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Original Article

Clinical Signs and Treatment of New-Onset Bone Marrow Failure Associated SARS-CoV-2 Infection in Children: A Single Institution Prospective Cohort Study

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Abstract. ***Background*: Viral infections can cause direct and indirect damage to hematopoietic stem cells. The objectives of this study were to identify the frequency and severity of aplastic anemia in children infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as well as recognize the response to treatment.**
***Methodology*: 13 children with newly diagnosed severe aplastic anemia were enrolled in this prospective clinical trial. Blood samples were obtained from all patients to detect SARS-CoV-2 antibodies, and nasopharyngeal swabs were collected for reverse-transcription Polymerase Chain Reaction to detect SARS-CoV-2 viruses. According to the laboratory results, patients were classified as having SARS-CoV-2 positive antibodies and SARS-CoV-2 negative antibodies. Both groups received combined cyclosporine (CsA) + Eltrombopag (E-PAG). The hematological response, either complete response (CR) or partial response (PR), no response (NR), and overall response (OR) rates of combined E-PAG + CsA treatment after 6 months were evaluated.**
***Results*: Four children were recognized to have aplastic anemia and SARS-CoV-2 positive antibodies. Two patients fulfilled the hematological criteria for CR and no longer required transfusion of packed red blood cells (PRBCs) or platelets, and one had PR and was still PRBC transfusion-dependent but no longer required platelet transfusion. The remaining patient showed NR, and he had died before reaching the top of the HSCT waiting list. Moreover, six patients in the SARS-CoV-2 negative antibodies group had CR, while three patients had PR. The difference in ANC, Hg, and platelet counts between both groups was not significant.**
***Conclusion*: The SARS-CoV-2 virus is added to several viral infections known to be implicated in the pathogenesis of aplastic anemia. Studies are needed to establish a definitive association and determine whether the response of bone marrow failure to standard therapy differs from that of idiopathic cases.**

Keywords: Bone marrow failure; SARS-CoV-2 infection; Children.

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Introduction. Acquired aplastic anemia (AAA) in childhood is a serious disorder characterized by pancytopenia and hypocellular bone marrow.[1] It is a rare disorder with an incidence of about 2 per 1,000,000 children per year in North America and Europe and 2–3 fold higher in Asia with an equal male-to-female ratio.[2]

It has to be differentiated from inherited bone marrow failure syndromes (IBMFS), which are more common in children.[3]

The pathophysiology of AAA is unknown; it has been suggested to be immune-mediated. Several studies have verified the increased cytokine expression, low CD4 T regulatory cells, oligoclonal CD8 cytotoxic T cells, and expansion of specific CD4 cell subsets in the bone marrow (BM).[4-6]

Viral infections can cause direct and indirect damage to hematopoietic stem cells (HSCs). King and Goodell demonstrated four different mechanisms by which viruses can alter HSC biology.[7] Two mechanisms act via direct effects on HSCs, including direct infection or direct recognition of a pathogen. Two indirect mechanisms act through pro-inflammatory cytokines released by other cells or through changes in the BM microenvironment.

Viral infections that directly altered the HSCs include Epstein–Barr virus (EBV), varicella-zoster virus (VZV), cytomegalovirus (CMV), human immunodeficiency virus (HIV), human herpes virus 6 (HHV-6), hepatitis A and C viruses (HAV and HCV), dengue and parvovirus B19.[8-11]

Indirect damage of the HSCs resulting from acute or chronic viral infection has often been attributed to the immune response against viruses, with IFNγ and CD8+ T cells having a key role. The hematopoietic cells are the target of oligoclonal CD8+ T cells, which produce IFNγ and TNFα and cause its death. Otherwise, constant secretion of these pro-inflammatory cytokines can also deplete HSCs, thus leading to aplastic anemia[12]. Nevertheless, bone marrow pathologies are rare and frequently related to changes in gene regulation of cytokines, effector, and MHC molecules, which suggest a genetic basis for abnormal T cell activation in BM failure.[11,13] AAA is presenting clinically with easy bruising or petechiae, epistaxis, and menorrhagia in postmenarchal girls due to thrombocytopenia. Anemia may manifest as pallor and fatigue, while neutropenia may predispose to infections.[14]

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel coronavirus. It is named COVID-19 and infected millions of people worldwide since its outbreak in 2019.[15]

It is a systemic disease and classically presents with flu-like systemic and/or respiratory symptoms. Severe cases presented with acute respiratory distress syndrome (ARDS) and Multi-organ failure, which is the major cause of COVID-19-related death.[16] Moreover, one-third of patients with COVID-19 are asymptomatic.[17] SARS-CoV-2 infection begins by sticking the viral surface spike glycoprotein (S protein) to the angiotensin-converting enzyme-2 (ACE2) for initiation of cell entry. Also, it downregulates cell surface ACE2 expression, leading to loss of the enzyme mediate protective function.[18] The expression of ACE2 has been stated in a variety of cells, including HSCs.[19]

Several researchers have verified the profound effect of COVID-19 disease on the hematopoiesis system either by direct invasion of HSCs by SARS-CoV-2, which may explain the mechanism of hypoxia or hyper-inflammation and cytokine release, which might be related to activation and proliferative of the hematopoietic system.[20,21] All this leads to significant changes in the hematopoietic system, including stress erythropoiesis, lymphopenia, neutrophilia, and thrombocytopenia.[20,21]

To the best of our knowledge, no study has been piloted to confirm the existence of aplastic anemia in children with COVID-19 infection. Hence, the objectives of this study were to identify the frequency and severity of aplastic anemia in COVID-19-infected children and how it is related to the severity of infection, as well as recognition of the response to treatment.

Materials and Method. This study was a prospective, single-center clinical trial conducted at Assiut University Children's Hospital in Egypt. It enrolled all children with newly diagnosed aplastic anemia admitted to the clinical hematology unit between Jun 2021 and December 2022. All enrolled children fulfilled the eligibility criteria[22] and met the modified Camitta criteria for severe aplastic anemia (SAA).[23] According to these criteria, a diagnosis of SAA may be made if bone marrow cellularity is <25% and/or at least two of the following criteria are met: (i) the absolute neutrophil count is below 0.5×10^9/L, (ii) the platelet count is below 20×10^9/L, (iii) the reticulocyte count is below 20×10^9/L.

The exclusion criteria were inherited bone marrow failure, myelodysplasia, underproduction anemias secondary to B12, folate or iron deficiency, or with other reversible causes.

All patients were subjected to a detailed history regarding drug intake, radiation exposure, and recent history of infections. All patients subjected to complete medical history, physical examination, and laboratory evaluations included, including a complete blood count (CBC) with differential, serum chemistry, bone marrow aspiration and biopsy, viral serology, immunological tests, flow cytometric tests, a diepoxybutane clastogenic stress assay, and HLA typing. All patients were assessed for an inherited bone marrow failure syndrome, including chromosomal breakage examination for Fanconi anemia.

Blood samples were obtained from all patients to

detect SARS-CoV-2 antibodies, and nasopharyngeal swabs were collected for reverse-transcription Polymerase Chain Reaction (RT-PCR) to detect SARS-CoV-2 viruses.

The study was permitted by Assiut University's Ethical Committee for Clinical Research, and informed consent was obtained from the guardians of trial participants before the study.

PCR. All patients were subjected to PCR-RNA for SARS-CoV-2 (COBAS6800, Roche, India Qiagen, Germany) for use on the cobas® 6800 System. Nasopharyngeal and oropharyngeal swab samples were collected on 0.9% physiological saline. Selective amplification of target nucleic acid from the sample was achieved using specific forward and reverse primers for ORF1 a/b non-structural region that is unique to SARS-CoV-2. The pan-Sarbecovirus detection sets also detected the SARS-CoV-2 virus. Amplification of RNA Internal Control was achieved using non-competitive sequence-specific forward and reverse primers, which have no homology with the coronavirus genome. DNA polymerase enzyme was used for amplification.

Detection of antibodies to SARS-CoV-2. A three ml blood sample was obtained from all participants on gel and clot activator tubes for separation of sera for SARS-CoV-2 antibody testing. Detection of SARS CoV 2 total antibody was performed by Elecsys Anti SARS CoV 2 kit Lot No. 49546401 (Germany) supplied by Roche based on electrochemiluminescence immunoassay "ECLIA" using cobas e 411 immunoassay analyzers.

Treatment plan. According to the PCR and antibodies to SARS-CoV-2 detection results, patients were categorized into two groups: SARS-CoV-2 positive antibodies and SARS-CoV-2 negative antibodies. Because of the long list of patients in need of hematopoietic stem cell transplantation (HSCT) in our country and the anti-thymocyte globulin (ATG)is unavailable for economic reasons, both groups received combined cyclosporine (CsA) + Eltrombopag (E-PAG). The initial oral dose of E-PAG was 50 mg once daily. The dose was escalated by 25 mg every two weeks in all patients and then maintained at the maximum dose when it was reached. The maximum dose was 150 mg.[25] Adjustments and reductions of the E-PAG dose were made where necessary based on the pharmacokinetic data for ITP.[24] Oral CsA was initiated at 5–10 mg/kg/day, and the dose was adjusted to maintain trough levels of 170–270 ng/ml.[24] Supportive therapy was allowed for both groups during the study when essential. It included granulocyte colony-stimulating factor (G-CSF), iron chelation, or platelet transfusion (if the count was <10,000/μL with an apparent bleeding tendency or <20,000/μL with fever) and red blood cell (RBC) transfusion (if hemoglobin was <7 g/dL or in the presence of significant symptoms, such as exertional dyspnea or anemic heart failure). The hematological response, either complete response (CR)or partial response (PR), no response (NR), and overall response (OR) rates of combined E-PAG + CsA treatment after 6 months were evaluated, using the standard guidelines for the diagnosis and treatment of pediatric SAA.[25]

Response criteria. A hematological response was defined as a platelet count increase of at least 20 000/μL and/or platelet transfusion independence for a minimum of eight weeks, a hemoglobin level increase of at least 1.5 g/L or a reduction in the number of PRBCs units transfused by at least four for eight consecutive weeks (compared with transfusion requirements during the eight weeks preceding study treatment onset) and an increase of absolute neutrophil count (ANC) of >500/μL in patients with a pre-treatment count <500/μl. A PR was defined as a blood count no longer meeting the Camitta criteria[22] for SAA and no transfusion dependence for platelets or red blood cells. A CR was defined as Hb levels of ≥100 g/l, a platelet count ≥100 × 10^9/L, ANC of ≥1 × 10^9/L, and transfusion and growth factor independence. Overall response rates included all PR and CR within each group.

Statistics. Statistical Package for Social Science version 20. A T-test calculator for 2 Independent Means was used to detect the statistical differences between both groups. Descriptive statistics were expressed as frequencies and percentages for categorical data. Fisher Exact Test was used to detect the statistical differences in categorical variables. Data represented as means ±SD. The probability value of <0.05 is considered statistically significant.

Results. Thirteen patients (6 boys and 7 girls) fulfilled the inclusion criteria. The age at presentation ranged from 4.4 to 17.7 years (median 9.3 years) (**Table 1**).

Four children (30.7%) without prior hematologic diseases or SARS-CoV-2 vaccination were recognized to have aplastic anemia and SARS-CoV-2 positive antibodies (**Table 2**).

The first patient was a 15-year-old boy who presented with ecchymosis, epistaxis, and mild fever and was found to have pancytopenia. He did not have any prodromal symptoms before the onset of pancytopenia. His workup showed negative SARS-CoV-2 PCR and negative hepatitis, cytomegalovirus (CMV), Epstein-Barr virus (EBV), HIV, and PNH panel. Additionally, no nutritional deficiencies were found. SARS-CoV-2 total antibodies were the only test positive test in this patient.BM biopsy revealed severe hypoplasia (5%).

The second was a 7-year-old boy; he had a high-grade fever, sore throat, cough, and abdominal pain nine weeks

Table 1. Demographic characteristics and complete blood count (CBC) parameters of aplastic anemia patients included in the study.

	Age	Sex	WT	HB	ANC	PLTs	SARS CoV 2 total antibody	RT-PCR
1	2.5	M	9	4.5	125	14	N	N
2	14	M	40	5.8	435	8	N	N
3	12	F	23	4.9	654	27	N	N
4	4	F	12	7.1	432	26	N	N
5	15	M	50	6.2	350	13	Post	N
6	5	F	17	4.8	435	18	N	N
7	16	F	40	3.9	509	22	N	N
8	2	F	10	6.8	453	55	N	N
9	3	M	16	5.8	123	12	N	N
10	15	F	46	7.1	211	12	Post	post
11	13	M	30	5	498	22	Post	N
12	7	M	20	4.6	361	8	Post	N
13	10	F	42	5	298	32	N	N
	9.12±5.4		27.3 ±14.8	5.5 ±1.1	355.07 ±125	21.07±12.6		

Table 2. Clinical and laboratory parameters for SARS-CoV-2 positive antibodies groups.

	1St Patient	2nd Patients	3rd Patient	4th Patient
Sex	M	M	F	M
Age	15	7	15	13
Prior hematologic diseases	IDA	NO	No	NO
Prodrome	Absent	Fever, cough, abd pain	Fever, URI, GI	Absent
Interval to onset of AA	-	9 weeks	Synchronous	-
Initial presenting symptoms	ecchymosis, epistaxis, mild fever	Severe pallor, fatigue, gastrointestinal bleeding	Menorrhagia, ecchymosis and pallor	pallor and fatigue
Severity of aplasia	SAA		SAA	
BMB cellularity	5%	10-20%	<10%	10%
HIV	- ve	- ve	- ve	- ve
CMV	-ve	-ve	-ve	-ve
EBV	-ve	-ve	-ve	-ve
PNH clones	Negative	Negative	Negative	Negative
History of autoimmune disease	Negative	Negative	Negative	Negative
U/S	Normal	Normal	Normal	Normal
SARS CoV 2 total antibody	Positive	Positive	positive	Positive
SARS-CoV-2 PCR	Negative	Negative	positive	Negative

IDA, Iron deficiency anemia. URI, upper respiratory infection.

before the onset of pancytopenia. This patient presented to the emergency unit with severe pallor, fatigue, and gastrointestinal bleeding, which necessitated urgent transfusion of PRBCS and platelets. CBC revealed pancytopenia, and other investigations (liver function, renal function, abdominal ultrasound, CRP, ESR, were unremarkable. A bone marrow biopsy verified hypocellularity (10%-20%) with all lineages without signs of malignancy or megaloblastic changes (**Table 2**).

The third patient was a 15-year-old girl. She had a fever, chest infection, and mild gastroenteritis for 3 days, followed by massive menorrhagia, ecchymosis, and pallor. She had not experienced a similar attack previously. She had Synchronous pancytopenia at the time of diagnosis of SARS-CoV-2 infection by positive PCR testing and positive total immunoglobulins. She was diagnosed with SAA according to the results of the bone marrow biopsy (< 10 % cellularity).

The fourth patient was a 13-year-old male with absent prodromal symptoms before cytopenia, but he had a positive family history of SARS-CoV-2 infection. He presented with marked pallor and fatigue. Bone marrow biopsy results confirmed the diagnosis of severe aplastic anemia (<10 % cellularity).

No PNH clone or evidence of inherited bone marrow failure syndrome was recognized in all SARS-CoV-2 antibodies-positive children. Additionally, all were negative for other viral testing.

Table 3. CBC, Transfusion rate and response after 6 months of treatment in the SARS-CoV-2 positive antibodies.

	1St Patient	2nd Patients	3rd Patient	4th Patient
Hg (g/dl)	9.6	10.9	11.3	6,4
PLT (109/L)	67	123	112	19
ANC (cell/ul)	986	1432	1232	645
RPCs transfusion rate	Every 6 ws	No need	No need	Every 4 weeks
Platlets transfusion	- No need	No need	No need	- Every 3 weeks
Response	PR	CR	CR	NR

Table 4. CBC Follow up and response rate before and after 6 months of treatment in both groups.

	SARS-CoV-2 positive antibodies (N=4)			SARS-CoV-2 Negative antibodies (n=9)			*P- Value*
Before treatment							
Hg (g/dl)	5.72±3.91			5.4±9			*0.3*
PLT (109/L)	13.75±5.9			23.78±14			*0.1*
ANC (cell/ul)	355±117.25			384.8±174.42			*0.32*
After 6 months of treatment							
Hg (g/dl)	9.55 ±1.9			8.8±1.97			0.29
PLT (109/L)	80.3±41			102.6±30.7			0. 170
ANC (cell/ul)	1073.75 ±293.6			1417.7±1101			0. 29
Response	CR N (%)	PR N (%)	NR N (%)	CR N (%)	PR N (%)	NR N (%)	
	2(50%)	1(25%)	1(25%)	6 (66.6%)	3 (33.3%)	0	
Overall survival at 6 ms	3 (75%)			9(100%)			*0.307

CR complete response, PR partial response, Or overall response, NR; no response. p-Value is calculated to compare both groups using T-Test Calculator for 2 Independent Means. *P-Value is calculated to compare the CR rate in both cohorts using Fisher Exact Test.

Transfusion rate and Treatment results after 6 months. The hematological response of the SARS-CoV-2 positive antibodies after six months of treatment was recorded in **Table 3**. Two patients (50%) fulfilled the hematological criteria[26] for CR and no longer required transfusion of packed red blood cells (PRBCs) or platelets. One (25%) more patient had a partial response (PR) and were still PRBC transfusion-dependent but no longer required platelet transfusion. The remaining patient (25%) did not meet any of the response criteria, and he had died before reaching the top of the HSCT waiting list.

Moreover, six patients in the SARS-CoV-2 negative antibodies group had CR (66.6%), while three patients (333.3%) had PR. The difference in ANC, Hg, and platelet counts between both groups was not significant (**Table 4**).

Discussion. Acquired aplastic anemia is a rare disorder characterized by damage to progenitor cells caused by chemicals, drugs, ionizing radiation, viral infections, or autoimmune destruction. The majority of aplastic anemia cases (70-80%) are idiopathic, with viral infections representing only a small portion of these cases.[27,28]

Among the 13 cases of newly diagnosed aplastic anemia admitted to our unit within 18 months duration, we report 4 cases associated with COVID-19 infection. A recent large case series of new-onset AA in adults associated with preceding SARS-CoV-2 infection was reported.[29]

In another case series conducted with Avenoso et al., they reported that three adults were diagnosed with AA a few weeks after SARS-CoV-2 infection.[30] Hock et al. also reported severe aplastic anemia in a 21-year-old man infected with SARS-CoV-2.[30,31]

Moreover, Rohini Chakravarthy et al. confirmed the diagnosis of SAA in a 12-year-old girl and an 18-year-old male. Both patients received anti-thymoglobulin (ATG) and cyclosporine and became transfusion independent.[32]

Viral-induced bone marrow suppression is well established, and different mechanisms have been proposed for its occurrence, including directly influencing the replication of hematopoietic stem and progenitor cells (HSPCs) or indirectly by inducing different patterns of cytokines and chemokines such as IL-1ß, IL-6, TNF-α, and INF-γ, which cause Fas-

mediated apoptosis. Direct destruction of HPSC by viruses has been observed as well.[33]

The SARS-CoV-2 infection has a significant impact on the host's immune system. Variable degrees of lymphopenia (CD3 + T, CD4 + T, or CD8 + T cells) are observed in mild to moderate COVID-19 phenotypes; however, the decline in T lymphocyte count considerably worsens in severe cases.[33-35]

In most cases, pancytopenia is mild and transient and does not necessitate bone marrow examination.[36]

In the present study, one patient did not show any response and was dependent on transfusion until death. Ranjima et al.[36] reported that a 4-year-old girl developed aplastic anemia after COVID, which did not respond satisfactorily to treatment, and became a candidate for bone marrow transplantation. Fatemeh et al.[37] described a 16-year-old girl who developed severe aplastic anemia with COVID infection and did not respond well to treatment, even though she received supportive treatment and immunosuppression.

Poor response to treatment is predicted by low CD8 + T and B cell numbers and a high CD4/CD8 ratio. In the phenotypes of serious diseases, the production of interferon-gamma (IFN-γ) by CD4 + T lymphocytes is also diminished by.[38,39]

Another rare outcome of SARS-CoV-2 in children associated with abnormal innate and adaptive immune responses is known as multisystem inflammatory syndrome (MIS), which is characterized by a cytokine storm.[40,41] Even though the effects of SARS-CoV-2 infection on host immune responses are well documented, bone marrow-induced aplasia (BM) is less well understood. The exact mechanism behind COVID-19 could induce bone marrow aplasia has not yet been fully explained, but it is multifactorial.

Numerous cases of severe central pancytopenia related to COVID-19 have been reported.[42-44] While most cases were transient and did not require bone marrow biopsies, the four cases in our study, with observation, did not demonstrate spontaneous resolution of peripheral cytopenias and marrow hypocellularity, arguing against a diagnosis of viral myelosuppression. Some other case series have shown that SARS-CoV2 may cause bone marrow failure requiring immunosuppressive therapy (IST) or even hematopoietic stem cell transplantation (HSCT).[30-32]

The role of autoimmune cytotoxic T-cell-related hematopoietic cell destruction causing aplastic anemia in COVID-19 patients is still undiscovered. For COVID-19 to trigger immune-mediated bone marrow failure, infection should precede pancytopenia by weeks to months. Half of our patients had an unknown duration between the onset of infection and pancytopenia (Patients 1 and 4). These patients had negative PCR but positive SARS-CoV-2 antibodies, suggesting distant infection. One of the remaining two patients had an infection 9 weeks before pancytopenia, while the last one (Patient 3) had pancytopenia 3 days after the onset of COVID-19 symptoms.

Regarding management and follow-up, all of our patients were treated with immunosuppression (Cyclosporin) plus Eltrombopag with variable response. Two patients had variable responses. Two other cases (Patients 2 and 3) showed a complete response, one patient showed a partial response (Patient 1), and one did not respond at all and died (Patient 4).

The response to Cyclosporin and whether the addition of eltrombopag improves the treatment results in these patients are also issues. It has been assumed that eltrombopag can potentiate the effect of thrombopoietin (TPO) in vivo, overcoming the suppressive effect of IFN-γ upon TPO signaling in hematopoietic stem cells.[45]

Overall, the epidemiological data and prior SARS-CoV-2 infection estimated to have preceded the development of pancytopenia by weeks to months support the theory that SARS-CoV-2 may be causally associated with AA. Besides, regardless of disease phenotype (even in asymptomatic cases), SARS-CoV-2 infection could be associated with severe aplastic anemia. This study does not establish a mechanistic link between COVID-19 infection and marrow failure; however, the clinical course and response to Cyclosporin have led to our hypothesis that this novel coronavirus may mediate an immune response or, less likely, a direct marrow toxicity that contributes to the pathogenesis of SAA.

Conclusions. We report one of the largest case series to date of the new onset of SAA in paediatrics, presumably associated with preceding SARS-CoV-2 infection and their clinical outcomes. Considering the results of this study, the virus is added to several viral infections known to be implicated in the pathogenesis of aplastic anaemia. More studies are needed to establish a definitive association and determine whether the natural history and response of bone marrow failure to standard therapy differ from that of idiopathic cases.

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