Case Report

Symptoms of Acute Appendicitis Masquerading Overlapping with Distal Intestinal Obstruction Syndrome in Adult Cystic Fibrosis

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Commented [A1]: Thanks for providing this opportunity to assist you with this manuscript. I have edited the text for language, grammar, and improved clarity. I have also checked the manuscript for conformance with the formatting guidelines provided. In the cases where additional information is required from you, I have added comments to bring them to your attention. Should you have any concerns, please feel free to get back to me. My best wishes for your success with the manuscript.

Commented [A2]: Please note that Case Reports in Gastrointestinal Medicine encourages authors to use the CARE checklist to improve the reporting of their case report. Therefore, please consider following this checklist. I have included comments where information could be included as per these guidelines.

Commented [A3]: I have revised this according to the reviewer’s comment. However, I have also included “symptoms of” at the beginning to make the title clearer.

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Abstract

Overshadowed by Sino-pulmonary infections, With the improved life expectancy in Cystic Fibrosis (CF) patients, there has been an increase in gastrointestinal organ manifestations because of secretory and motility dysfunction. Infrequently, these changes can result in Distal Intestinal Obstruction Syndrome (DIOS), an increasingly diagnosed gastrointestinal condition in adult CF patients. We present the case of a 22-year-old male who presented to our hospital with right lower quadrant abdominal pain. Despite the suspicion of acute appendicitis, the patient and was subsequently diagnosed with DIOS. Our case highlights the importance of considering DIOS as a differential diagnosis for right lower quadrant abdominal pain in CF patients, especially for physicians working at community hospitals that may not have a CF care program available.

1. Introduction

Cystic Fibrosis (CF) is a genetic disease that affects multiple organs. With advancements in the management of CF patients, patients can now often survive to adulthoods [1]. However, the improved life expectancy among adult CF patients has led to an increase in extrapulmonary, notably gastrointestinal, manifestations, which previously were uncommon. Distal Intestinal Obstruction Syndrome (DIOS) continues to be a rising complication in adult CF patients, presenting as acute abdominal pain like and mimicking an acute abdominal emergency.

We report the case of a 22-year-old man with a history of CF who presented to our hospital with right lower quadrant abdominal pain. Despite the initial suspicion of acute appendicitis, he was subsequently diagnosed with DIOS.

2. Case Report Presentation

A 22-year-old Turkish-origin male with a past medical history of Cystic Fibrosis CF presented with a one-day
history of right lower quadrant abdominal pain. He described a sharp periumbilical pain that continued to worsen, which then shifted to the right lower quadrant of the abdomen. Prior to the onset of the abdominal pain, he reported experiencing nausea and anorexia for three days. His last bowel movement was two days prior to admission. The patient was also diagnosed with Cystic Fibrosis at the age of four, and the disease progressed to exocrine pancreatic insufficiency, which was being treated with pancreatic enzymes. Upon reviewing the patient’s past history, it was noted that he had several episodes of pneumonia, for which he was appropriately treated with antibiotics. Notably, no history of constipation or recurrent abdominal discomfort was reported prior to this. At home, the patient was prescribed Albuterol inhalation as needed, Pseudomona Alfa inhalation, Aztreonam lysine nebulization, 500 mg Azithromycin three times a week, Lansoprazole, Lumacaftor-Ivacaftor twice a day, Lipase-protease-amylase capsule three times a day, and a multivitamin capsule once a day. The patient was also diagnosed with Cystic Fibrosis at the age of four and his disease progressed to exocrine pancreatic insufficiency, which was being treated with pancreatic enzymes. On abdominal examination, he was found to have diminished bowel sounds and tenderness on right lower quadrant with equivocal rebound tenderness on the right lower quadrant. Laboratory analysis showed leukocytosis (white blood cell count WBC 13.0 mm/Kg, neutrophils count, 62%) with a normal differential. He had no electrolyte imbalances. Computed tomography (CT) of the abdomen revealed thickening and edema around the terminal ileum, inflammatory changes in the colon, with inflammatory changes, free fluid in the right paracolic gutter adjacent to the cecum, an appendix measuring 5.3x4.6 mm, and reactive lymph nodes (Figures 1 and 2).
FIGURE 1: Axial abdominal computed tomography scan depicting thickening around the terminal ileum and colon (yellow arrows) along with extraluminal fluid and reactive lymph nodes.

FIGURE 2: Coronal view computed tomography scan showing thickening of the ileum with a distended appendix (yellow arrows).

Due to extraluminal fluid and cecal wall edema with inflammation, early acute appendicitis could not be excluded as a possible diagnosis. Surgical intervention was performed, which revealed a ruptured microperforation of a cecal diverticulum and a distended appendix in chronic adhesions, for which he required an appendectomy and partial cecectomy with an intact ileocecal valve. Postoperatively, he was diagnosed with DIOS and was subsequently started on Polyethylene glycol. The patient made an unremarkable recovery and was discharged home to be...
followed up in the outpatient clinic without and did not have any recurrence of any symptoms.

3. Discussion

Due to the improved life expectancy of CF patients, DIOS is now being increasingly diagnosed in adult patients with CF. Distal Intestinal Obstruction Syndrome (DIOS) was called a Meconium Ileus equivalent in the past, described by the collection of viscid fecal material within the lumen combined with sticky mucoid intestinal content adherent to the intestinal wall of the terminal ileum and cecum [1]. Perez-Aguilar et al. reported that the prevalence of DIOS was 19.5% (mean age 20.6 years) among 46 CF patients in a retrospective analysis, while Dray et al. conducted a cross-sectional study reporting a 15.8% (mean age 28.9 years) prevalence in 171 CF patients in a cross-sectional study [2, 3]. Despite the fact that there continues to be a limited assessment of the prevalence of DIOS in adult CF patients, DIOS is considered more common among adults compared to children due to increased disease progression.

5. Distal Intestinal Obstruction Syndrome (DIOS), previously known as was called a Meconium Ileus equivalent, in the past, described is characterized by the collection of viscid fecal material within the lumen combined with sticky mucoid intestinal content adherent to the intestinal wall of the terminal ileum and cecum [1]. Defective intestinal chloride and water secretions into the gut, luminal acidity, and loss of bile salt all contribute to the development of DIOS [1]. These patients characteristically present with right lower quadrant pain, nausea, abdominal distension, and failure to pass stools or flatus [1, 3]. In some patients, a palpable right lower quadrant mass may be appreciated present that may be confirmed on abdominal radiography X-ray [1]. Though abdominal X-rays are recommended to aid in the diagnosis of DIOS, they are inadequate in differentiating ileus from other causes of abdominal pathologies that may present in Cystic Fibrosis CF patients [4]. Due to the proximity of the anatomical locations, as well as the overlapping clinical presentations, appendicitis and intussusception may mimic DIOS. This which further leads to diagnostic uncertainty. Overlap of several intra-abdominal pathologies in CF increases the risk of misdiagnosis, especially with acute appendicitis, as these patient’s underlying pathologies may be masked in CF patients with pulmonary infections using antibiotics, as seen in our case [5, 6].

Commented [A18]: As per CARE guidelines, consider including follow-up diagnostic and intervention adherence if possible.

Commented [A19]: Please note, while the discussion section discusses the known facts about the condition, there has been no mention of the present case and how the case relates to the existing literature on the topic. I have therefore included this here to put the case in context to the literature. Please review this addition. Ideally, this discussion of the characteristics of the condition should be included in the Introduction section and this section should discuss this case in the context of previously reported cases.

Commented [A20]: I have added this sentence from the introduction to add some context about DIOS before discussing its prevalence. Please review this change and ensure that you include the relevant citation here.

Commented [A21]: I believe this sentence would be more appropriate here as this paragraph describes the characteristics and diagnosis of DIOS.

Commented [A22]: I have deleted this sentence as it was a repetition of the previous sentence (i.e. overlapping pathologies between appendicitis and DIOS leading to misdiagnosis). Please review these changes to ensure you agree with them.
Osmotic laxatives are the cornerstone of bowel regimens for the treatment of DIOS. The most commonly prescribed laxative is polyethylene glycol (PEG) administered at a dose of 20–40 ml/kg/h, up to a maximum of 1 L/kg/h for a total of 8 hours, resulting in achieving fecal effluent consisting of clear fluid, along with the resolution of abdominal pain and constipation [1, 6]. If the diagnosis remains unclear, and thus, requires surgical intervention, ileocecal valve resection should be considered to prevent the development and recurrence of intestinal obstruction sequelae and growth, especially in adolescents [7].

With the increase in immigration of foreigners into America, inner-city and community hospitals may not be sufficiently equipped with a Cystic Fibrosis CF care center; moreover, these hospitals may not have programs in provision, with expertise available to other clinicians involved in patient care. Therefore, our case highlights the significance of considering DIOS as a differential diagnosis in patients with a history of CF presenting with right lower quadrant abdominal pain and constipation, particularly in hospitals without a CF care program available.

**Consent**

Informed consent was obtained from the patient for this case report.

**Conflicts of Interest**

**Authors’ Contributions**

All authors contributed to the revision and approval of the manuscript.

**Acknowledgements**

**References**


Title: Acute Appendicitis Masquerading Distal Intestinal Obstruction Syndrome in Adult Cystic Fibrosis

Thank you for submitting the above manuscript. Below please find a list of comments and changes to be made to the manuscript. Please make all necessary revisions and email us the revised manuscript.

We encourage you to send your revision within 45 days.

When submitting your revision please include the following items:

- A rebuttal letter that responds to each point brought up by the academic editor and reviewer(s) as a 'Response to Reviewers' file.
- A clean revised manuscript as your 'Manuscript' file.
- A marked-up copy of the changes made from the previous article file as a 'Revised Manuscript with Track Changes' file. This can be done using 'track changes' in programs such as MS Word and/or highlighting any changes in the new document.

Thank you for your thoughtful suggestions and insights, which have enriched the manuscript and produced a better and more balanced account of the research. The manuscript has been rechecked and appropriate changes have been made in accordance with the suggestions. The responses to the comments have been prepared and are given below. We hope that the revised manuscript is now suitable for publication in your journal.

Major points:

1. The title of the case is misleading. Since the patient had both appendicitis and DIOS, there was no ‘masquerading’.
   Response: Thank you for your comment. We have revised the title to “Symptoms of Acute Appendicitis Overlapping with Distal Intestinal Obstruction Syndrome in Adult Cystic Fibrosis”

2. The novelty of the case is not apparent from the Introduction. If DIOS is already associated with acute abdominal pain and abdominal emergency, what is the novelty here? Response: Thank you for your comment. Our case is novel because we used the patient’s cystic fibrosis (CF) history to diagnose distal intestinal obstruction syndrome (DIOS), despite the initial diagnosis of appendicitis. Therefore, we were able to successfully treat the patient accordingly. We would like to highlight that physicians should consider a diagnosis of DIOS for abdominal pain in CF patients.

3. There is no clear conclusion to the case. Please add a concluding sentence to the manuscript.
   Response: Thank you for your suggestion. We have added the following sentence at the end of the Discussion of the revised manuscript.
   “Therefore, our case highlights the importance of considering DIOS as a differential diagnosis in patients with a history of CF presenting with abdominal pain and constipation.”

4. Was consent obtained from the patient for publishing this case? Response: Thank you for your query. We have included a section on patient consent in the manuscript.

5. The Introduction doesn’t flow well into the Case Report. End of the Introduction section should have introduction of case.
   Response: Thank you for your suggestion. We have added the following sentence at the end of the

Commented [A30]: Thank you for sending in your responses to the reviewer comments for editing. I have edited the responses for language, checked that they adequately answer the reviewers’ questions, and included comments where additional information is needed. I have also checked the manuscript to ensure that the required changes have been made to the manuscript. I have also prepared a response to the general comments for you to use during resubmission.

Commented [A31]: I have changed the title of the manuscript accordingly and included “symptoms of” at the beginning of the title for better clarity.

Commented [A32]: Is this the first case to report on this? If so then please consider stating this, as this would highlight its novelty.

Commented [A33]: I have included this in the revised manuscript.

Commented [A34]: Please specify whether the patient consented to the publication of this case report e.g. “Informed consent was obtained from the patient for the publication of this case report” in the manuscript. I have included a placeholder in the manuscript for where this information should be included.
Introduction of the revised manuscript: "We report the case of a 22-year-old man with a history of CF who presented to our hospital with right lower quadrant abdominal pain. Despite the initial suspicion of acute appendicitis, he was subsequently diagnosed with DIOS."

Minor points:
1. The yellow arrows in the figure are not visible. Please use thicker arrows to point to the relevant sections.
Response: Thank you for this suggestion. We have updated the figures.

Commented [A35]: I have included this in the revised manuscript.
Commented [A36]: Please consider specifying how the figures have been updated i.e. were the yellow arrows made more visible?
Methylmalonic acidemia (MMA) is a rare inherited metabolic disorder caused by deficiency of the enzyme methylmalonyl-CoA mutase. The disease is characterized by early infancy by lethargy, vomiting, failure to thrive, and encephalopathy and is fatal if left untreated. A 5-years-old boy presented with poor weight gain and recurrent episodes of fever, feeding problems, and lethargy, since from the age of 11 months, and poor weight gain. He was admitted to our hospital and evaluated for metabolic disorders; he was suspected and diagnosed with methylmalonic acidemia (MMA). He was treated with vitamin B12 and carnitine supplements and has been on followed up for the last three years. Mutation analysis by next generation sequencing (NGS), and supplemented with Sanger sequencing, revealed two novel variants in exon 5 and 3 of the MUT gene responsible for the MMA in exon 5 and exon 3. Recently, he developed dystonic movements including orofacial dyskinesia. Our results indicate the necessity of genetic testing if MMA is suspected; it can confirm the diagnosis, aid in selecting treatment options, and may enrich the panel of responsible gene variants.

1. Introduction

Methylmalonic acidemia (MMA) is a rare inherited metabolic disorder caused by deficiency of the enzyme methylmalonyl-CoA mutase. MMA presents with lethargy, acidosis, hypoglycemia/hyperglycemia, ketosis, and recurrent episodes. MMA due to mutations in the MUT gene muts, which encodes the enzyme methylmalonyl-CoA mutase, usually leads to severe phenotypes, and around 35–40% of the cases are due to novel mutations [1, 2]. There can be missense or nonsense mutations, deletions, insertions, and so on in the MUT gene and so on leading to a clinical phenotype, and these...

Variables of the MUT gene encoding the enzyme methylmalonyl-CoA mutase are responsible for about 60% of MMA cases; mut0 and mut- phenotypes are caused by complete and partial enzyme deficiency, respectively. Variations in the MMAA, MMAB, MMADHC, and MCEE genes are required for normal functioning of methylmalonyl-CoA mutase.

Here, we report the case of a 5-years-old boy with MMA who presented with poor weight gain and recurrent episodes of fever, feeding problems, and lethargy, since from the age of 11 months, and poor weight gain. This case is novel because we who was found that the patient had to have two previously unreported mutations in the MUT gene.

Abstract

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2. Case Presentation

A 5-year-old boy presented for the first time at the age of 11 months, with complaints of fever, vomiting, poor feeding, and lethargy since the age of 11 months. We observed that the patient had pallor and tachypnea and was drowsy. Laboratory tests suggested that the patient had high anion-gap metabolic acidosis with ketonuria (urine ketones 3+) and ketonuria (urine ketones 3+) and normal electrolytes, blood sugar (94 mg/dL), vitamin B12, and homocysteine levels. Plasma ammonia and plasma lactate were 118 units and plasma lactate was 2.9 units, respectively. Transcranial magnetic simulation results were normal, but urine gas chromatography mass spectrometry (GC-MS) analysis of urine revealed elevated 3-OH propionic acid [12.39 retention time (RT)] and as well as elevated methyl malonic acid levels.[16.92 RT, Suppl. Figure 1, in Supplementary Material available online at https://doi.org/10.1155/2017/8984951]. Since then, the patient was on a low-protein diet, carnitine, biotin, thiamine, and vitamin B12 injections. Child was thereafter admitted to the hospital on seven multiple occasions (7 times) with acute decompensation and managed as per protocol. Mutational analysis was sent for MMA which showed a single heterozygous missense variant c.976 A>G (p.Arg326Gly) in exon 5 of the MUT gene (genomic coordinates: chr 6: 49421405); as a variant of uncertain significance. Chromosomal microarray analysis done did not reveal any major deletion or duplication that could disrupt the gene. Since exon 3 and exon 6 were not adequately covered by next generation sequencing (NGS), further evaluation by Sanger sequencing for targeted exons was performed, and a second mutation in exon 3 c.753 G>A (p. =) was identified. The variants were predicted to be as damaging by the SIFT database score (Suppl data) and as deleterious by Polyphen-2 and Mutation Taster, but they were absent and not found in the ExAC database. Brain MRI brain of the patient (done at the age of 4 years) was showing multifocal cystic encephalomalacic changes with surrounding gliosis in deep white matter predominantly in frontoparietal regions (Figure 1). During the latest admission of the patient to the hospital, we observed child was found to have fresh neurological findings in the form of perioral tremors, generalized hypertonia, and generalized dystonia with clonus with exaggerated deep tendon reflexes. The patient was treated with intravenous dextrose and sodium bicarbonate and was continued on carnitine and injection of vitamin B12 injections. Plasma ammonia and plasma lactate levels were 18 units and lactate level was 4.9 units, respectively. Brain magnetic resonance image MRI brain of the patient was repeated and revealed bilateral basal ganglia hyperintensities, suggestive of metabolic stroke. After the subsidence of acute crisis, he was discharged on carnitine, injection of vitamin B12 injections, and trihexyphenidyl. The parents were counseled regarding the patient's prognosis and possible prenatal diagnosis for subsequent pregnancies.
3. Discussion

The advent of NGS technology has enabled better characterization of mutations in several populations. In this study, we used NGS and Sanger sequencing to identify mutations in the genes linked to MMA development and revealed two novel variants of the MUT gene. In a Saudi study on 60 MMA patients, nonsense, missense, and frameshift mutations were detected across the MUT gene [3]. Another study in 43 Chinese patients identified 8 recurrent mutations and 10 novel mutations in the MUT gene [4]. A previous Indian study involving 15 patients with clinically diagnosed MMA identified one novel exon 12 mutation in the MUT gene with predicted pathogenicity. Here, we identified two novel variants, one in exon 3 and another in exon 5 of the MUT gene, both labelled as variants of unknown significance (VUS). The exon 3 variant is a synonymous variant, and a different nucleotide change c.753 G>C (p.Lys251Asn) has been reported earlier in ClinVar. Some synonymous variants can also affect the splicing or protein function and lead to clinical phenotypes. The identified exon 5 variant is novel, but another close variant c.977 G>A (p.Arg326Lys) has been reported in ClinVar. The variants were found to be deleterious on bioinformatic analysis and were absent not found in the ExAC database. Both variants identified in the present case could be responsible for possibly explaining the MMA phenotype of MMA in the child. MUT-related MMA has poor prognosis in most cases. Specialized diet and supplements may not improve the outcomes, even if MMA is diagnosed early. Early recognition and appropriate treatment of acute crises are necessary. Metabolic stroke can sometimes occur in the absence of acute metabolic decompensation; thus, meticulous neurological examination at each visit is important. The treatment options for MMA include early liver transplantation [5], and possibly gene therapy could also be used in the future. Genetic counseling and prenatal diagnosis could help these families in making informed reproductive decisions.
Conflicts of Interest
The authors declare that there are no conflicts of interest regarding the publication of this article.

Acknowledgments
The authors would like to acknowledge Dhiti Omics Technologies Pvt Ltd for help in mutation analysis.

References


The report presents an interesting case of methylmalonic acidemia (MMA) potentially caused by two newly identified mutations in the MUT gene. The study contributes to the array of mutations relevant to MMA and deserves publication. However, the manuscript has several flaws, which should be addressed before re-submission.

**Major issues**

**Abstract:** the background and clinical implications are missing.

In the background, the subject of the study should be introduced in 1-2 short sentences explaining what MMA is, including disease causes and clinical symptoms. The Abstract should end with the take-home message indicating the importance of genetic testing in cases suggestive of MMA; it should be patient-oriented rather than method-oriented. The statement “With advent of NGS, judicious use of NGS with Sanger sequencing can help identify causative possibly pathogenic mutations” is trivial because it is obvious that without sequencing (either NGS or conventional Sanger), mutations cannot be identified; rather, it should be concluded that if MMA is suspected, genetic testing is necessary to identify the type of the disease, which would aid in applying the most appropriate treatment.

**Response:** As per the reviewer’s suggestion, we added the following introductory sentences:

“Methylmalonic acidemia (MMA) is a rare inherited metabolic disorder caused by deficiency of the enzyme methylmalonyl-CoA mutase. The disease is characterized in early infancy by lethargy, vomiting, failure to thrive, and encephalopathy and is fatal if left untreated.”

The conclusion of the abstract was modified as follows: “Our results indicate the necessity of genetic testing if MMA is suspected; it can confirm the diagnosis, aid in selecting treatment options, and may enrich the panel of responsible gene variants.”

**Introduction is totally missing.**

Every case report must be preceded by a concise Introduction section (1-2 paragraphs) presenting the disease and stating the importance of the case. The Introduction should describe MMA as an inherited autosomal recessive disorder caused by deficiency in the conversion of methylmalonyl coenzyme A (CoA) to succinyl CoA, resulting in the accumulation of methylmalonic acid in the blood to toxic levels. The manifestations of the disease should be described, including symptoms, age at the onset, severity range, treatment algorithms, and prognosis. The frequency of this disorder in the general population should also be indicated.

In the next paragraph, the genetic spectrum of the disease should be characterized, including the affected genes and related metabolic changes. Thus, it should be mentioned that variants of the MUT gene encoding the enzyme methylmalonyl-CoA mutase are responsible for about 60% of MMA cases; mut0 and mut- phenotypes caused by complete and partial enzyme deficiency, respectively, should be mentioned considering that the described case probably presented the mut- phenotype responsive to B12 supplementation. Variations in the MMAA, MMAB, MMADHC, and MCCE genes required for normal functioning of methylmalonyl CoA mutase should also be mentioned as they account for the rest of MMA cases.

Finally, the novelty of the present MMA case should be introduced by stating that the patient had two previously unreported mutations in the MUT gene.
Response: We thank the reviewer for the recommendation. Accordingly, in the revised manuscript, we have added the Introduction section wherein we have explained the genetic nature of MMA, mentioned the genes involved, and described the relevant biochemical mechanisms. Clinical manifestations, age at onset, incidence of the disease, and treatment regimens have also been described in this section. The presentation of the case has been justified by stating that our patient demonstrated MMA symptoms, which could have been caused by novel variants of the MUT gene.

Case presentation is incomplete.
Response: Thank you for pointing this out. In the revised manuscript, we have presented the results indicative of high anion gap. Units for plasma ammonia and lactate should be provided. Currently, it is difficult to assess the status of these parameters as they can be expressed in conventional units or SI units; accordingly, the numerical values can differ. I recommend the authors to convert all test results to SI units for consistency and provide reference ranges for all biochemical parameters.

Response: As per your suggestion, we have converted the levels of plasma ammonia and lactate into SI units and have indicated the reference ranges.

Actual concentrations of propionic acid and methylmalonic acid should be provided.
Response: In the revised manuscript, concentrations of propionic and methylmalonic are provided.

It should be explained how the diagnosis of MMA was made and whether alternative diagnoses such as propionic acidemia were considered, as both conditions are manifested by ketones in the urine and high blood ammonia levels; furthermore, a high level of propionic acid was detected.

Response: The symptoms observed in the child (tachypnea, recurrent vomiting, poor feeding, lethargy) were characteristic of MMA and were confirmed by biochemical testing. Propionic acidemia was ruled out as we did not find mutations in the PCC genes, which encode propionyl-CoA carboxylase. There is no explanation of specific treatment decisions. In particular, B12 injections should be justified, considering that there are two types of MMA differing in the sensitivity to cobalamin supplementation, responsive and nonresponsive, and that B12 levels in the patients were normal, i.e., there was no B12 deficiency.

Response: Patients with all forms of MMA (mut0, mut-, and cbl types) are routinely treated with a low-protein diet and respond well to carnitine supplements, as they typically develop carnitine deficiency. Our patient also had severe lactic acidosis, most likely due to thiamine deficiency, and elevated levels of propionic acid, which is indicative of dysfunctional propionyl-CoA carboxylase requiring biotin; therefore, he was administered thiamine and biotin. Although not all forms of MMA are responsive to cobalamin, we prescribed B12 supplements to the patient, as it is considered the first-line treatment for MMA.

NGS results: MMA is a recessive condition, i.e., only homozygous variants result in the phenotype, whereas heterozygous variants are asymptomatic. Therefore, the reported heterozygous missense variant c.976 A>G (p.Arg326Gly) in exon 5 of the MUT gene cannot be responsible for MMA in this patient. This fact should be discussed.
Response: According to the recommendation of the reviewer, we have added the following sentences: “Since MMA is a recessive autosomal disorder, the newly found heterozygous missense variant in exon 5 of the MUT gene cannot be responsible for the symptoms of the patient. However, it is known that over 180 missense mutations in the MUT gene have been linked to the severe (mut0) MMA form; therefore, the newly identified missense mutation should be further investigated for its functional significance.”

Line 45: The test results of plasma ammonia and lactate should be converted to the SI units and interpreted. Plasma ammonia levels significantly decreased but lactate levels increased in parallel with deterioration of neurological conditions of the patient.

Response: According to the recommendations of the reviewer, we have mentioned in the manuscript that plasma ammonia level significantly decreased, whereas plasma lactate level increased as compared to that in the original test results, and the overall condition of the patient worsened.

Lines 50-51: What was the prognosis for this patient? It should be indicated. Considering that the parents must be carriers of the MMA-causing mutation(s), were they asked to undergo genetic testing?

Response: Monitoring of the patient for several years – from 11 months to 5 years – revealed the progression of the disease. This was evident from the appearance of additional neurological symptoms at the age of 4 years; therefore, the prognosis was not favorable. We recommended the parents to undergo genetic testing and counseling before considering the next pregnancy. This information has been added to the revised manuscript.

Lines 52-55: This paragraph describing MMA should be moved to the Introduction.

Response: We thank the reviewer for the recommendation. The paragraph has been modified and moved to the Introduction.

Lines 61-64: The description of comparative advantages of NGS and Sanger sequencing is trivial and not directly relevant to the subject of the case report, which is identification of new mutations relevant to MMA. This paragraph should be reduced to the statement that the mutations were identified by NGS as well as by Sanger sequencing.

Response: According to the recommendation of the reviewer, this part was shortened as follows: “In this study, we used NGS and Sanger sequencing to identify mutations in the genes linked to MMA development and revealed two novel variants of the MUT gene.”

Line 77: The variant in exon 5 is heterozygous and cannot be responsible for MMA symptoms as MMA is a recessive inheritance pattern and the mutation should be homozygous to cause the phenotype. The statement regarding the exon 5 variant should be corrected.

Response: We modified the sentence as follows: “Since MMA is a recessive autosomal disorder, the newly found heterozygous missense variant in exon 5 of the MUT gene cannot be responsible for the observed clinical manifestations. However, it is known that over 180 missense mutations in the MUT gene have been linked to the severe (mut0) MMA form (Keyfi et al., 2016); therefore, this variant should be further investigated in in vitro and in vivo models to determine its functional effects.”

Implications of the study based on the specific findings of this case should be mentioned. How does the case contribute to the diagnosis and treatment options of MMA?

Response: Analysis of this case revealed that although the identified mutations do not seem
to cause severe deficiency in the production of any functional enzyme linked to MMA pathogenesis, the symptoms presented by the patient are serious and the prognosis is not favorable, suggesting that the genotype and clinical phenotype associated with MMA are not always concordant. Nevertheless, in cases when MMA is suspected, genetic testing is important to confirm the diagnosis and the most appropriate treatment should be prescribed. These considerations have been added at the end of the Discussion.

5. The language is poor. There are multiple grammar and stylistic mistakes, which must be corrected. The spelling should be consistent either with the American or British English but not be the mixture of the two.
Response: The manuscript has been revised by a professional English-speaking editor and all language-related errors have been corrected.

Minor issues:

- All abbreviations must be defined at the first mention both in the Abstract and the main text.

Response: All abbreviations have been defined, as recommended.
- Gene symbols ($MUT$) should be italicized.

Response: In the revised manuscript, all gene symbols are italicized.
Abstract
In this study, the present paper reports the use of $^{31}$P NMR spectroscopy to predict the isomer structures of bis-[4-methoxy-phenyl]-[3-(4-methoxy-phenyl)-allylidene]-amine-bis(triphenylphosphine)ruthenium(II) complex, also known as bis(di-p-anisole)-1,4-azabutadiene-bis[triphenylphosphine]ruthenium(II), complex, was synthesized using (di-p-anisole)-1,4-azabutadiene (compound 1), triphenylphosphine (PPh$_3$), and ruthenium chloride in the ratio of 2:2:1 for 5 h. The formation of the in addition, ruthenium(II) complex were was confirmed by also characterized using FTIR and UV–Vis spectroscopic analysis to support the formation of ruthenium(II) complexes. $^{31}$P NMR spectroscopy study on ruthenium(II) complexes suggested indicated the presence of that there are three isomers present after the complexation reaction. All the isomers of the ruthenium complex had demonstrate octahedral geometry.

Keywords: $^{31}$P NMR spectroscopy; FTIR spectroscopy; UV–Vis spectroscopy; Ru complex; Isomers; Structure prediction

1 NMR, nuclear magnetic resonance; FTIR, Fourier transform infrared; UV–Vis, ultraviolet–visible
2. Methodology

The ruthenium complexes were characterized using UV/Vis, FTIR, and 31P NMR spectroscopy. The IR spectra were recorded using a Thermo Scientific Nicolet iS10 spectrophotometer as KBr discs. The 1H NMR spectrum of compound 1 and 31P NMR spectrum of the ruthenium(II) complexes were recorded using a JEOL JNM-ECA 500 spectrometer with TMS as the internal standard. The UV-Vis spectra were recorded with a Jasco V-630 UV-Vis spectrophotometer.

2.1. Preparation of 4-Methoxy-phenyl)bis{[3-(4-methoxy-phenyl)allylidene]amine or (di-p-anisole)1.4-azabutadiene(1)

4-Methoxycinnamaldehyde (1.62 g, 10.00 mmol) was dissolved in 10 mL of ethanol, and followed by the addition of 10 mL of methanol, and then added to solution. The reaction mixture was stirred for 4 hours, and a yellow solid was obtained. The solid was filtered, washed with 5 mL of ethanol, and dried in vacuo.

2.2. Preparation of (Bis{[3-(4-methoxy-phenyl)allylidene]amine or (di-p-anisole)1.4-azabutadiene)-triphenylphosphine)ruthenium(II) Complexes

For the synthesis of bis{[di-p-anisole]1.4-azabutadiene-bis(triphenylphosphine)ruthenium(II) complex, RuCl3·3H2O (2.070 g, 1.0 mmol) and PPh3 (0.525 g, 2.0 mmol) were added to a round-bottom flask containing 10 mL of ethanol, and the mixture was then refluxed for 5 h. Compound 1 (0.316 g, 2.0 mmol) was then added to the round-bottom flask, and the mixture was refluxed for another 5 h. The reaction mixture was poured into ice, and the precipitate was filtered and dried. The resulting precipitate was identified as the complex.

Commented [A12]: I have deleted the section heading as the introduction, Experimental, Results and Discussion sections should be combined into a single untitled section.

Commented [A13]: The objective of the current study is not clear from the introduction. Please state a sound reason for the interest in synthesis of ruthenium(II) complexes. Also, please explain why the structure elucidation of these complexes is so important.

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Commented [A15]: The in-text citations are in the style prescribed by the journal.

Commented [A16]: In the present study, a Ru(II) complex is synthesized, the complexation is confirmed by UV-vis and FTIR spectroscopies, and the isoamides are identified by NMR spectroscopy. In general, this is a study on the synthesis and characterization of the complex by different techniques, and NMR is alone is not discussed exclusively (and neither in detail). Hence, suggest that the introduction be made more general. The focus should be on the importance of these complexes and the criticality of their structural elucidation rather than on NMR. Along with NMR, a brief description of UV-Vis and FTIR can also be included.

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577 (Ru-N), 2′P NMR (202.5 MHz, CDCl₃) δ: 49.7 (d, 1P, H₂), 47.4 (d, 1P, H₃), 47.1 (d, 1P, H₄), 39.7 (d, 1P, H₅), 35.1 (s, Ph-P=O), and 29.9 (s, 1P). UV-Vis (DCM) λ 321 and 382.

On the other hand, the IR binding of compound 1 to the ruthenium(II) metal center exists can be confirmed using FTIR and UV-Vis spectroscopy. The comparison of the IR spectra of compound 1 and the ruthenium complex reveals the vibrations of C=N and C=C stretching bands of compound 1 which are shifted with respect to those in the ruthenium complex. The C=N stretching band is up-shifted from 1627 cm⁻¹ to 1661 cm⁻¹ in the spectrum of the ruthenium complex. In contrast, the C=C stretching band is at 1610 cm⁻¹ in the spectrum of compound 1, but it is not clearly shown in the spectrum of the ruthenium complex because the IR bands of aliphatic and aromatic C=C bands are shifted due to the aromatic nature of the complex. The C=C bands of compound 1 are shifted to 1610 cm⁻¹ in the spectrum of the ruthenium complex.

The complexation of compound 1 to the ruthenium(II) metal center is not observable in the UV-Vis spectra of compound 1, while in the IR spectra, they are located on the aliphatic and aromatic regions at 1576 cm⁻¹. Nevertheless, the two additional peaks are present at 577 and 654 cm⁻¹ in the fingerprint region of the spectrum. This indicates the formation of the respective Ru-N and Ru-C bonds.

The characterization of the ruthenium complexes was done using UV-Vis, FTIR, and 31P NMR spectroscopy. The UV-Vis data was analyzed in Figure 5.3. In the case of compound 1, two absorption bands are observed at 273 and 372 nm, which are assigned to the transition of the benzene ring. The 31P NMR spectrum of compound 1 shows a single broad Ru–P=O band centered at 1576 cm⁻¹. Nevertheless, the two additional peaks are present at 577 and 654 cm⁻¹ in the fingerprint region of the spectrum. The IR spectra confirm the formation of the respective Ru-N and Ru-C bonds.

Figure 4: IR spectra of (a) compound 1 and (b) the ruthenium(II) complexes.

The singlet at 29.88 ppm reveals the presence of two PPh₃ units that are magnetically equivalent in the ruthenium(II) complex. There can be three possible structures based on this singlet. In the first case, the two PPh₃ units are located at the axial positions. In the second case, they are located on the equatorial plane, which is the most probable structure given the presence of the C=N and C=C stretching bands. In the third case, they are located on the apical sites in the octahedral complex. The presence of these units in the complex is supported by the IR spectrum of the ruthenium complex, which shows the absorption bands of the aliphatic and aromatic regions at 1576 cm⁻¹. The UV-Vis spectrum further supports this, as the bands are shifted due to the binding of the complex.

Figure 5: UV-Vis spectra of (a) compound 1 and (b) the ruthenium(II) complexes.

Figure 6: Results and Discussion

Characterization of the ruthenium complexes was done using UV-Vis, FTIR, and 31P NMR spectroscopy. The IR spectra confirm the presence of C=N and C=C stretching bands. The UV-Vis spectra of the complexes show absorption bands in the fingerprint region, indicating the presence of the ruthenium(II) center. The 31P NMR spectrum of the ruthenium complex shows a single broad Ru–P=O band centered at 1576 cm⁻¹. Nevertheless, the two additional peaks are present at 577 and 654 cm⁻¹ in the fingerprint region of the spectrum. The IR spectra confirm the formation of the respective Ru-N and Ru-C bonds.
The pair of doublets at 41.84 and 39.74 ppm with a coupling constant of 21 Hz is assigned to the cis isomer of the ruthenium(II) complex shown in Figure 5(a). Lastly, the other, another pair of doublets at 49.80 and 47.36 ppm with a coupling constant of 38 Hz is assigned to the trans-ruthenium(II) complex shown in Figure 5(b). It is evident that the two absorption bands shift up on the UV-Vis spectrum has shown the presence of three isomers of the bis(di-p-anisole)-1,4-azabutadiene]-bis(triphenylphosphine)ruthenium(II) complex. The single peak observed at 33.14 ppm is attributed to the presence of the triphenylphosphine oxide [138].

On the other hand, the binding of compound 1 to ruthenium(II) metal centre can be further supported by the IR and UV-Vis data as shown in Figure 5. For compound 1, two absorption bands were observed at 231 and 321 nm which are assigned to transition of the benzene ring and transition of the azine group [12]; respectively. After the complexation, both absorption bands shift to 231 and 323 nm, respectively. Significant shifts of these two absorption bands have proven compound 1 was successfully bound to ruthenium(II) metal centre via the nitrogen atom from C=N and the carbon atom from C=C-azine group in compound 1. This, in turn, has weakened the bond in C=N-C=C of compound 1. The other absorption bands were due to the backbonding of electrons from Ru to the antibonding orbitals of C=C-N moiety in compound 1. This, in turn, has weakened the bond in C=C-C=N of compound 1.

The UV-Vis spectra revealed the evidence from NMR spectrum has shown the presence of three isomers of the bis(di-p-anisole)-1,4-azabutadiene]-bis(triphenylphosphine)ruthenium(II) complex in the 1:1:1 ratio of Ru(II)-cis, trans, cis. Two of these isomers are those shown in Figure 5, i.e., one cis and one trans isomer, while the third isomer could be any one of those shown in Figure 5. In addition, the data from IR and UV-Vis revealed that compound 1 has bound to ruthenium(II) metal centre.
HIGHLIGHTS

- Three isomers were detected for a phosphine-bearing Ru complex using $^{13}$P NMR.
- Formation of Ru-N and Ru-C bonds were confirmed by FTIR spectroscopy.
- At least one cis isomer and one trans isomer of the complex were formed.
RESPONSES TO REVIEWER COMMENTS

This is a relatively simpler and brief report on structure identification of an organometallic complex using $^{31}$P NMR. There are certain missing gaps in this study. Though the topic of the study fits into the scope of the journal, I do not recommend its publication in the current form, however, the manuscript can be reconsidered for the same after major revisions.

Response: We thank you for your thoughtful suggestions and insights; the manuscript has benefited from these insightful suggestions. We have rechecked the manuscript and made the necessary changes in accordance with your suggestions (the revised portions have been marked in red font in the revised manuscript). Our responses to your comments are given below.

1. Some information is missing in experimental section describing the syntheses of compound 1 and the Ru complex. Details on the stirring of the reaction mixture are not complete. Particularly, the duration and temperature of stirring is critical in many syntheses, and this should be precisely mentioned for the easy reproducibility of experimental data. And, was the yield or nature of the product affected by the stirring speed/temperature/reaction time?

Response:
Thank you for noting this critical point. The reaction mixture for the synthesis of compound 1 was stirred for $4 \text{ h}$, while that for the synthesis of the Ru complex was stirred for $5 \text{ h}$. In both the cases, the temperature was maintained at $60 \degree \text{C}$. Two different stirring speeds (250 and 150 rpm) were tested for both the reaction mixtures. However, no significant difference in the yield or nature of the products was observed.

2. Corresponding to the singlet at 29.88 ppm, the authors mention the existence of two probable structures, i.e., a structure in which phosphines are located at the two axial positions or a structure in which the phosphines are located on the equatorial plane and are trans to the carbon atom from C=N. I think there could be another probable structure corresponding to this peak: a structure in which phosphines are located on the equatorial plane but are trans to N=C bonds. Please discuss this aspect.

Response:
Thank you for your insightful comments. We analyzed the spectrum again and found that the peak at 29.88 ppm could also correspond to a structure in which the phosphines are on the equatorial plane and trans to N=C. This corresponds to a cis configuration of the complex. We have now included this in the manuscript and also in Fig. 2. However, we still cannot assign any specific structure based on this singlet.

3. In the structures shown in Figure 2a and Figure 3b, the two phosphine ligands are trans to each other. Despite this, only one peak is observed for the former, while a doublet is observed for the latter. Please clarify this.

Response:
We thank you for this valuable comment. The magnetic fields of the two phosphine ligands shown in Fig. 3b are different because the two (di-p-anisole)-1,4-azabutadiene ligands are trans to each other on the equatorial plane. That is, the phosphine ligands shown in Fig. 2a are magnetically equivalent, while the ones shown in Fig. 3b are not. Thus, a singlet is observed for the former, while a doublet is observed for the latter. This aspect has now been discussed in the revised manuscript.

4. What solvent did the authors used for UV-vis spectroscopy? Although, bathochromic shift confirmed the bond formation to the metal center, authors have to mention probable reason for such a bathochromic shifting , particularly in the terms of electronic distribution. In addition, the shift of the first peak (273 nm to 321 nm) was significantly high than that of second peak (372 to 382 nm). Can authors give probable reason for this based on the bond formation to the metal center ?

Response:
All the samples for the UV–Vis spectroscopic measurement were prepared in water. After a careful survey of the literature, we have concluded that the bathochromic shift resulted from the backbonding of electrons from Ru to the antibonding orbitals of C=C-C=N in compound 1. That is, the addition of electrons to the antibonding orbitals weakens the C=C-C=N bonds, leading to the bathochromic shift. We have now discussed this aspect in the revised manuscript.

Commented [A37]: This comment has been appropriately addressed in the manuscript.
5. The authors may refer to the studies by Dharmaraj et al., Grushin et al., and Ahmed et al. on the metal complexes and cite them accordingly. In the title for the paper in the reference 13, “Complexation of bis-2-(benzylideneamino)phenol to cobalt(II) and zinc(II), and their spectroscopic studies,” studies should be studies.

Response:
We thank you for suggesting these valuable references. The information provided in these references has improved our understanding of the formation of metal complexes. We have now cited these references at relevant places in the manuscript. The title in reference 13 has also been corrected now.

6. The geometry of the complexes is not mentioned in the text.

Response:
We apologize for not mentioning the geometry of the isomers of the Ru complex. All the complexes were octahedral. We have mentioned this in the revised manuscript.

Commented [A49]: This has been appropriately addressed in the revised manuscript.

Commented [A50]: Changes were made here to improve the clarity and readability of this part. Please check whether the revised part retains the intended meaning.

Commented [A51]: Please note that the geometry was mentioned only in the abstract. I have now included this information in the main text too.