephrol*. (2019) 14:557–66. doi: 10.2215/CJN.11470918
17. George JN. The thrombotic thrombocytopenic purpura and hemolytic uremic syndromes: overview of pathogenesis (experience of the Oklahoma TTP-HUS registry, 1989-2007). *Kidney Int Suppl*. (2009) 112:S8–10. doi: 10.1038/ki.2008.609
18. Hassan S, Westwood JP, Ellis D, Laing C, Mc Guckin S, Benjamin S, et al. The utility of ADAMTS13 in differentiating TTP from other acute thrombotic microangiopathies: results from the UK TTP Registry. *Br J Haematol*. (2015) 171:830–5. doi: 10.1111/bjh.13654
19. Cavero T, Arjona E, Soto K, Caravaca-Fontan F, Rabasco C, Bravo L, et al. Severe and malignant hypertension are common in primary atypical hemolytic uremic syndrome. *Kidney Int*. (2019) 96:995–1004. doi: 10.1016/j.kint.2019.05.014
20. Timmermans S, Abdul-Hamid MA, Vanderlocht J, Damoiseaux J, Reutelingsperger CP, van Paassen P, et al. Patients with hypertension-associated thrombotic microangiopathy may present with complement abnormalities. *Kidney Int*. (2017) 91:1420–5. doi: 10.1016/j.kint.2016.12.009
21. Le Quintrec M, Lionet A, Kamar N, Karras A, Barbier S, Buchler M, et al. Complement mutation-associated *de novo* thrombotic microangiopathy following kidney transplantation. *Am J Transplant*. (2008) 8:1694–701. doi: 10.1111/j.1600-6143.2008.02297.x
22. Vaught AJ, Braunstein EM, Jasem J, Yuan X, Makhlin I, Eloundou S, et al. Germline mutations in the alternative pathway of complement predispose to HELLP syndrome. *JCI Insight*. (2018) 3:e99128. doi: 10.1172/jci.insight.99128
23. Miyata T, Fan X. A second hit for TMA. *Blood*. (2012) 120:1152–4. doi: 10.1182/blood-2012-06-433235
24. Kraft S, Bollinger N, Bodenmann B, Heim D, Bucher C, Lengerke C, et al. High mortality in hematopoietic stem cell transplant-associated thrombotic microangiopathy with and without concomitant acute graft-vs.-host disease. *Bone Marrow Transplant*. (2019) 54:540–8. doi: 10.1038/s41409-018-0293-3
25. Izzedine H, Perazella MA. Thrombotic microangiopathy, cancer, and cancer drugs. *Am J Kidney Dis*. (2015) 66:857–68. doi: 10.1053/j.ajkd.2015.02.340
26. Lechner K, Obermeier HL. Cancer-related microangiopathic hemolytic anemia: clinical and laboratory features in 168 reported cases. *Medicine*. (2012) 91:195–205. doi: 10.1097/MD.0b013e3182603598

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CASE REPORT
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Case Report: Microangiopathic Hemolytic Anemia With Normal ADAMTS13 Activity

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Thrombotic microangiopathies (TMAs) include a heterogeneous group of diseases characterized by abnormalities in the vessel walls of arterioles and capillaries resulting in microvascular thrombosis that typically presents with a microangiopathic hemolytic anemia (MAHA) and severe thrombocytopenia. We describe here the case of an 82-year-old woman, who came to our attention for a clinical condition consistent with thrombotic microangiopathy. Even if initially highly suggestive for a thrombotic thrombocytopenic purpura (TTP), the elevated ADAMTS13 activity together with the alteration of the main coagulation parameters (D-dimer elevation, fibrinogen consumption, slightly prolonged prothrombin time), induced us to consider several other diseases in the differential diagnostic process. The case evolved toward a suspected overlapped secondary hemophagocytic syndrome, though the hyperferritinemia was finally interpreted within the frame of a cytokine storm. After a complex diagnostic workup, the clinical and biochemical parameters guided us toward the diagnosis of a cancer-related microangiopathic hemolytic anemia (CR-MAHA) secondary to a relapsing breast cancer with multiple metastatic localizations. Prednisone 1 mg/kg body weight was started, and several units of fresh frozen plasma were infused, obtaining a good control of the hemolysis. No specific oncological therapies were, however, possible, due to the older age and the critically compromised general condition of the patient; therefore, after clinical stabilization, the patient was discharged for treatment in a palliative care Hospital.

Keywords: thrombotic microangiopathy, breast cancer, ADAMTS13, thrombotic thrombocytopenic purpura, disseminated intravascular coagulation, hemolytic anemia

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INTRODUCTION

Cancer-related microangiopathic hemolytic anemia (CR-MAHA) is a rare pathologic condition and often represents a very challenging enigma for the physician at the frontline while facing a critically ill patient. A broad and fast differential diagnostic process is mandatory to achieve the correct diagnosis for the most appropriate and potentially successful treatment.

The present case prompted us to review the pathophysiology of thrombotic microangiopathies and the most recent literature on this multifaceted cluster of diseases (1, 2). In describing this case, we have an intent of highlighting how crucial it is to dedicate extreme attention to

the several coagulative and hemachrome parameters for the achievement of a correct diagnosis of such complex pathologies.

Written informed consent was obtained from the patient for the publication of this case report.

CASE DESCRIPTION

An 82-year-old woman was referred in December 2019 to our University Hospital Internal Medicine Unit with a diagnosis of thrombotic thrombocytopenic purpura, from a nearby community hospital where she had been admitted few days before for an episode of mild epistaxis.

No neurological or major hemorrhagic/thrombotic events were reported. From collection of her clinical history emerged a type II diabetes mellitus treated with metformin and a history of ductal infiltrating breast cancer. The breast carcinoma had been diagnosed in 2006 and firstly treated with quadrantectomy and radiotherapy (QUART) and tamoxifen and, after documented relapse in April 2019, with radical mastectomy and axillary lymph nodes dissection after which she restarted hormone therapy with anastrazole. The most recent follow-up tests were negative for disease relapse. As shown in **Table 1**, when she was admitted her blood tests showed a mild normochromic normocytic anemia (Hb 9.3 g/dL) with increased reticulocyte count (325,000 per μl), and a severe thrombocytopenia (platelet count 32,000 per μl); lactate dehydrogenase (LDH) was increased (1200 U/L) as it was total bilirubin (2.5 mg/dL). Coagulation tests highlighted a slightly prolonged prothrombin time (INR 1.4) with a normal activated partial thromboplastin time and a severely reduced plasma concentration of fibrinogen (0.5 g/L) and markedly elevated plasma concentrations of D-dimer (7000 U/L). Direct and indirect antiglobulin tests were negative. Renal function indexes and serum electrolytes concentrations were within the normal range. A peripheral blood smear showed the presence of an elevated number of schistocytes (about 20%).

Given the diagnosis of thrombotic thrombocytopenic purpura hypothesized the day prior to referral to our Internal Medicine Unit, the patient had been infused with 800 cc of fresh frozen plasma (FFP).

Right after admission to our Internal Medicine Unit, a sample for ADAMTS13 activity detection was obtained, whose results came to our attention few hours after admission and showed a normal activity (104%).

Even if PLASMIC score, that is, a score that predicts ADAMTS13 deficiency, in suspected thrombotic thrombocytopenic purpura (TTP) with high discrimination was high-intermediate, several aspects did not fit with a presumptive diagnosis of TTP, which was therefore put into doubt. In fact, the markedly reduced fibrinogen and the elevated plasma D-dimer concentrations, together with the normal ADAMTS13 activity, even if taking into account that the patient had undergone FFP infusion before testing are not typical of TTP. Plasma exchange (PEX) was, therefore, not performed, but oral therapy with prednisone 1 mg/kg body weight was started.

TABLE 1 | Biochemical parameters at the time of admission and dismission from the Internal Medicine Unit.

Biochemical parameters	At admission	At dismission
Hemoglobin (g/dl)	9.3	11.3
Mean corpuscular volume (fl)	96	94
Platelets count (per μl)	32,000	118,000
Reticulocyte count (per μl)	325,000	–
Total bilirubin (μmol/liter)	80	16
LDH (U/liter)	1200	872
Creatinine (μmol/liter)	96.8	57.2
Aptoglobin (g/liter)	<0.08	–
D-dimer (ng/ml)	800	–
INR	1.42	1.18
aPTT	1.07	1.03
Fibrinogen (g/liter)	0.5	1.57
ADAMTS13 activity (%)	104	101
Direct Coombs test	Negative	–
Indirect Coombs test	Negative	–
Blood smear for schistocytes count	20%	–

Considering also, in differential diagnosis, the hypothesis of a subacute disseminated intravascular coagulation (DIC), even if not fulfiling all the criteria, FFP infusion at low dosage (10 mL/kg/day) was started. Fresh frozen plasma was performed for four consecutive days and then, because of the normalization of PT-INR and aPTT with persistently reduced fibrinogen levels (<1 g/L), fibrinogen infusions were started and maintained for 1 week and eventually stopped, given the absence of either major or minor bleeding events.

To make a correct differential diagnosis of Coombs negative hemolytic anemias, other diagnostic hypotheses such as sepsis, endocarditis, and rheumatic diseases (*in primis* antiphospholipid antibody syndrome and systemic lupus erythematosus), paroxysmal nocturnal hemoglobinuria and folate/vitamin B12 deficiency needed to be excluded. Other primary thrombotic microangiopathies were not clinically probable, given the advanced age of the patient, the absence of prodromal diarrhea, and the normal renal function.

ADAMTS13 activity was subsequently repeated after 1 week, and it was confirmed to be normal. Also, ADAMTS13 inhibitors resulted not detectable.

Remarkably, elevated serum ferritin concentrations were observed (2900 μg/L) and therefore, given the presence of persistently reduced fibrinogen levels, elevated natural killer (NK) cells count (>1000 per μl, even if it is clearly not the right test to demonstrate an altered NK cells function), a bone marrow biopsy was performed showing a certain grade of hemophagocytic phenomenon.

Abbreviations: MAHA, microangiopathic hemolytic anemia; TTP, thrombotic thrombocytopenic; ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; QUART, quadrandectomy and radiotherapy; DIC, disseminated intravascular coagulation; PT, prothrombin time; aPTT, activated partial thromboplastin time; INR, international normalized ratio; MCV, mean corpuscular volume; vWF, von Willebrand factor; FFP, fresh frozen plasma; PEX, plasma exchange; PNH, paroxysmal nocturnal hemoglobinuria; GPI, glycosylphosphatidylinositol; NK, natural killer; PET, positron emission tomography; FDG, fludeoxyglucose; US, ultrasonography; CT, computed tomography; CD, cluster of differentiation; LDH, lactate dehydrogenase.

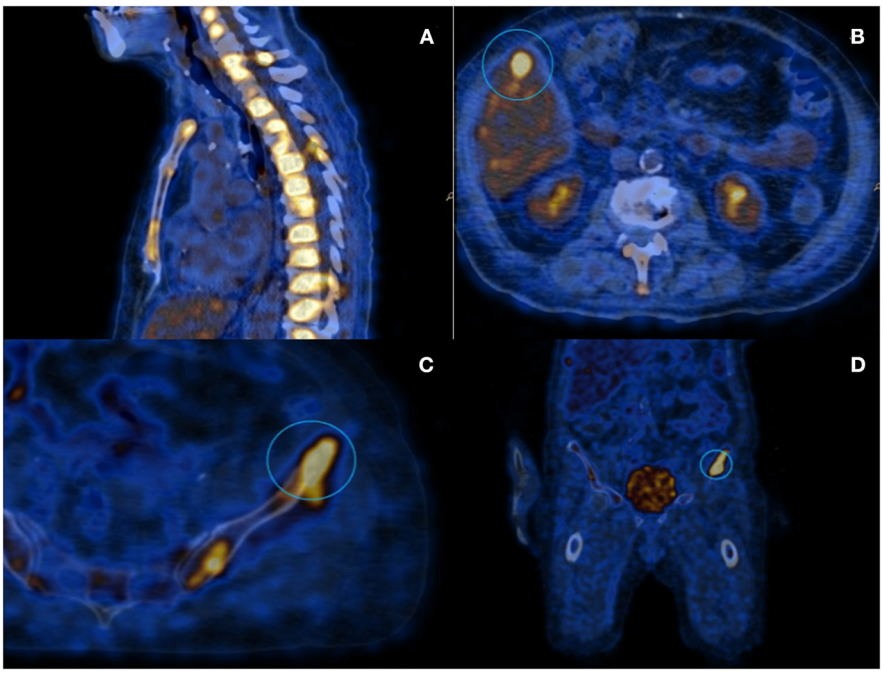

FIGURE 1 | PET scanning with 18-FDG: **(A)** Image showing diffuse hypermetabolism located at the spinal column; **(B)** Hepatic lesion of about 1 cm diameter; and **(C,D)** Iliac lesion that underwent to CT-guided needle-biopsy.

Even if neoplastic markers, including Ca-15.3, were normal, concerns arose for the presence of a diffuse relapse of breast carcinoma, and therefore scar and bilateral axillary ultrasonography, along with contralateral mammography, were performed, all of which were negative for neoplastic localizations. A PET/CT scan was also performed, which showed instead a diffuse fludeoxyglucose hypercaptation in almost all vertebral bodies together with other multiple focal bone lesions, and a unique hepatic lesion of about 1 cm diameter (**Figure 1**).

The hepatic lesion was too small to possibly perform a US-guided biopsy; therefore, with the support of our interventional radiologist, we decided to perform a CT-guided needle biopsy of the left iliac crest (**Figures 1C,D**), which resulted positive for the presence of neoplastic dedifferentiated cells expressing keratins 8-18-19 and GATA-3 at immunohistochemistry, compatible with metastatic breast carcinoma localizations (**Figure 2**).

The global performance status of the patient progressively and rapidly declined during the period of hospitalization, precluding the possibility of specific oncological therapies; hence, the patient was lastly dismissed to another hospital for palliative care, maintaining low dose of oral prednisone therapy (**Table 1**).

DIFFERENTIAL DIAGNOSIS

When approaching thrombotic microangiopathy (TMA) syndromes, terminology is a key element to understand the entire diagnostic process. Microangiopathic hemolytic anemia (MAHA) is a descriptive term for non-immune hemolysis (Coombs-negative hemolysis) resulting from intravascular red blood cell fragmentation that produces schistocytes on the peripheral blood smear where characteristic laboratory data include increased serum LDH, increased indirect bilirubin, and low serum haptoglobin (1, 2).

Thrombotic microangiopathies describes a specific pathologic lesion in which abnormalities in the vessel wall of arterioles and capillaries lead to microvascular thrombosis. Not all MAHA are caused by a TMA, but nearly all TMAs cause MAHA and thrombocytopenia (1, 2).

Thrombotic microangiopathies is a pathologic diagnosis made by tissue biopsy, typically a kidney biopsy; however, it is commonly inferred from the observation of MAHA and thrombocytopenia in the appropriate clinical setting.

Primary TMA syndromes include TTP (hereditary or acquired), Shiga toxin-mediated haemolytic uremic syndrome (ST-HUS), drug-induced TMA (DITMA) syndromes, complement-mediated HUS (hereditary or acquired), and rare hereditary disorders of vitamin B12 metabolism or factors involved in hemostasis (1).

As for the case of our patient, the presence of a microangiopathic hemolytic anemia and thrombocytopenia is, at a first glance, indicative of TTP.

However, the normal ADAMTS13 activity is a key element to exclude TTP, in which it is almost always below 10% (3, 4). Complement-mediated hemolytic uremic syndrome (HUS) typically presents with an impaired renal function, and so does Shiga-toxin-associated HUS; in the latter case, TMA is usually preceded by diarrhea, abdominal pain, and fever. No new drugs had been recently introduced by the patient, so even the diagnosis of a drug-induced TMA was unlikely.

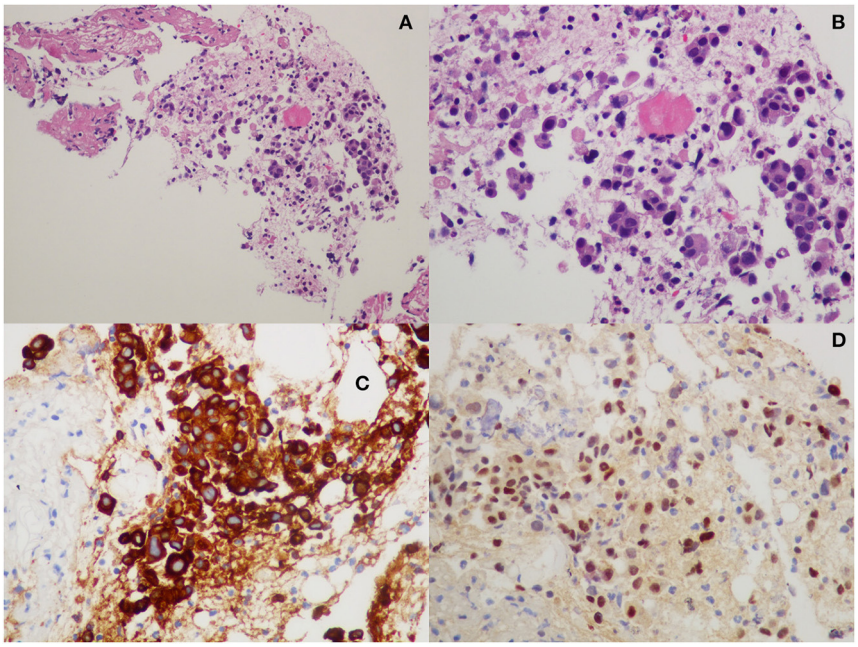

FIGURE 2 | Histopathological examination from iliac bone biopsy: **(A,B)** hematoxylin-eosin staining; **(C)** cytokeratin-8 immunohistochemistry; and **(D)** GATA-3 immunohistochemistry.

In megaloblastic anemia, ineffective erythropoiesis mimics MAHA, because of the elevated indirect bilirubin, elevated LDH, and reduced aptoglobin concentrations. In such a case, the increased MCV is the key factor to guide the correct diagnosis. Thrombocytopenia and leucopenia are also generally present, but the degree of thrombocytopenia is usually not as severe as in TTP. Dosage of serum vitamin B12 plus autoantibodies to intrinsic factor in the case of pernicious anemia and folate concentration are then confirmatory.

Hemolytic anemia in Evans syndrome and antiphospholipid antibody syndrome is not due to microangiopathy, and its pathogenesis is invariably autoimmune then featuring a Coombs-positive hemolytic anemia.

In the case of paroxysmal nocturnal hemoglobinuria, in which a Coombs-negative hemolytic anemia is present, thrombocytopenia is due to a reduced medullary production in the context of an aplastic anemia rather than a peripheral consumption; in such a case the diagnosis is confirmed by flow-cytometry for glycosylphosphatidylinositol (GPI)-linked proteins is the confirmatory test.

Moreover, in TTP, clot formation is very rarely dependent by the coagulation cascade, being the clot is almost entirely built of platelets and the von Willebrand factor (vWF). The prolonged PT-INR, the reduced fibrinogen, and the elevation of D-dimer plasma levels are suggestive of DIC, but DIC is not generally associated with the presence of schistocytes in the peripheral blood smear (5). Moreover, in this case, the antithrombin-III (AT-III) levels were normal, making the differential diagnosis even more challenging.

Taking into consideration all of these biochemical parameters, together with the diagnosis of metastatic breast cancer relapse, we think that the diagnosis of cancer-related microangiopathic hemolytic anemia (CR-MAHA) is the most appropriate to solve the case.

Finally, some case reports of hemophagocytic syndrome presenting as thrombotic microangiopathy are reported in the literature, and the possible overlap even of a secondary form of hemophagocytic syndrome made really hard the right interpretation of this aspect (6, 7).

The elevated ferritin levels were interpreted as the epiphenomenon of a cytokine storm, a very complex life-threatening systemic inflammatory syndrome involving elevated blood concentration of cytokines and a hyperactivation of immune cells triggered by several clinical conditions, such as monogenic and autoimmune disorders, among which are *in-primis* hemophagocytic lympho-histiocytosis (HLH), infectious diseases (including COVID-19), autoimmune pathologies, various therapies, and even cancers (8). In the case described here, the hemophagocytic phenomena observed at the bone marrow biopsy were very limited, and in the absence of more specific tests, such as the dosage of soluble CD25 and the evaluation of NK cells activity, the diagnosis of a secondary HLH could not be formulated with absolute confidence.

DISCUSSION AND LITERATURE REVIEW

Endothelial dysfunction in cancer is an established event (9), and CR-MAHA, especially in cases of widespread metastasis of a malignant tumor, is a rare but well-described condition (10). Cancer-related microangiopathic hemolytic anemia has been reported in association with several types of carcinomas as

observed in a report of 168 cases of CR-MAHA, where gastric cancer was the most frequent, followed by breast, prostate, and lung cancer (10, 11). Even if CR-MAHA in breast cancer does not seem to be associated with a specific histological type, ductal infiltrating carcinoma is the most commonly reported (10). The pathophysiology behind MAHA in malignancy is yet not well-understood, even if it is thought to be rather a paraneoplastic syndrome than an independent condition co-existing with cancer disease. Two mechanisms have been proposed: red cell fragmentation against tumor emboli within blood vessels and an increased vWF multimers release from bone marrow (the latter in response to altered angiogenesis and secondary myelofibrosis) (12). Cancer-related microangiopathic hemolytic anemia must not be confused with chemotherapy-induced MAHA, which is a specific form of drug-induced TMA, particularly frequent when using gemcitabine and mitomycin, and can be both immune and non-immune mediated.

In a 2011 case of a series of eight breast-cancer associated TMAs, ADAMTS13 activity was normal or slightly reduced, and basic coagulation parameters (PT-INR and aPTT) were only minimally altered, exactly as in the case of our patient (13). von Willebrand factor levels were elevated, but it is known that vWF is also an acute phase reactant and that vWF plasma levels are elevated in case of inflammation and many other physical stressful conditions.

The most effective treatment in CR-MAHA is cancer-specific chemotherapy, while PEX is almost useless, and the prognosis is generally very poor (10). Similarly, treatment of secondary hemophagocytic syndrome is based on the specific oncologic treatment, which was unfortunately not possible for our patient due to a degraded performance status, so the choice was directed toward an adequate palliative care.

CONCLUSIONS

We presented the case of an 82-year-old woman with CR-MAHA secondary to a metastatic breast cancer relapse. The main clinical challenge related to this case was to collect in a unifying diagnosis the ample and diverse set of all symptoms and laboratory findings presented by the patient together with the urgency of a prompt diagnosis to decide the most appropriate therapy for a critically ill patient.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because this is a case report and a dataset has not been generated. Requests to access the datasets should be directed to simonetta.friso@univr.it.

ETHICS STATEMENT

Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

NO and SF conceived the manuscript, performed analysis and interpretation of clinical data, and wrote the manuscript. GB, MG, LG, EP, AS, AP, MC, FP, and OO performed interpretation of clinical data and contributed to manuscript writing. All authors contributed to the article and approved the submitted version.

REFERENCES

1. George JN, Nester CM. Syndromes of thrombotic microangiopathy. *N Engl J Med*. (2014) 371:654–66. doi: 10.1056/NEJMra1312353
2. Shen Y. Clinical evaluation of thrombotic microangiopathy: identification of patients with suspected atypical hemolytic uremic syndrome. *Thromb J*. (2016) 14(Suppl. 1):19: 151–8. doi: 10.1186/s12959-016-0114-0
3. Bendapudi PK, Li A, Hamdan A, Uhl L, Kaufman R, Stowell C, et al. Impact of severe ADAMTS13 deficiency on clinical presentation and outcomes in patients with thrombotic microangiopathies: the experience of the Harvard TMA Research Collaborative. *Br J Haematol*. (2015) 171:836–44. doi: 10.1111/bjh.13658
4. Bendapudi PK, Hurwitz S, Fry A, Marques MB, Waldo SW, Li A, et al. Derivation and external validation of the PLASMIC score for rapid assessment of adults with thrombotic microangiopathies: a cohort study. *Lancet Hematol*. (2017) 4:157–64. doi: 10.1016/S2352-3026(17)30026-1
5. Lesesve J, Martin M, Banasiak C, Andr E. Schistocytes in disseminated intravascular coagulation. *Int J Lab Hematol*. (2014) 36:439–43. doi: 10.1111/ijlh.12168
6. Bae MN, Kwak DH, Park SJ, Choi BS, Park CW, Choi YJ, et al. Acute kidney injury induced by thrombotic microangiopathy in a patient with hemophagocytic lymphohistiocytosis. *BMC Nephrol*. (2016) 17:1–6. doi: 10.1186/s12882-015-0217-z
7. Chiang WC, Wu M-S, Tsai C-C, Lin S-L, Tsai T-J, Hsieh B-S. Microangiopathy in hemophagocytic syndrome: a Case report. *J Formos Med Assoc*. (2002) 101:362–7.
8. Fajgenbaum DC, June CH. Cytokine storm. *N Engl J Med*. (2020) 383:2255–73. doi: 10.1056/NEJMra2026131
9. Blann AD. Endothelial cell activation markers in cancer. *Thromb Res*. (2012) 129:122–6. doi: 10.1016/S0049-3848(12)70031-2
10. Takabatake D, Oishi K. Microangiopathic hemolytic anemia associated with metastatic breast cancer: case report and literature review. *Springerplus*. (2016) 5:684. doi: 10.1186/s40064-016-2312-4
11. Lechner K, Obermeier HL. Cancer-related microangiopathic hemolytic anemia. *Medicine*. (2012) 91:195–205. doi: 10.1097/MD.0b013e3182603598
12. Lee EH. Hemolytic anemia of malignancy: a case study involving signet ring cell metastatic breast cancer with severe microangiopathic hemolytic anemia. *Case Rep Oncol*. (2019) 12:104–8. doi: 10.1159/000494753
13. Regierer AC, Kuehnhardt D, Schulz C-O, Flath B, Jehn CF, Scholz CW, et al. Breast cancer-associated thrombotic microangiopathy. *Breast Care*. (2011) 6:441–5. doi: 10.1159/000335201

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