ESTIMATION OF PRE &POST-OPERATIVE LEVELS OF SERUM IRON AND ZINC IN PATIENTS WITH MUCORMYCOSIS AND TO EXPLORE THE POSSIBILITY OF THESE BEING USED TO PREDICT THE DISEASE ACTIVITY

THESIS

Submitted to

All India Institute of Medical Sciences, Jodhpur

In partial fulfilment of the requirement for the degree of

MASTER OF SURGERY (MS)

OTORHINOLARYNGOLOGY

JULY, 2020
AIIMS, JODHPUR

DR. HAGE DUNIYA

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DECLARATION

I, hereby declare that the work reported in the thesis titled - "**Estimation of pre & post-operative levels of serum iron and zinc in patients with mucormycosis and to explore the possibility of these being used to predict the disease activity**" embodies the result of an original research work done by me in the Department of Otorhinolaryngology, All India Institute of Medical Sciences, Jodhpur. I further state that no part of the thesis has been submitted, in part or in full, to any other University or Institute for the award of any other degree.

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CERTIFICATE

This is to certify that the thesis titled "**Estimation of pre & post-operative levels of serum iron and zinc in patients with mucormycosis and to explore the possibility of these being used to predict the disease activity**" is the bona fide work of Dr. Hage Duniya carried out under our guidance and supervision, in the Department of Otorhinolaryngology and Department of Biochemistry, All India Institute of Medical Sciences, Jodhpur.

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Certified that the project titled "**Estimation of pre & post-operative levels of serum iron and zinc in patients with mucormycosis and to explore the possibility of these being used to predict the disease activity**" is the record of research done in this department by **Dr. Hage Duniya**. She has fulfilled all the necessary conditions for the submission of this research work.

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Dr. Hage Duniya

CONTENTS

LIST OF ABBREVIATIONS

CAM	COVID Associated Mucormycosis
CAROCM	COVID Associated Rhino Orbito Cerebral Mucormycosis
ROCM	Rhino Orbito Cerebral Mucormycosis
RTPCR	Reverse Transcriptase Polymerase Chain Reaction
CTRI	Clinical Trial Registry - India
DNE	Diagnostic Nasal Endoscopy
TIBC	Total Iron Binding Capacity
UIBC	Unsaturated Iron Binding Capacity
TSAT	Transferrin Saturation
DFO	Desferrioxamine
ROC	Receiver Operating Curve

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INTRODUCTION

Mucormycosis, which is also referred to as zygomycosis, is a potentially fatal angio-invasive fungal infection that most frequently affects people who are predisposed by underlying comorbidities that affect immune system function, such as diabetes mellitus in ketoacidosis, other types of metabolic acidosis, treatment with corticosteroids and immunosuppressive medications, primary or secondary immunodeficiency, haematological malignancies, solid organ transplantation, iron overload, deferoxamine therapy, trauma and burns etc (1,2).

It is caused by fungi of the class Zygomycetes, specifically by organisms from the order Mucorales. The order Mucorales has 262 species in 55 genera, 38 of which have been linked to human illnesses (3). The species Rhizopus oryzae (Rhizopus arrhizus)is by far the most prevalent source of infection, accounting for around 70% of all cases of mucormycosis (1). Other isolated fungi come from the genera Lichtheimia, Mucor, Rhizo mucor, Cunning hamella, Saksenaea, etc. There are many mucormycosis agents, depending on the area. Among the genera examined in India, Apophysomyces species were the second most prevalent after Rhizopus (4).

Mucorales are widespread fungi that are typically found in tainted foods, compost, decomposing organic materials, and soil (5). They infect the host either through inhalation, ingestion, and/or direct inoculation of fungus spores through abraded skin as a result of burns or trauma. In medical settings, it has been established that the primary causes of primary cutaneous mucormycosis are contaminated surgical dressings and nonsterile adhesive tape (1). The organism enters the host by adhering to extracellular matrix proteins and/or possibly adhering to epithelial cells, escaping destruction by local phagocytes, and then progressing to infect the blood vessel and causing hematogenous spread (6).

It may affect various body systems and is thus classified as rhino cerebral, rhino orbital, sino nasal, pulmonary, gastrointestinal, cutaneous, disseminated and other rare forms such as renal, osteomyelitis, endocarditis, peritonitis etc (7).

The condition was initially identified in 1876 by Fürbinger, who wrote about a German cancer patient whose right lung had a haemorrhagic infarct with fungal hyphae and a few sporangia(7). Later Arnold Paltauf described the first instance of widespread mucormycosis in 1885, naming it "Mycosis mucorina" (7).

With the advent of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that caused coronavirus disease 2019 (COVID-19), there has been an emergence of a variety of opportunistic bacterial and fungal diseases (8). Over 169 million people worldwide have been affected by the coronavirus disease pandemic of 2019 (COVID-19), including over 27 million in India alone (9). It was also discovered that the exponential rise in the incidence of mucormycosis was linked to the soaring second wave of Covid-19 infection(10), especially among patients with uncontrolled hyperglycaemia, has been observed in many countries. The prevalence of mucormycosis in India was about 0.14 instances per 1000 people, which was nearly 80 times higher than the frequency of the disease in affluent nations(7). The second wave of Covid-19 pandemic attack has proven too much for India's healthcare system to handle. Atleast over 400,000 instances of Covid-19 were being recorded every day at its height in early May 2021(11).

Risk factors differ greatly depending on the location. In studies from Europe, haematological malignancy was the most prevalent underlying condition, but Diabetes Mellitus was prevalent in India, Iran, and Mexico(12). Haematological malignancies, with acute myeloid leukaemia are the most prevalent underlying diseases in developed nations and rising diabetes continues to be the primary underlying disease in developing nations. Although intravenous drug use is the primary risk factor for isolated cerebral mucormycosis, post-pulmonary TB and chronic renal disease are growing risk factors. It has also been observed that a considerable percentage of immunocompetent patients have developed mucormycosis in whom trauma or burns are typically the predisposing factors, leading to cutaneous illness. The trauma can range in severity from mild to major, including injection sites, animal bites, gardening, and surgery (13).

Numerous investigations have revealed a connection between the infection location and the underlying illness. The most common cause of pulmonary mucormycosis and neutropenia are linked to sinusitis and rhino cerebral mucormycosis in people with diabetes mellitus while cutaneous mucormycosis with trauma. Depending on the host's immune condition, the severity of the infection, and the organs implicated, mucormycosis patients can present with a variety of clinical symptoms, ranging from localised to disseminated illness. Immunosuppressed patients (such as those with haematological malignancies, and hematopoietic stem cell transplantation) are more likely to develop pulmonary or disseminated diseases, while diabetic patients are more likely to develop rhino-orbital-

cerebral diseases and patients who have experienced trauma are more likely to develop cutaneous diseases (7).

Diagnostic methods that take a clinical approach have poor sensitivity and specificity. Despite lack of specificity, some of the clinical symptoms may have a strong predictive value. Tissue necrosis brought on by thrombosis and angioinvasion is the defining feature of mucormycosis, but the lack of necrotic eschar does not rule out the diagnosis. Although molecular techniques are advancing and novel fungal genetic targets are being investigated, histopathology, direct observation, and culture remain crucial tools of diagnosis as of now (7).

The conventional treatment for invasive mucormycosis entails the reversal of the underlying predisposing conditions (if possible), urgent, extensive surgical debridement of the infected region, and concomitant antifungal therapy. The most recent recommendations strongly encourage the use of liposomal Amphotericin B (>=5mg/kg) as the first-line therapy in conjunction with surgery whenever possible. Amphotericin B, the first antifungal to be identified in 1953, is derived from Streptomyces Nodosus (14). It is an amphoteric polyene macrolide with 4–7 conjugated double bonds and an internal cyclic ester. In aqueous conditions, it is weakly soluble due to the hydrophobic polyene domain. Four commercially available formulations are available for the parenteral method of administration. These are: conventional amphotericin-b, amphotericin-b colloidal dispersion, liposomal amphotericin-b, and amphotericin-b lipid complex (15).

However, Amphotericin B's nephrotoxicity has led to the adoption of lipid formulations of the same, which are less nephrotoxic and can be given for a longer period of time. And isavuconazole and posaconazole given intravenously or with a delayed release have continued to be second-line treatments(16).

Unfortunately, despite disfiguring surgical debridement and concurrent antifungal medication, the death rate of mucormycosis is substantially higher than 50% overall and nearly 100% in patients with severe disease or protracted neutropenia (16).

Micronutrients like iron and zinc are essential cofactors in a variety of cellular processes in all living organisms. Except for a few unusual bacterial species, they are indispensable for every living organisms(17).

The pathogenetic process of the responsible organisms depend on the uptake of iron. However, serum iron is so tightly coupled to carrier proteins like transferrin in mammals, and is not readily available (18). Rhizopus oryzae fungus infects patients who have elevated levels of accessible serum iron (19). Therefore, inhibiting fungal iron uptake by sequestration of iron by serum can be one important host defensive method against R. oryzae in particular and can be a possible treatment approach to improve clinical outcomes of this fatal illness (6).

The second-most prevalent transition metal in the human body is zinc, but, like iron, only very little of it is available to microbial pathogens (13). Zinc is an essential trace element for all aspects of life. The expression of genes, correct protein folding, enzyme activity, and intracellular signalling are just a few biological activities that depend on it. Most microbial depend on these micronutrients for development and pathogenicity. So higher levels of elements like iron and zinc in serum are far more likely to be associated with pathogenic infestations like mucormycosis(20). But there are specific membrane transporters or proteins that bind zinc and mediate zinc uptake or storage, which helps to keep the zinc quota in check. And there is evidence that host cells use zinc sequestration to prevent microbial development in conditions where zinc levels are low. There are other suggested hypotheses, including the role of zinc supplementation and iron overload states in CAM that augments the growth of fungus (21).

With this study, we want to evaluate the fluctuations in the level of serum iron and zinc in mucormycosis patients in connection to the disease activity. The study will also investigate whether levels of iron and zinc can be utilised to forecast the activity of the disease.

REVIEW OF LITERATURE

Coronavirus disease 2019 (COVID-19) is a contagious disease caused by a virus, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)which quickly spread worldwide resulting in a Pandemic. The first known case was identified in Wuhan, China in December 2019. The virus had a zoonotic origin and spread via direct and contact transmission. Fever, cough, and myalgia are the main symptoms of the symptomatic phase, which sometimes progressed to various multisystem problems such as shortness of breath, anosmia, ageusia, diarrhoea, acute cardiac injury and secondary infections. The diagnosis was verified by Reverse transcriptase PCR (RTPCR) test. And the main treatment was supportive therapy and, in more severe cases, mechanical ventilation was given. Along with effective illness isolation and community containment, preventive methods play a vital role in decreasing the public transmission of viruses (22).

By April 2021, as India was dealing with the second wave of the COVID-19 pandemic, a sudden rise in the prevalence of mucormycosis cases, a typically rare disease in COVID patients who were either still sick or had recently recovered from the virus attracted significant attention and became a public health threat. Due to the association of mucormycosis with COVID-19 disease, it has been termed COVID-19-associated mucormycosis (CAM). Because of angioinvasion and subsequent tissue necrosis caused, it gave the tissue a black appearance, and so the infection was more commonly recognised as black fungus. The mortality rate varied between 40% and 80% and was based on the infection site and underlying health issues (23).

Mucormycosis is an angio-invasive fungal infection caused by fungal species belonging to Rhizopus, Apophysomyces, Rhizomucor, Lichtheimia, Cunninghamella, Mucor and Saksenaea. Globally, the most common causative agent of mucormycosis is *Rhizopus arrhizus*. Other Rhizopus species involved in causing mucormycosis infection are R. homothallicus and R. microsporus. In addition to Rhizopus, Apophysomyces is considered as the second most common fungal Genus causing mucormycosis infection in Asia(24). Various other species, including Lichtheimiaramosa, Thamnostylumlucknowense, and Mucor irregularis have also been reported in India(25).

Edeas M et al reported that Mucorales spores by producing reactive oxygen species and anti-microbial peptides, such as defensins. Furthermore, the presence of specialized iron-binding

proteins, which sequester iron in serum and endothelial cells that regulate permeability, controls mucormycosis. Mucormycosis infections are rare in immune-competent hosts and typically occur in the setting of trauma. However, in immune-compromised hosts, these defence mechanisms break down and Mucorales multiply rapidly upon encountering a favourable environment. The impairment of host defence mechanisms, such as decreased phagocytosis during immune-compromising conditions, including hyperglycaemia and glucocorticoid treatment, aids fungal growth and leads to disease progression (26).

In diabetic ketoacidosis (DKA), the acidic pH of the serum causes the dissociation of free iron from sequestering proteins, and free iron contributes to the rapid growth and proliferation of pathogens. The interaction of Mucorales spores containing CotH7 with the β1-integrin receptor present in epithelial cells, followed by endothelial invasion via CotH3 and GRP78 (glucose-regulated protein) receptor interaction, is a crucial step in the pathogenesis of mucormycosis. Mucorales proliferation causes the occlusion of blood supply (thrombosis), which results in tissue necrosis and endothelial cell damage. Endothelial cell damage leads to fungal angioinvasion and further dissemination (25).

The research findings communicated that Mucormycosis is a critical infection among COVID-19 patients. The use of monoclonal antibodies, steroids and antibiotics for the management of COVID-19 illness can enhance the possibility of fungus infection in the patient. Mucormycosis prevalence has appeared as a surge among patients with the disease that can be related to more utilization of corticosteroids (27) immune-suppressed conditions inflicted by COVID-19 or states like diabetes (28). The death rate associated with mucormycosis is significantly high. Patients with diabetic ketoacidosis, transplantation of organs, HIV, long-term corticosteroid use, neutropenia, and iron overload are considered in a high-risk group for mucormycosis(1). Furthermore, it was found that patients with decreased phagocytic activity of white blood cells due to immunosuppression (SARS-CoV-2 mediated, steroid-mediated or background comorbidities) coupled with several other shared risk factors including prolonged hospitalization with or without mechanical ventilators are at higher risk of COVID associated Mucormycosis(29).

Prevalence of COVID-19-Associated Mucormycosis

John TM et al stated that a rise in CAM cases has been observed worldwide amid the SARS-CoV-2 pandemic in both developed and developing countries. However, compared to developed nations, mucormycosis cases are more prevalent in developing countries and a majority of CAM cases (incidence rate: 0.14 per 1000 people) have been reported in India(30).

According to Honavar et. al warning signs suggestive of Rhino-orbital-cerebral mucormycosis are as follows (31)-

- Nasal stuffiness
- Foul smell
- Epistaxis
- Nasal discharge - mucoid, purulent, blood-tinged or black
- Nasal mucosal erythema, inflammation, purple or blue discoloration, white ulcer, ischemia, or eschar
- Eyelid, periocular or facial oedema
- Eyelid, periocular, facial discoloration
- Regional pain- orbit, paranasal sinus or dental pain
- Facial pain
- Worsening headache
- Proptosis
- Sudden loss of vision
- Facial paraesthesia, anaesthesia
- Sudden ptosis
- Ocular motility restriction, diplopia
- Facial palsy
- Fever, altered sensorium, paralysis, focal seizures.

STAGING SYSTEMS

A staging strategy for Mucormycosis was proposed by Honavar SG et al, which stages scores individually for disease extent (31). This categorisation has not however been verified.

HONAVAR RADIOLOGICAL STAGING OF DISEASE (31)

Stage1: Involvement of nasal mucosa

1a: Limited to the middle turbinate

1b: Involvement of the inferior turbinate or ostium of the nasolacrimal duct

1c: Involvement of the nasal septum

1d: Bilateral nasal mucosal involvement

Stage 2: Involvement of paranasal sinuses

2a: One sinus

2b:Twoipsilateral sinuses

2c: >two ipsilateral sinuses and/or palate/oral cavity

2d: Bilateral paranasal sinus involvement or involvement of the zygoma or Mandible

Stage 3: Involvement of the orbit

3a: Nasolacrimal duct, medial orbit, vision unaffected

3b: Diffuse orbital involvement (>1 quadrant or >2 structures) vision unaffected

3c: Central retinal artery or ophthalmic artery occlusion or superior ophthalmic vein thrombosis; involvement of the superior orbital fissure, inferior orbital fissure, orbital apex, diminution or loss of vision.

3d: Bilateral orbital involvement

Stage4:Involvementof the CNS

4a: Focal or partial cavernous sinus involvement and/or involvement of the cribriform plate

4b: Diffuse cavernous sinus involvement and/or cavernous sinus thrombosis

4c: Involvement beyond the cavernous sinus, involvement of the skull base, internal carotid artery occlusion, brain infarction

4d: Multifocal or diffuse CNS disease

Soni et al proposed another staging system as a part of treatment strategy adopted during the second wave of the pandemic- The proposed clinico-radiological classification of ROCM (AIIMS Jodhpur ROCM Staging Protocol) (32).

Stage	Clinical profile		Radiological Findings
	Symptoms	**Signs**	
I	Nasal obstruction, nasal stuffiness, purulent/black tinged/ haemorrhagic nasal discharge. Cheek swelling with facial pain	Pale/blackish nasal mucosa. Blackish nasal crusts. Cellulitis/edema over the cheek.	Mucosal thickening of the nasal mucosa and/or para nasal sinuses without bony erosion/rarefaction.
II	Absent/reduced sensation over the cheek, loosening of teeth.	Hypoaesthesia/Anesthesia over the cheek or palatal mucosa	Bony involvement of septum, turbinates, Bony erosion along antero-lateral or medial wall or floor of maxillary sinus or erosion of the floor of orbit, alveolar arch and presence of sinus over alveolus. Perineural involvement of infra-orbital nerve.
III A	Restriction of eye movement in at least one quadrant but normal vision. Periorbital swelling.	Ophthalmoplegia	Erosion of posterior wall of maxillary sinus, extension of disease into the pterygomaxillary fissure/pterygopalatine fossa/sphenopalatine foramen/infratemporal fossa or extraconal intraorbital extension of disease
III B	Diminution/loss of vision, restriction of eye movements in all quadrants. Protrusion of eyeball.	Chemosis, proptosis, Complete ophthalmoplegia, Hemifacial hypoesthesia/anesthesia, Relative Afferent Pupillary Defect (RAPD)	Involvement of intraconal compartment of the orbit, erosion of pterygoid wedge, pterygoid plates, limited involvement of greater wing of sphenoid, CRAO (Central Retinal Artery Occlusion), trigeminal nerve (V1,2,3) involvement

Stage	Clinical profile		Radiological Findings
	Symptoms	Signs	
IV A	Fever, neck rigidity, headache, vomiting, skin discoloration/ulceration, facial asymmetry, hoarseness, aspiration, trismus	Necrotic involvement of skin, facial paralysis, absent gag reflex, vocal cord palsy, deviation of tongue, positive Kernig's/Brudzinski sign.	Bony erosion of lesser wing/extensive involvement of greater wing/body of sphenoid/clivus/cribriform plate/ soft tissue neck space involvement (parapharyngeal space/masticator space, muscles of mastication), mandible. Cavernous sinus involvement, orbital apex/superior orbital fissure involvement/skull base foramina involvement, other cranial nerves, dural involvement, skin and extensive subcutaneous tissue involvement
IV B	Altered sensorium Focal seizures Weakness/loss of power of upper and/or lower limbs, Focal neurological deficit	Hemiparesis/Hemiplegia/ Seizures	Internal carotid artery thrombosis/cerebritis/cerebral infarcts/brain abscess, direct parenchymal extension

Hussain S et al. conducted a meta-analysis for estimating the global prevalence of CAM (COVID Associated Mucormycosis), by pooling a sample size of 52,196 COVID-19 patients. They reported that CAM incidence was 50 times higher (7 per 1000 COVID-19 cases) than the previously available highest-incidence data (0.14 per 1000 population). A high mortality rate (26.9%) was found to be associated with CAM infection (33).

Chavan R.P et al conducted a study to assess the symptoms of mucormycosis infection among COVID-19 patients in a tertiary hospital. The study reported the appearance of symptoms of mucormycosis within 7 days after the onset of COVID-19 infection in about 34.7% of patients, while in the majority (64%) it appeared after 15 days of COVID-19 infection(34). It might even occur after 2 months in some cases. Therefore the patients should

be the most vigilant for any signs and symptoms during the 2–6-week period after testing positive for COVID (21).

A systematic review conducted by Kamat et al. reported rhino-orbital mucormycosis in the majority of CAM cases. More than 80% of cases were diabetic and showed a history of steroid administration. A mortality rate of 25.6% was reported, even after the administration of antifungal drug therapy (35).

COVID-19–associated rhino-orbital-cerebral mucormycosis (CAROCM) is the most common type observed during the epidemic, followed by the pulmonary form. Prior to the COVID-19 pandemic, mucormycosis mortality was 50%. However, during the pandemic situation in India, it has increased to 85%, mainly due to crowded hospitals, unavailability of healthcare resources, overburdened healthcare workers, and poor diagnostic quality. Also for India, a low doctor/patient ratio and practise by unqualified or nonregistered healthcare professionals are other key hurdles that the country faces to tackle CAM along with a lack of awareness and inadequate health infrastructure which caused additional burden (36).

In another study, Walia S et al conducted a study in a tertiary eye care centre to evaluate the COVID-19-associated mucormycosis. In the study, 540 proven cases of mucormycosis were included. Most common age group affected was 41–50 years with male preponderance (69%). Sino nasal was the most common site of involvement in mucormycosis (100%), followed by orbital (51.85%), cerebral (9.44%), cutaneous (1.85%), and pulmonary (0.18%). Most common presentation was periocular and facial swelling (28%). 97.96% of patients had associated diabetes and 89.44% of patients had history of COVID-19 with concurrent steroids use (84.85%), higher antibiotics (82.59%), oxygen therapy (52.40%), remdesivir (28.89%), and biological agents (2.56%). Duration from COVID-19 positivity to presentation of mucormycosis was 22.56 days, while 4.44% of patients had coexisting COVID-19 with mucormycosis. The mortality rate was 9.25% (50 out of 540) (37).

Necrosis and thrombosis during mucormycosis can result in poor delivery of antifungal drugs. Therefore, the elimination of affected/necrosed tissue may serve as a critical form of care to completely eradicate the infection. The preferred surgical approach depends upon the patient's disease status. Open surgeries are performed when the disease is extensive, whereas endoscopic surgeries are done when the disease is limited. Surgical management has been

reported to give improved results over treatment without surgery and leads to local control of the infection (38).

Hoenigl et al. stated that surgical intervention and systemic antifungal therapy were associated with improved outcomes compared to antifungal therapy alone for patients with COVID-19 affected by rhino-orbital cerebral mucormycosis without central nervous system (CNS) involvement (39).

According to Madhavan Y et al., surgical debridement is a crucial component of the therapy plan for mucormycosis because of its angio-invasive nature. And in order to treat mucormycosis, sinus debridement must be done frequently, intensely, and regularly eliminating all black, necrotic tissues for a better prognosis (40).

IRON AND MUCORMYCOSIS

Iron, one of the most abundant metals on earth is an essential micronutrient in the human body. It plays important roles in oxygen transport, regulation of cell growth and differentiation, mitochondrial respiration, DNA synthesis and many other metabolic processes. However, both iron deficiency, as well as an excess of iron in the human body is a problem. The most common nutritional deficiency worldwide is iron deficiency (41).

IRON ABSORPTION AND METABOLISM

The majority of iron is absorbed in the duodenum. And the absorption depends on the body's needs. Iron absorption is increased in active red cell production and/or iron deficiency and is decreased in iron overload states and systemic inflammation. In the duodenum, the ferric iron is reduced by duodenal cytochrome b reductase to ferrous iron which is then transported into the intestinal villous cell by the Divalent metal transporter (DMT). Ferroportin (iron efflux pump that mediates the export of iron from the enterocyte) in association with hephaestin and ceruloplasmin (having ferroxidase activity that converts $Fe^{2+} \rightarrow Fe^{3+}$) oxidizes the iron back to the ferric form, which is later exported across the basolateral membrane of the duodenum and is transported by plasma apo-transferrin to blood circulation and to various tissues (42).

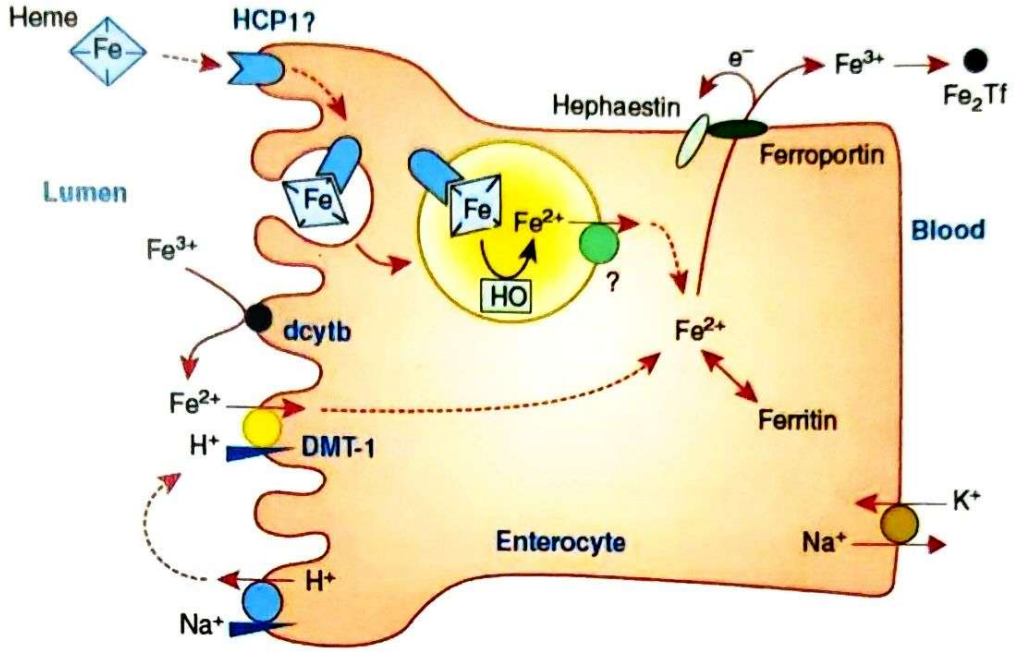


FIGURE 1: Schematic diagram of iron uptake

Transferrin is the principal transport protein for iron throughout the body. It is a free peptide or protein which is synthesized exclusively in the liver and secreted into the blood. It transports ferric iron from the blood to various tissues, such as the liver, spleen, and bone marrow and controls iron metabolism. It has a high affinity to ferric iron and functions as the most critical ferric pool in the body (43).

The normal iron content of the body is about 3 to 4grams. Of this, about 2.5 g is contained in the haemoglobin in circulating red cells and in developing erythroblasts. Approximately, 400 mg of iron is contained in iron-containing proteins such as myoglobin, cytochromes, catalase etc. And about 3-7 mg of iron is bound to plasma transferrin (44).

The rest of the iron in the body is stored as ferritin. Ferritin is an iron-binding protein that exists in both intracellular and extracellular compartments and is a major iron storage protein, essential for iron homeostasis. Hepatocytes are the major site for ferritin synthesis. It is involved in a wide range of physiologic and pathologic processes besides iron storage, which includes maintenance of cellular iron concentrations, iron sequestration from invading pathogens, and oxidative stress protection (45).

Iron is stored commonly in the bone marrow, liver, and spleen. The primary physiologic source of reserve iron in the body is the iron stores of the liver. Adult men have approximately 1 g of iron in storage. Adult women have fewer iron stores due to loss through menstruation, pregnancy and lactation. Only a small amount of iron enters and leaves the body on a daily basis. Most iron in circulation is recycled from the breakdown of old red blood cells by macrophages of the reticuloendothelial system (46).

Iron is one of the key elements in basal metabolism. It is an important component of heme. It acts as the active site for electron transport in cytochromes and cytochrome oxidase and is involved in energy generation in mitochondria. The heme moiety in haemoglobin and myoglobin binds with O2 thereby transferring O2 from the lungs to the various tissues and storing it. Heme is the active site in peroxidases. It reduces peroxides to water and generates microbicidal hypochlorite in granulocytes and thus is involved in the protection of cells from oxidative injury (47).

IRON EXCRETION

Our body conserves iron effectively. About 1mg of iron is lost via faeces everyday secondary to desquamation of intestinal epithelial cells. Other smaller losses occur through skin exfoliation and dermal appendages and sweating. In women, menstruation leads to negative iron balance. Average total iron loss per day in males is 1mg, whereas in menstruating female it is 2mg. During iron overload states daily loss can be as much as 4mg(48).

IRON AND INFECTION

Iron plays a pivotal role during infection because it functions as a critical cofactor in a variety of physiological metabolic reactions and is an essential nutrient for bacteria. Thus, during bacterial infections, both the host and bacteria compete for iron to thrive. In the human body, most iron is sequestered by the haemoglobin (49). Iron-binding proteins, antibodies and complement proteins in the body also function jointly to retain iron (50). Nevertheless, both free iron and protein-bound iron can be captured by bacteria through various mechanisms, such as siderophores (50,51) which are secreted by pathogens and possess an affinity to iron that is higher than that of the host transport proteins (52). However, free iron is toxic because it readily accepts or donates electrons, leading to the generation of reactive oxygen species (53).

The iron level throughout the body is tightly controlled by systems that elaborately regulate iron absorption, systemic transport and cellular uptake and storage(54). Because nearly all fungi and protozoa require a continuous supply of host iron to successfully sustain an infection, humans have evolved powerful strategies, such as the immune system and various iron-binding proteins, to restrict the availability of iron to pathogens. Hosts, including humans, have developed mechanisms to make iron as inaccessible to microorganisms as possible, especially during infection and concomitant inflammation (increased production of hepcidin and natural iron chelators being some of them), which probably reduces the virulence and pathogenicity of bacterial and fungal pathogens. Therefore, there is a constant competitive relationship between hosts and microorganisms, including fungal pathogens, with respect to iron (55).

TRANSFERRIN- Studies have shown that free iron in tissue fluids is less than 10^{-24} M which is an exceedingly low concentration and is maintained predominantly by the iron-binding protein transferrin. Hence transferrin possesses a powerful bactericidal or bacteriostatic effect as they help to maintain the concentration of free iron at such low levels that are restrictive for the survival of the invading pathogen (56). Moreover, serum transferrin levels <150 mg/dl were associated with an increased risk of sepsis in burn patients along with other nutritional deficits like serum albumin, and total lymphocyte counts(57).

Higher iron levels independently facilitate the pathogenesis of fungal infection. The excess iron which directly facilitates recurrent infections may cause iron-related mortality. Serum ferritin levels more than or equal to 500 ng/ml were found to have higher cumulative incidences of overall infections. Moreover, iron catalyses the chemical production of reactive oxygen species, such as the hydroxyl anion and superoxide, which contributes to the development of multiorgan failure(58).

Although the diagnosis of iron overload can be confirmed by various invasive and non-invasive (imaging) methods, the simplest, most widely used, but not the most accurate method is to determine serum ferritin values; a serum ferritin value of 1000 µg/L or more is mainly used in haematology as a cut off value, which indicates treatment with iron chelators(59,60)

Iron serves as an essential nutrient for metabolic pathways in both humans and microorganisms. Pathogenic microorganisms, including bacteria, fungi, and protozoa, require

iron for growth and proliferation. As a defence strategy, hosts have developed numerous mechanisms to reduce the availability of iron to invading pathogens. Therefore, the decision to supplement iron in patients with infections requires careful consideration of risk vs benefit (61,62).

Iron and COVID infection

Iron ions are among the nutrients necessary for viral replication, so they favour its own growth by ensuring iron repletion to the infected host cell. But when a host is infected with the virus, hepcidin is released from the liver and lowers serum iron levels to limit iron availability. Therefore, lowering iron levels engages host tolerance responses to protect itself from the infective pathogen(63).

Several reports have shown that a decreased serum iron level is observed in patients with severe COVID-19, and serum iron levels can be a prognostic marker. However, the association between host iron-lowering response and disease severity is not clear(64,65).

Kang Zhao et al conducted a study with aim to assess serum iron in predicting severity and mortality of COVID-19. Study result shows that out of the 50 patients, 45 (90%) patients had abnormally low serum iron levels (<7.8 μmol/L). The severity of COVID-19 was negatively correlated with serum iron levels before and after treatment and was positively correlated with C-reactive protein, serum amyloid A, D-dimer, lactate dehydrogenase, urea nitrogen, and myoglobin levels. Decreased serum iron levels could predict the transition of COVID-19 from mild to severe and critical illness. Low serum iron concentration was an independent risk factor for death in COVID-19 patients(66).

A study was conducted to find if ferritin levels could be a crucial factor influencing the severity of COVID-19. In one study with 20 COVID-19 patients, it was found that the individuals with severe and very severe COVID-19 exhibited increased serum ferritin levels, showing serum ferritin significantly higher in the very severe COVID-19 group than in the severe COVID-19 group (67).

In agreement with this, another study revealed that in patients who died from COVID-19, ferritin levels were high upon hospital admission and throughout the hospital stay. The median values of serum ferritin levels after day 16 of hospitalization exceeded the upper limit of detection in these patients, suggesting that ferritin levels increased to much extent (68).

Also, Chen et al. analysed the clinical characteristics of 99 patients, in which 63 of them had serum ferritin way above the normal range. Elevated ferritin levels were found also in autopsies of 12 patients whose cause of death was SARS-CoV-2 infection. An analysis of the peripheral blood of 69 patients with severe COVID-19 revealed elevated levels of ferritin compared with patients with the non-severe disease. Therefore, it was concluded that serum ferritin levels were closely related to the severity of COVID-19 (69)

Iron and fungal infection

Alternatively, iron can be stored as part of iron-rich proteins (ferritins). To date, the only fungi identified that store iron in ferritins are members of the class Zygomycetes. Also, fungi can store iron as part of small proteins called siderophores, which specialize in obtaining iron from the environment. This mechanism of storage is common among fungi belonging to the ascomycetes and basidiomycetes classes (70).

Siderophores are low-weight, high-affinity iron-chelating molecules produced in response to iron deficiency. Rhizopus takes up iron from the host with the help of siderophores. It takes up the iron with the help of its endogenous siderophore rhizoferrin in a receptor-mediated, energy-dependent process. It also takes up iron from the exogenous siderophore desferrioxamine (DFO). Iron uptake from DFO is energy-dependent which requires a reductive step. It interacts with binding sites different from those to which rhizoferrin attaches to the fungus. The intact DFO complex does not enter the fungal cell but iron uptake is by intracellular transport of the reduced iron without deferoxamine internalization. This transport is likely mediated by high-affinity iron permeases. These high-affinity iron permeases are present in fungi as part of a reductive system containing redundant surface reductases that reduce ferric into the more soluble ferrous form. The iron bound to rhizoferrin is taken up by serum apo transferrin, therefore making iron unavailable to Rhizopus. Whereas, the iron within DFO is not trapped by serum apo transferrin and it is easily utilized by Rhizopus. Thus deferoxamine, in contrast to other iron chelators can lead to iron accumulation by Rhizopus for its growth and ultimately lead to fungal infections (71).

Toni Valkovi et al(2022) conducted a study to find out the link between iron and iron overload with susceptibility to invasive fungal infections. They cited that invasive fungal infections are an important problem in the treatment of patients with haematological malignancies, and they are associated with significant morbidity and mortality. The role of

iron and its metabolism in the virulence and pathogenesis of various invasive fungal infections is intriguing, and so far, there is only some evidence linking invasive fungal infections to iron or iron overload. They cited that Ferritin is a protein that stores iron in the body in a non-toxic form. It is mainly located intracellularly, although a small proportion of this protein is found in the serum and correlates with the body's total iron stores. When the concentration of ferritin in the body (serum) is higher than normal, then it is called iron overload. The latter can result from various pathological conditions, such as primary hemochromatosis or frequent blood transfusions, which are common in some haematological diseases. Elevated serum ferritin levels also occur with other infections or liver damage. In iron overload conditions excess iron can chemically interact with hydrogen peroxide, creating reactive oxygen species that can cause tissue damage, inflammation, and fibrosis in affected organs. Therefore iron overload can damage organs such as the liver, the heart, and the endocrine glands, while the impact of this disorder on the immune system is less well understood. And the invasive fungal infections are susceptible to patients with impaired innate immunity (a reduced number and function of neutrophils and macrophages), and also impaired T cell immunity (72).

Álvarez F et al. cited that upon a fungal infection, hosts restrict iron availability in order to limit the growth and virulence of the pathogen. Fungi possess common and species-specific mechanisms to acquire iron and to control the response to iron limitation. Upon iron scarcity, fungi activate a wide range of elegant strategies to capture and import exogenous iron, mobilize iron from intracellular stores, and modulate their metabolism to economize and prioritize iron utilization. Hence, iron homeostasis genes represent remarkable virulence factors that can be used as targets for the development of novel antifungal treatments(73).

Ibrahim AS et al cited that agents of mucormycosis are uniquely susceptible to variations in environmental iron concentrations. Therefore, abrogation of iron acquisition by the agents of mucormycosis is a promising therapeutic strategy to impact the clinical outcomes of this deadly disease. They studied other experimental iron chelators like Deferiprone and Deferasirox in addition to deferoxamine in vitro against R. oryzae where they found that, unlike Deferoxamine, other iron chelators did not allow the organism to take up iron and did not support its growth in vitro in the presence of iron. Therefore they suggested the possibility of utilizing these iron chelators as adjunctive antifungal therapy(74).

Kumar H et al conducted a case–control study enrolling COVID-19 participants with and without mucormycosis and compared the serum iron indices in them. They compared the baseline serum iron indices (iron, ferritin, total iron-binding capacity [TIBC], unsaturated iron-binding capacity and percentage transferrin saturation) between CAM cases and COVID-19 controls. The mean serum iron values (33 vs. 45 μg/dl, p = .03) and TIBC (166.6 vs. 201.6 μg/dl, p = .003) were significantly lower in COVID-19-associated mucormycosis cases than controls. The case fatality rate of COVID-19-associated mucormycosis was 73.9%. The iron indices were not significantly different between CAM survivors and non-survivors. The conclusion of the study was that COVID-19-associated mucormycosis is associated with lower TIBC levels than COVID-19 subjects without mucormycosis, suggesting dysregulated iron metabolism in its pathogenesis(75).

ZINC AND MUCORMYCOSIS

Zinc is the second most abundant trace element (after iron) essential for all living organisms. Zinc exists as a divalent cation (Zn^{+2}) and is not redox active under physiological conditions, which explains why zinc performs multifarious physiological roles in a variety of biological processes(76).

Health evaluators such as the National Institute of Health (NIH) directed daily consumption of 8 mg of Zinc for adult females and 11 mg for adult males. The requirement of Zn in malnourished children is 2–4 mg/kg body weight, which is higher than that in healthy children (0.17 mg/kg) in the age group of 1–3 years, owing to the higher Zn loss and reduced Zn absorption in the former cohort(77).

ABSORPTION OF ZINC

Zinc is absorbed in the small intestine by a carrier-mediated mechanism. Under normal physiologic conditions, transport processes of uptake are not saturated. The fraction of zinc absorbed is difficult to determine because zinc is also secreted into the gut. Zinc administered in aqueous solutions to fasting subjects is absorbed efficiently (60-70%), whereas absorption from solid diets is less efficient and varies depending on zinc content and diet composition. Generally, 33% is accepted as the average zinc absorption in humans. More recent studies have suggested different absorption rates for different population groups based on their type of diet and phytate. Phytate is a naturally occurring substance found in common foods like cereals, corn, and rice, has a significant detrimental impact on the absorption of zinc from

composite meals. The phytate forms of inositol hexaphosphate and pentaphosphate that cause these adverse effects are different from the lower phosphates, which have no or little impact on zinc absorption(78).

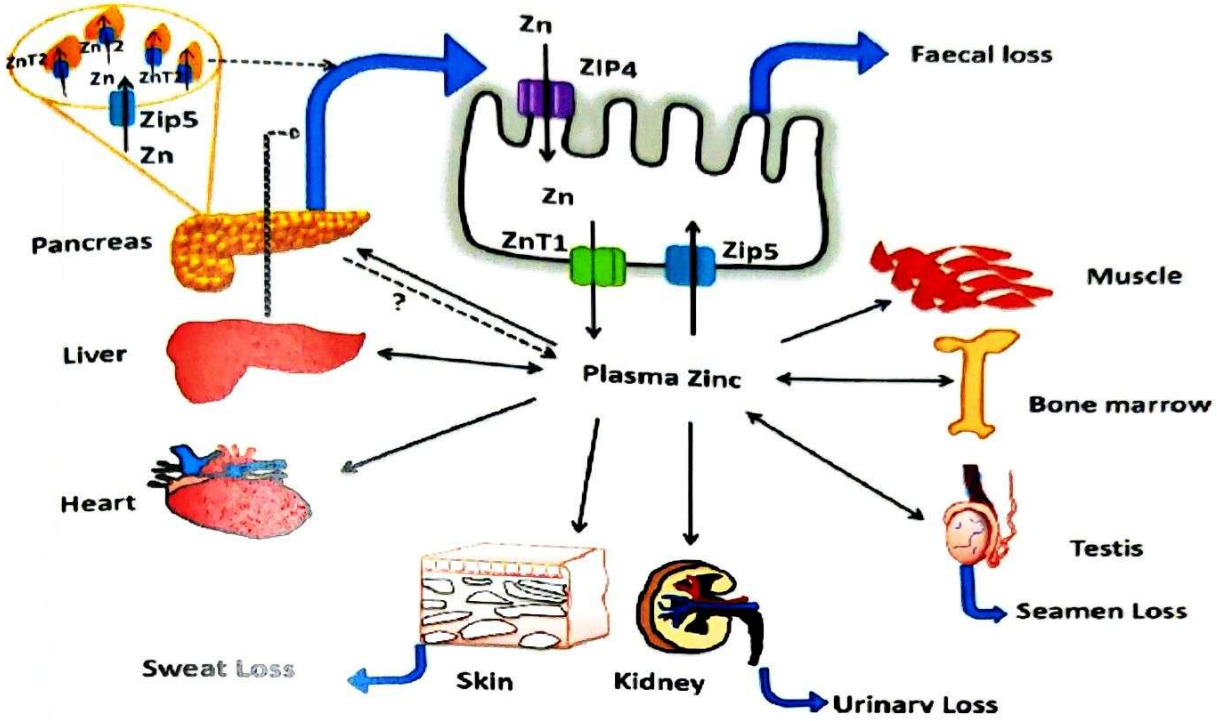


FIGURE 2: Zinc (Zn) absorption and homeostasis in human body(79)

Zinc absorption is concentration dependent and increases with increasing dietary zinc up to a maximum rate. In addition, zinc status may influence zinc absorption. Zinc-deprived humans absorb this element with increased efficiency, whereas humans on a high-zinc diet show a reduced efficiency of absorption(79).

The portal system carries absorbed zinc directly to the liver, and then released into systemic circulation for delivery to other tissues. About 70% of the zinc in circulation is bound to albumin, and any condition that alters serum albumin concentration can have a secondary effect on serum zinc levels. Although, serum zinc represents only 0.1% of the whole body zinc, the circulating zinc turns over rapidly to meet tissue needs(80). Clinically, Zn deficiency affects various organs including those in the gastrointestinal, epidermal, immune, central nervous, skeletal and reproductive systems. Reportedly, Zn supplementation has a positive impact on the reduction of cases caused by Zn deficiency such as diarrheal episodes and diarrhoea-induced morbidity, premature delivery, and acute infection of the lower respiratory tract; and it also builds on the linear growth and weight gain in children (81).

Zinc is a second messenger for immune cells, and its intracellular status is directly altered by an extracellular stimulus and then intracellular zinc participates in signalling events(82).

In the lungs, zinc has anti-inflammatory, antioxidant, and antiviral effects. It can inhibit cancer cell migration, regulate lipid metabolism and immune cells and exerts other protective effects. The comprehensive evaluation highlights the clinical and experimental effects of zinc in the pathogenesis of respiratory diseases(83).

Zinc, which was initially thought to be helpful in the prevention and treatment of COVID-19 owing to the hypothesized antiviral activity, was later found to be ineffective for the same. Long-term use of large doses of zinc has concerns of serious adverse effects like neurological deficits, and major guidelines have recommended against the use of zinc in doses above its recommended dietary allowance for the treatment of COVID-19. However, many regional guidelines in India continue to include high-dose zinc as a common treatment across several categories of COVID-19. Zinc being available as an over-the-counter medication has facilitated the general population to consume it in large quantities with the hope of protection against COVID-19 due to the widespread circulation of such guidelines through social media (84).

Zinc concentration is kept in close limits (0.1–0.5 mM)(85). The zinc quota is maintained by the activity of specific membrane transporters or by zinc-binding proteins that mediate zinc uptake or storage. Zinc-depleting conditions are known to reduce fungal growth (86)and evidence suggests that host cells employ sequestration of zinc to hamper fungal development. This is exemplified by the reduced zinc levels in macrophages infected with Histoplasma capsulatum (87), and neutrophils infected with Cryptococcus neoformans enhance the production of calprotectin, a zinc-binding protein. However, excess cellular zinc can generate an imbalance in oxidative metabolism. Indeed, macrophages have developed a strategy to kill phagocytosed bacterial cells by zinc overload in the phagosomal environment and the consequent generation of high levels of reactive oxygen species (ROS) in the invading microorganisms(88).

Zinc is known as a common growth factor for different pathological fungi, including Mucorales(89) Zinc influences diverse mechanisms of fungal pathogenesis by directly regulating fungal proteins required to infect mammalian hosts(90). Zinc deprivation by the

host is an important antifungal mechanism(86) and zinc chelators have been shown to inhibit different fungal growth, including Mucorales, both in vitro and in vivo(91,92).

Mansour et al (2022) conducted a cross-sectional study aimed to assess the association between the mean serum zinc level in COVID-19 Egyptian patients and its relationship with disease severity. The samples were divided into two groups according to clinical outcome, group 1 which included 30 intensive care unit (ICU) patients and group 2 which included 30 patients who were admitted to the ward. Mean serum levels of zinc were compared between the two groups. There was a statistically significant difference noted among study groups regarding the serum zinc level (p=0.039), where lower mean serum zinc levels were noted in ICU patients compared to ward patients. Study concluded that low serum zinc level is associated with severe outcome of COVID-19 infection (93).

PVSN, et al (2022) conducted a cross-sectional comparative study to analysis Serum Zinc, Copper and Magnesium Level and Their Relations in Association with Severity and Mortality in SARS-CoV-2 patients in one of the isolation centres dedicated for the COVID-19 patients in All India Institute of Medical Sciences, Jodhpur, India. A total of 150 individuals infected with COVID-19 and 50 healthy individuals were recruited. Cases were divided based on severity (mild, moderate and severe) and outcome (discharged or deceased). Serum Zn, Mg and Cu levels were analysed by direct colorimetric method. Both serum Cu and Zn levels were significantly decreased in cases when compared to those in controls ($p < 0.005$ and $p < 0.0001$). they concluded that severely ill patients showed decreased zinc levels and increased copper levels when compared to mild and moderate ill patients(94).

Muthu V et al (2021) conducted an experimental and clinical study to explore the association between zinc and mucormycosis. But the study did not conclusively support the hypothesis that zinc supplementation contributed to the pathogenesis of mucormycosis(95).

Kumar S et al (2022) conducted a case-control single-centre study to assess the association between level of Zinc and COVID-19-associated mucormycosis. The duration of the study was three months, from June 2021 to August 2021. A total of 55 cases and 50 controls were enrolled in the study. The use of zinc was found to be significantly associated with COVID-19-associated mucormycosis, with 89.1% of the cases having a history of zinc intake and only 52% of controls having a history of zinc intake(p-value <0.001). Researcher concluded that zinc might be the hidden culprit behind the sudden surge of COVID-19-associated

mucormycosis worldwide owing to the self-administration of zinc by the patients to acquire innate immunity and over-prescription of multivitamins by the treating clinicians. Intake of zinc was also found to be associated significantly with COVID-19-associated mucormycosis, therefore treating clinicians should restrict the use of multivitamins containing zinc and other metals, which might promote fungal growth(96).

Karoli et al (2021), conducted a retrospective, observational study to assess Clinical-epidemiological Profile of Coronavirus Disease 19 Associated Mucormycosis (CAM) and Relation with Zinc and Iron Levels. Demographic profile, clinical and laboratory parameters were recorded. Multidisciplinary treatment including antifungals and surgical interventions were noted. This study included 98patients of mucormycosis, diagnosed on the basis of clinical and radiological findings and later were confirmed by microbiological investigations. Out of 98 patients, 72 had rhino orbital, 24 had rhino-orbital-cerebral and 2 had pulmonary mucormycosis. Twelve had coinfection of covid 19 while 86 had developed mucormycosis within 3 weeks. Mucormycosis is uniformly associated with low iron levels but regarding role of zinc there is need of further study(97).

AIMS AND OBJECTIVES

AIM: To estimate the pre & post-operative levels of serum iron and zinc in patients with mucormycosis.

OBJECTIVES:

1. To assess the levels of iron and zinc in patients with mucormycosis pre-operatively and post-operatively.
2. To evaluate if the levels of iron and zinc change significantly with the disease activity.
3. To explore if the serum levels of iron and zinc can be used to predict the disease activity.

RESEARCH QUESTION: Can the Pre & Post-operative levels of Serum Iron and Zinc be used to predict disease activity in patients with mucormycosis?

Null Hypothesis: The levels of Serum Iron and Zinc cannot be used to predict the disease activity in patients with mucormycosis.

Alternate hypothesis: The levels of Serum Iron and Zinc can be used to predict the disease activity in patients with mucormycosis.

MATERIALS AND METHODS

STUDY DESIGN: Prospective Observational Study

STUDY SETTING: The study has been conducted at All India Institute of Medical Sciences, Jodhpur in the Department of Otorhinolaryngology.

DURATION OF STUDY: The study was done over a period of 9 months, following the procurement of the CTRI Registration number in December 2021.

STUDY SETTING: The study was conducted in the Department of Otorhinolaryngology in conjunction with the Department of Biochemistry and Microbiology at a tertiary care institute i.e. All India Institute of Medical Sciences, Jodhpur, Rajasthan.

SAMPLE SIZE: As there were no studies in the Medline database correlating the levels of iron and zinc levels in mucormycosis patients pre and postoperatively, this study was devised.

The sample size was taken as time-bound, limited by time and logistics.

However, by the end of the second wave of COVID- 19 pandemic, there was a decrease in cases of mucormycosis. Thus, a sample size of only 41 subjects was feasible.

INCLUSION CRITERIA: Patients of all age groups with diagnosed mucormycosis, admitted in AIIMS Jodhpur were included in the study.

EXCLUSION CRITERIA:

1. Patients with active COVID-19 infection.
2. Critical patients requiring post-COVID or post-mucor ventilatory support.

FUNDING:

No fund was received from any source for the completion of this study.

METHODOLOGY:

We conducted a Prospective observational study on patients who were diagnosed with Mucormycosis and were admitted and managed both surgically and conservatively, by collecting prospective data from December 2021 to August 2022.

A thorough history was taken regarding types of symptoms and their onset, prior history of COVID infection, known comorbidities etc and thoroughly noted.

A complete physical examination was done and any signs suggestive of mucormycosis were noted.

Blood samples for Iron and Zinc were sent along with other routine samples according to Institutional protocol during the first 24 hours of admission.

A diagnostic nasal endoscopic examination (DNE) was done and findings were noted.

Tissue samples were taken from suspicious areas of involvement from the nasal cavity i.e mostly from the middle turbinate and sent for microbiological examination (Microscopy as well as Fungal Culture).

At the same time, disease extent was also evaluated radiologically by Contrast-Enhanced Computed Tomography Scans (and MRI Scans wherever indicated as well).

The radiological staging was done as per Honavar et al(31).

Following these, the patients were immediately started on Systemic antifungals- Injection Amphotericin B (5mg/kg) along with Tablet Posaconazole daily.

These patients underwent Surgical debridement of the infected tissue.

Patients' status following the $15^{th,}$ 30^{th} and 90^{th} postoperative days and respective days' CT Scan was recorded and again evaluated & staged along with the blood serum estimation of iron and zinc and assessment done.

All surgeries performed and clinical features in these cases were recorded.

Details of medications given to the patients in the form of antifungal drugs were noted as well.

RADIOLOGICAL ASSESSMENT:

Imaging plays a very important and useful role for assessing the disease and the extent of involvement and complications. Therefore, all suspected cases of Mucormycosis immediately underwent Contrast Enhanced Computed Tomography (CECT) of the nose, paranasal sinuses (PNS), orbit and brain of 0.5–1 mm slice thickness in axial plane (followed by reconstruction into coronal and sagittal planes). Gadolinium-enhanced Magnetic Resonance Imaging (CEMRI) of brain, orbit and paranasal sinus was done for patients with suspected intracranial or intraorbital extension or extensive soft tissue disease or in cases with diagnostic dilemma. Thickened mucosal lining, opacification of the paranasal sinuses, and obliteration of the osteo-meatal complex are the principal radiographic findings concerning the sinuses during mucormycosis by CT scan.

The sinuses showing opacification on CT or MRI were recorded in each case. On post-contrast CT and MRI, the type of contrast enhancement and involvement of any extra sinus structures including orbit, face, pterygopalatine fossa, masticator space, brain and cavernous sinus were noted. Presence of bone involvement was evaluated on the CT. Any complications like arterial thrombosis were noted on CT and MRI. Fat stranding and soft tissue extension similar in appearance to the intra-sinus soft tissue was taken as evidence of orbital, retro-antral, masticator and pterygopalatine involvement. Orbital cellulitis was seen as stranding in the retrobulbar fat, without overt abscess formation. Cavernous sinus and internal carotid artery involvement were seen as thickening and non-enhancement on post-contrast scans with the presence of abnormal surrounding soft tissue. Patients with intracranial extension were evaluated for dural enhancement, presence of extradural collections, infarcts, cerebritis and intracerebral abscess. However, radiological findings may be non-specific initially thus serial radiological investigations are required to assess progression and extent(98).

In our investigation, the radiologic findings were evaluated and preoperatively classified using the Honavar et al grading system. And at successive follow-ups, the disease's progression was evaluated by contrasting the results of the initial scan with the presence of lingering mucosal or soft tissue thickening, fresh foci of involvement, fresh or increased bony erosions, increased vessel involvement, an extension of the disease etc and noted.

BLOOD SAMPLE COLLECTION:

- Under all aseptic precautions, 2ml each of venous blood was obtained by venepuncture of the median cubital vein and collected in yellow vacutainer for estimation of both iron and zinc in serum.

Procedure for separating Plasma and Serum from whole blood

Blood was left to sit for 30 minutes to one hour at room temperature to clot before centrifugation.

A delay in centrifugation may have a detrimental effect on the sample quality and may result in inaccurate results.

Universal precautions must be taken when working with blood.

Blood was collected in yellow vacutainer and centrifuged (4000 rpm) at 4 degrees centigrade for 8 minutes.

After centrifugation using a clean pipette technique, 1.0ml of serum was placed into 1.5ml Eppendorf tube labelled with the tracking/identity number of the patient.

Freeze immediately at –80 degree freezer.

FIGURE 3: Centrifugation Machine

IRON ESTIMATION:

- ON BECKMAN COULTER METHOD BY COLORIMETRIC METHOD:

A technique for the direct assessment of serum iron was first presented by Schade et al. in 1954 (99). The serum was incubated in a phosphate buffer containing ascorbic acid and terpyridine in order to measure the iron level. Using an acetate buffer and bathophenanthroline, Goodwin(100) proposed a direct approach for serum iron in 1966. These changes improved colour development by using a more sensitive iron chromogen and removed random iron contamination from phosphate buffers.

The chromogen used in this Beckman Coulter technique is TPTZ [2,4,6-Tri-(2-pyridyl)-5-triazine]. Transferrin-bound iron separates into free ferric ions and apo-transferrin in an acidic environment. Ferric ions are converted to ferrous ions with the use of sodium ascorbate and hydrochloric acid. The reaction between the ferrous ions and TPTZ results in the formation of a blue complex that may be detected bi-chromatically at 600/800 nm. The amount of transferrin-bound iron present is directly proportional to the rise in absorbance.

Method: Photometric test using Ferene.

Principle: Iron bound to transferrin is released in an acidic medium as ferric iron and is then reduced to ferrous iron in the presence of ascorbic acid. Ferrous iron forms a blue complex with Ferene. The absorbance at 595 nm is directly proportional to the iron concentration.

Reagents:

Components and Concentrations R1: Acetate buffer (pH 4.5, 1 mol/L), Thiourea- 120 mmol/L

R2: Ascorbic acid- 240 mmol/L, Ferene- 3 mmol/L, Thiourea- 120 mmol/L.

Measuring range:

The test has been developed to determine iron concentrations within a measuring range from 5 – 1000 µg/dL (0.9 – 179 µmol/L). When values exceed this value samples should be diluted 1 + 2 with NaCl solution (9 g/L) and the result multiplied by 3.

Specificity/Interferences:

No interference was observed by conjugated and free bilirubin up to 60 mg/dL, haemoglobin up to 100 mg/dL, lipemia up to 2000 mg/dL triglycerides, copper up to 200 µg/dL and zinc up to 400 µg/dL.

Sensitivity/Limit of Detection: The lower limit of detection is 5 µg/dL (0.9 µmol/L).

Reference Range for serum iron (101)

	S. IRON (µg/dL)
6months	28-135
1year	35-155
Women	
25years	37-165
40years	23-134
60years	39-149
Men	
25years	40-155
40years	35-168
60years	40-120

ZINC ESTIMATION: Zinc estimation was done by a colorimetric method by Diasys kit on Beckman Coulter Platform. The estimated reference range of serum zinc level is 60-120 µg/dL, where the range was 59-125 µg/dL for male and 50-103 µg/dL for female (102) .

Principle-

- Zinc forms a red chelate complex with 2-(5-Bromo-2- pyridylazo)-5-(N-propyl-N-sulfo-propyl-amino)-phenol. The increase in absorbance of this complex is proportional to the concentration of total zinc in the sample.

Linearity is up to 400 µg/dL (61.2 µmol/L)

Sensitivity/Limit of Detection - The lower limit of detection is 2.9 µg/dL.

Features and Benefits:

- Direct colorimetric method with 5-Br-PAPS
- Without deproteinization on serum, plasma and seminal fluid
- End-Point reaction in 5 min at 37 °C
- Linearity: 400 µg/dL
- Standard included in the kit
- Liquid Stable; Single reagent
- Economical and fast analysis
- No interferences by clinical samples.

The tests were performed at the Department of Biochemistry at AIIMS-Jodhpur.

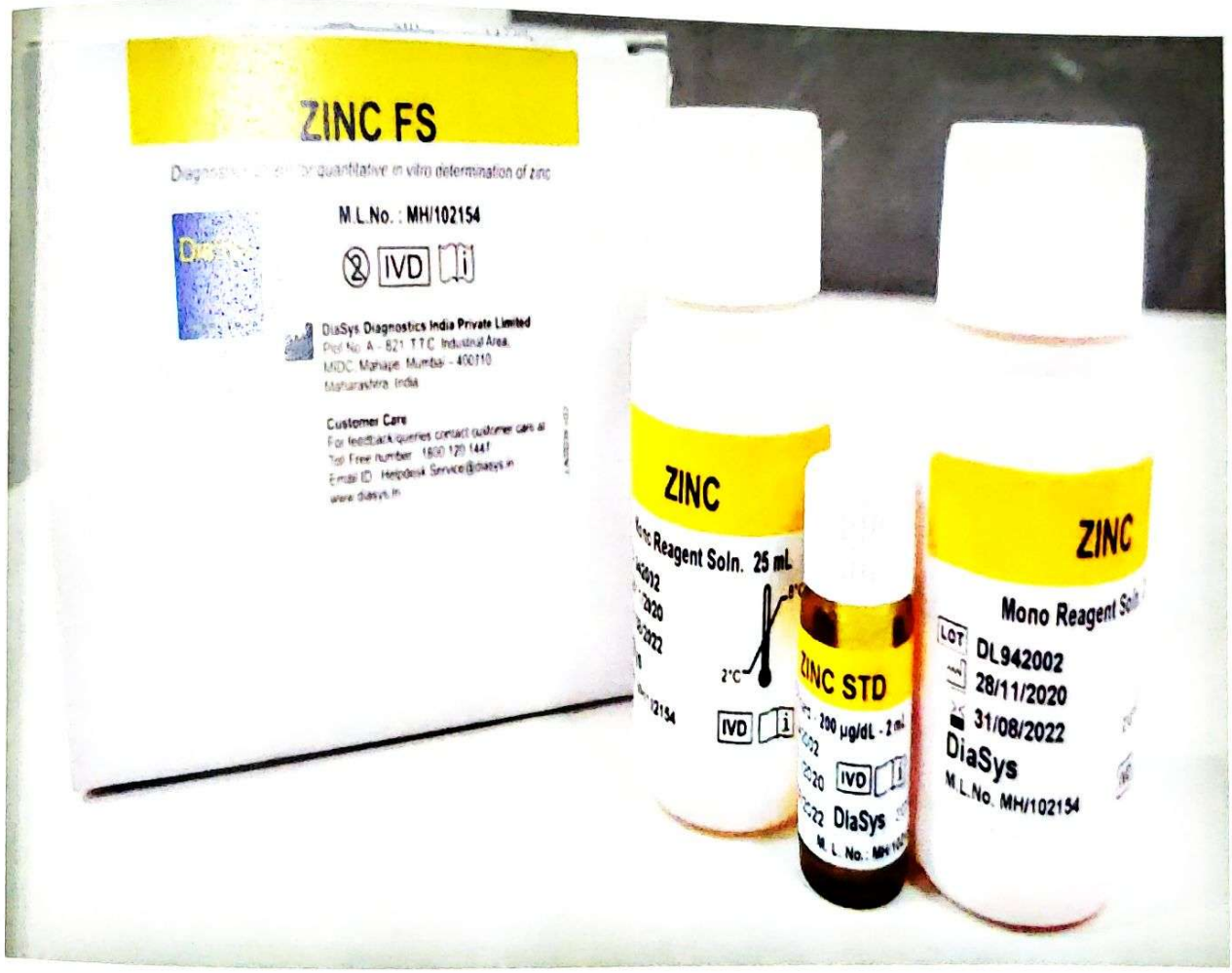


FIGURE 4: Zinc Reagents

FIGURE 5: Beckman Coulter Analyzer

FIGURE 6: -80°F Freezer

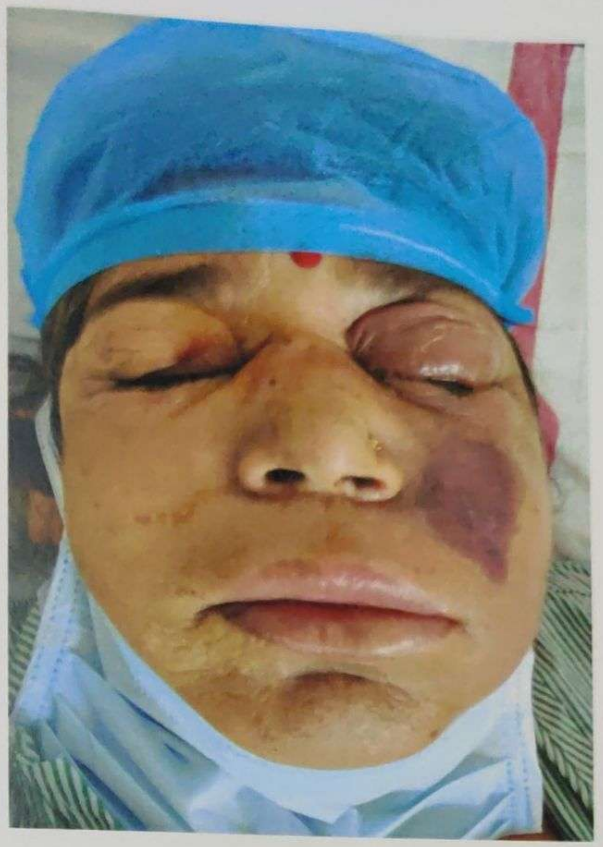


FIGURE 7: A lady with left periorbital swelling and skin discolouration over left cheek

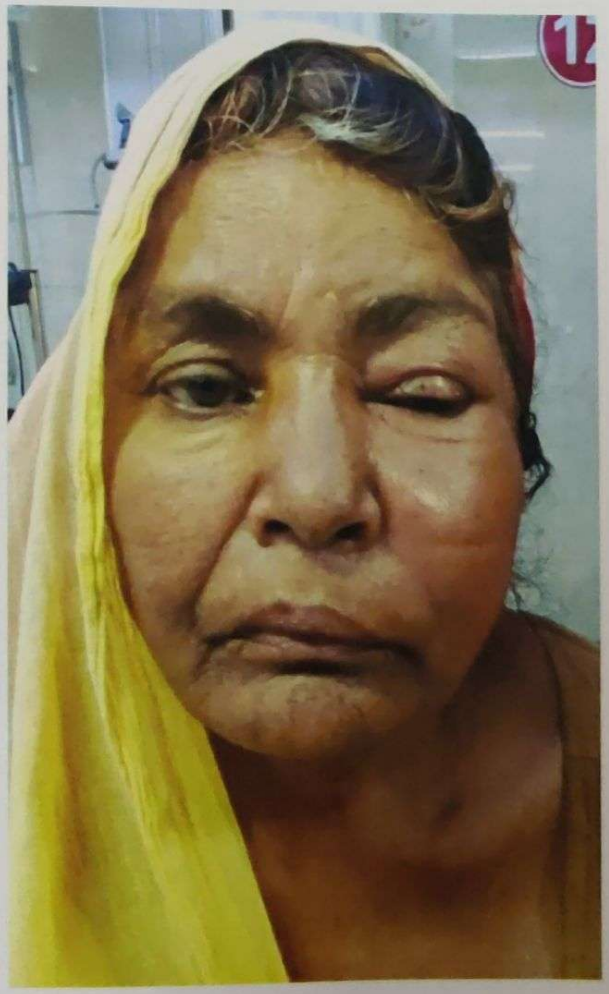


FIGURE 8: A lady with left cheek swelling

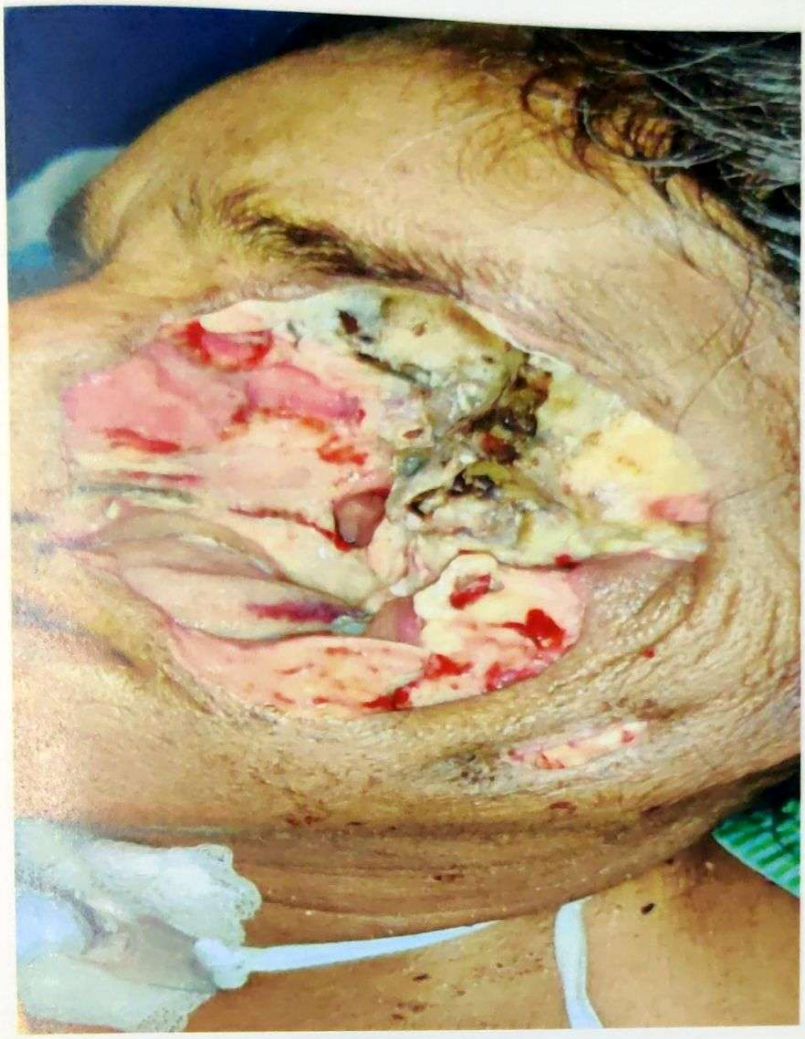


FIGURE 9: A lady with left cheek and eye defect with bone necrosis, status post debridement

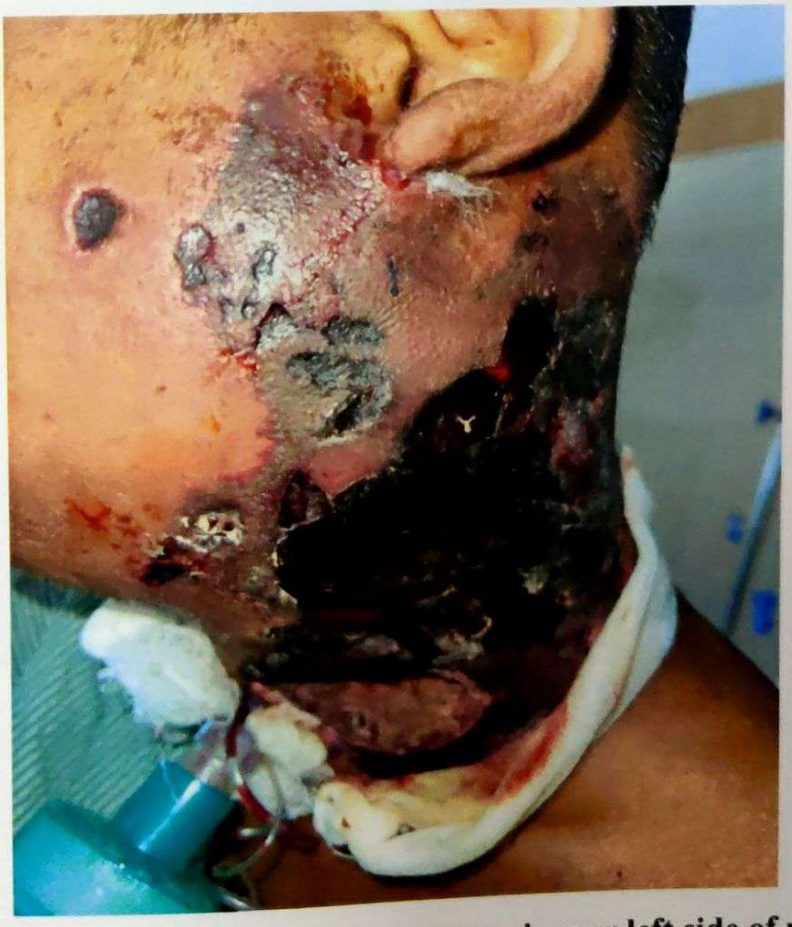


FIGURE 10: A boy with soft tissue necrosis over left side of neck

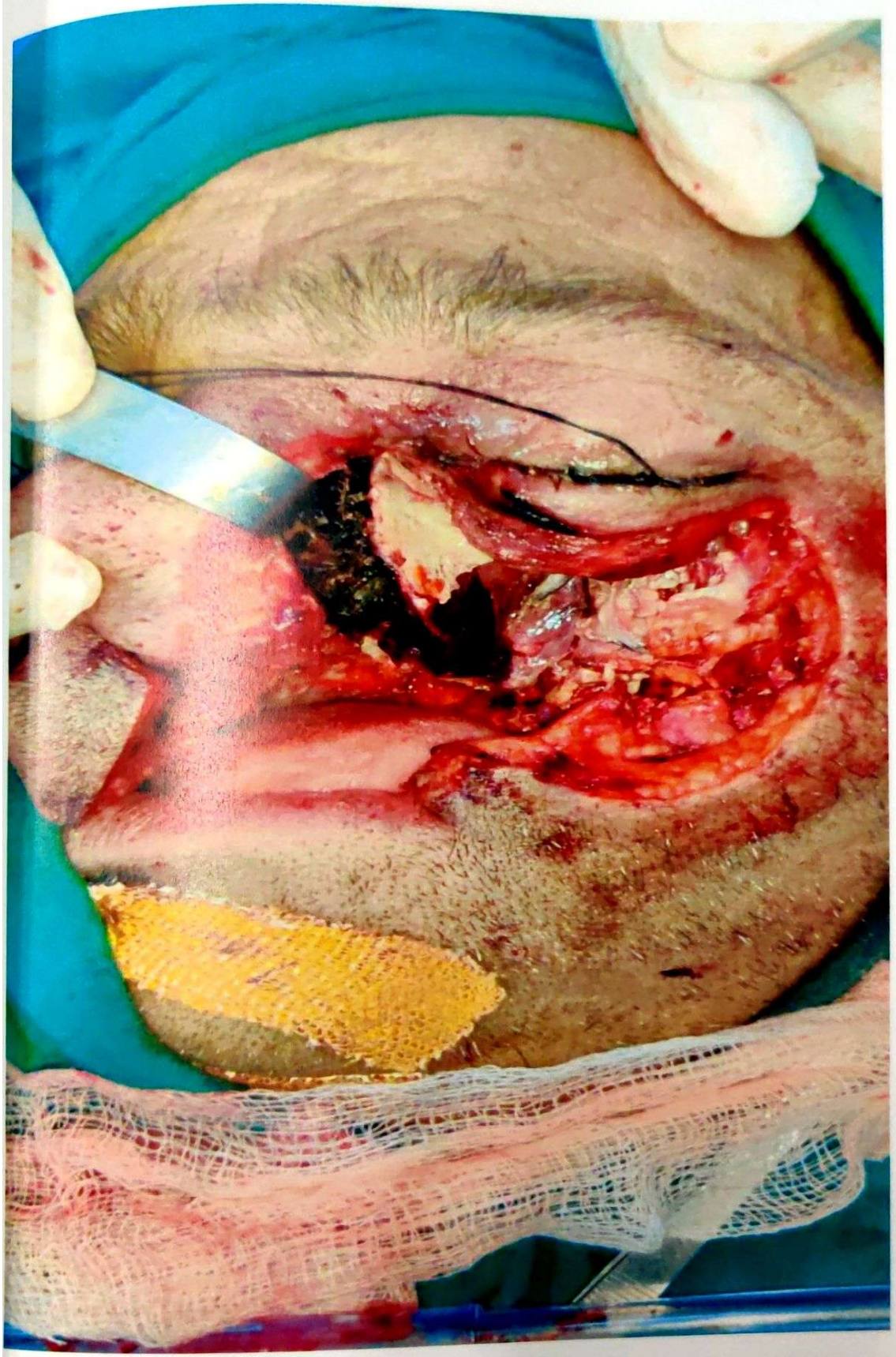


FIGURE 11: Surgical debridement

PRE-OPERATIVE WORKUP

Patients were subjected to a detailed clinical assessment, basic routine workups along with investigations relevant to the study.

The levels of iron and zinc were assessed preoperatively using Beckman Coulter method for iron and colorimetric method for zinc and recorded.

POST-OPERATIVE WORKUP

Levels of iron and zinc were assessed postoperatively using Beckman Coulter method for iron and colorimetric method for zinc on POD 15, 30 and 90 days and recorded.

SURGERY			PRE OPERATIVE		POST OPERATIVE						CLINICAL FEATURES	PER OPERATIVE DISEASE EXTENT	RADIOLOGICAL STAGING	DRUGS GIVEN	DOSAGE	REMARK
NO	DATE	PROCEDURE NAME	IRON	ZINC	IRON			ZINC								
					POD 15	POD 30	POD 90	POD 15	POD 30	POD 90						

STATISTICAL ANALYSIS

The data collected during the study was compiled on a Microsoft Excel spread sheet and analysed statistically using the statistical package for the MedCalc statistical software version 20.115 for windows. Qualitative data were presented as mean+/- SD and median. Comparison between groups was done by Chi-square test (X^2-test) and Fisher exact test, paired t-test and ANOVA. A p-value less than 0.05 was considered to be statistically significant. Data has been also presented by charts and graphs. SPSS statistical software was used for data analysis.

ETHICAL CLEARANCE

Approval to conduct this study was taken from the Institution Ethics Committee (IEC), certificate reference No. **(AIIMS/IEC/2021/3724, dated 11/09/2021)** and registration was done with Clinical Trial Registry-India **(CTRI Registration No. CTRI/2021/12/038446, obtained on 06/12/2021)**, AIIMS, Jodhpur. Informed and written consent in a language the patient and their attendees understand was obtained from them before their participation in the study.

There are reasonable ethical implications in this study:

i. All subjects have provided written informed consent.
ii. There were no added costs to the patient.
iii. Patient has to meet the aforementioned study inclusion criteria.

RESULTS

TABLE 1: Distribution of demographic and clinical variables among the subjects. (N=41)

Variables		N	%
Age (years)	51.80±11.88		
Gender	Male	32	78.05
	Female	9	21.95
Type of mucor	Rhino orbital cerebral	9	21.95
	Sino nasal	20	48.78
	Sino orbital	11	26.83
	Soft tissue	1	2.44
H/O COVID infection	Yes	14	34.15
	No	27	65.85
Comorbidities	Type 2 DM	12	29.27
	Type 2 DM with other comorbidities	14	34.15
	Non Diabetic with other comorbidities	6	14.63
	No comorbidities	9	21.95

As per above table, the mean age of subjects was 51.80±11.88 (40-65years). Out of 41, majority of subjects 78.05% were male followed by 21.95% female. Nearly half of the subjects (48.78%) were having sino-nasal mucormycosis followed by sino-orbital mucormycosis (26.83%). Only 34.15% subjects were having history of COVID infection. Most of the patients had associated comorbidities. Majority had a history of associated T2DM.

TABLE 2: Mean values of Serum Iron and Serum Zinc at different time intervals among mucormycosis patients (N = 41).

	Preop (Mean± SD)	15th Day (Mean± SD)	30th Day (Mean± SD)	90th Day (Mean± SD)
Iron	31.92±21.88	28.13±31.22	34.21±31.29	40.56±26.02
Zinc	94.54±50.84	116.66±57.84	102.51±56.21	94.05±43.86

The serum Iron levels were analyzed for descriptive statistics and the mean and standard deviation of serum iron levels were calculated and noted as in the above table. On` an average serum iron levels were found to be lower than normal in all four readings. The data was found to be skewed from a normal distribution using histogram, Shapiro Wilk test and Q -Q plot. Hence the values have been reported in the median and interquartile range [IQR] and analysis was done using non-parametric tests.

TABLE 3: Median values of Serum Iron and Serum Zinc at different time intervals among mucormycosis patients. (N = 41)

	Preop (n = 41) (Median [IQR])	15th Day (n = 41) (Median [IQR])	30th Day (n = 39) (Median [IQR])	90th Day (n = 39) (Median [IQR])	Friedman's test (*p*-value)
Iron	24 [11, 50]	17 [10, 33]	18 [8, 55]	33 [23, 45]	0.001
Zinc	90 [57, 127]	107 [70, 149]	100 [60, 136]	79 [60, 119]	0.01

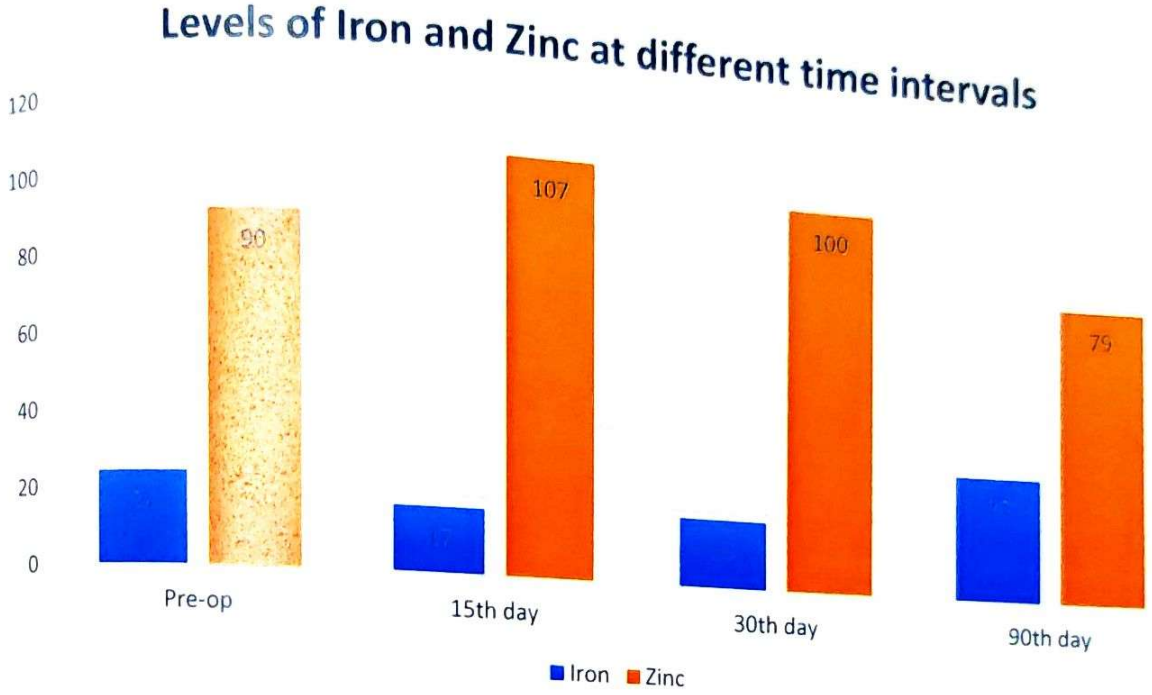


FIGURE 12: Levels of Iron and Zinc at different time intervals among patients.

The above table highlighted that the median level of serum Iron among mucormycosis was 24 [11, 50] µg/dl in the pre-operative phase. On day 15th, the iron level was 17 [10, 33] µg/dl followed by 18 [8, 55] µg/dl on the 30th day. Two patients were lost to follow due to death after 15th day. The level of serum Iron was increased on day 90th and it was 33 [23, 45] µg/dl. The Friedman test applied for four interval iron levels has been found significant (p = 0.001). Additionally, the present study also highlighted that the median level of serum Zinc among mucormycosis was 90 [57, 127] µg/dl in the pre-operative phase. On day 15th, the level was 107 [70, 149] µg/dl followed by 100 [60, 136] µg/dl on the 30th day. The level of serum Zinc on day 90th was 79 [60, 119] µg/dl. Two patients were lost to follow due to death after 15th day. The Friedman test applied for four interval zinc levels has been found significant (p = 0.01).

TABLE 4: Friedman's test for significance between repeated measures of serum iron levels. (N = 41)

Serum Iron levels		Friedman's test for repeated measures (p-value)
Preop	**15th day**	0.188
Preop	**30th day**	0.317
Preop	**90th day**	0.331
15th day	**30th day**	0.652
15th day	**90th day**	**0.001**
30th day	**90th day**	0.082

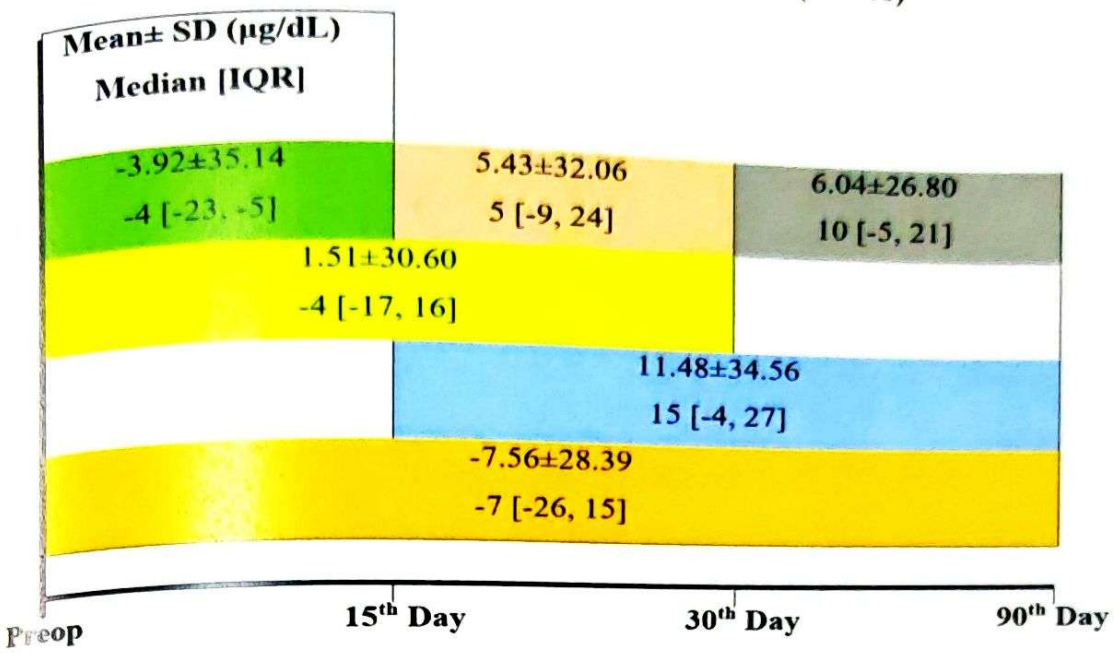
The above table is suggestive that of various combinations of serum iron levels taken between different time intervals only those in 15th day- 90th day was significant and could be taken for further analysis and prediction.

TABLE 5: Friedman's test for significance between repeated measures of serum zinc levels. (N = 41)

Serum Zinc levels		Friedman's test for repeated measures (p-value)
Preop	**15th day**	**0.029**
Preop	**30th day**	1.00
Preop	**90th day**	0.986
15th day	**30th day**	0.063
15th day	**90th day**	**0.016**
30th day	**90th day**	0.886

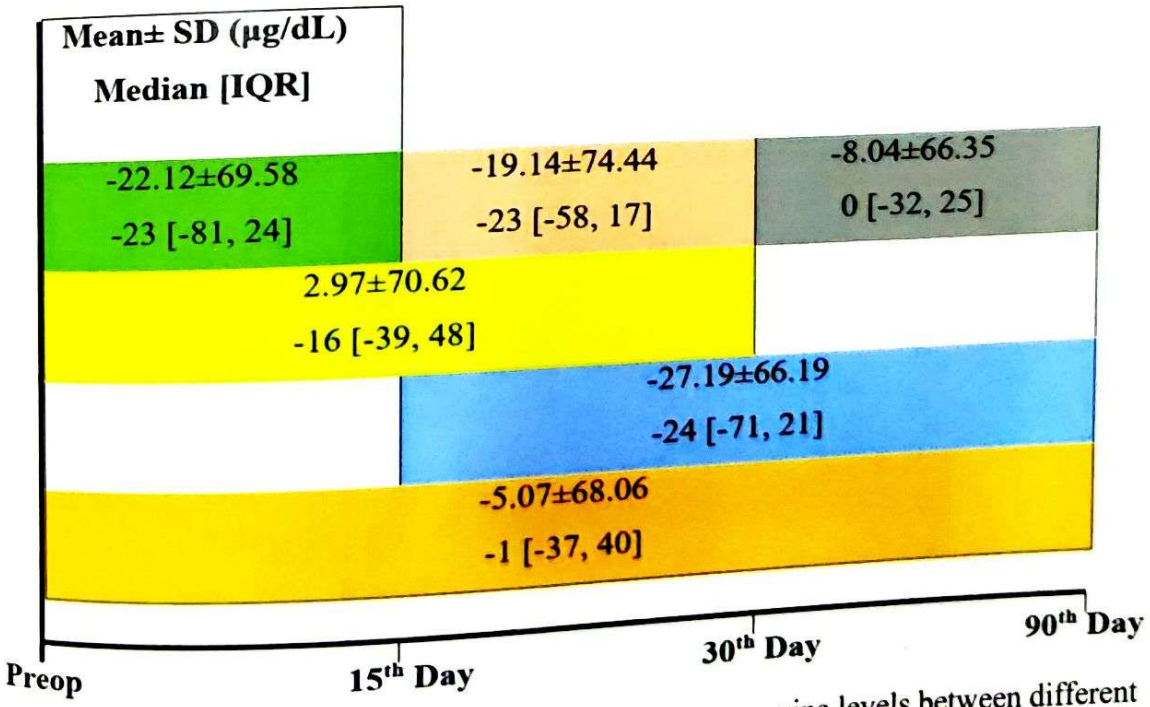
The above table is suggestive that of various combinations of serum zinc levels taken between different time intervals only those in Preop -15th day and 15th day - 90th day were significant and could be taken for further analysis and prediction.

TABLE 6: The difference in values of Serum Iron levels collected at different time intervals among mucormycosis patients. (N = 41)



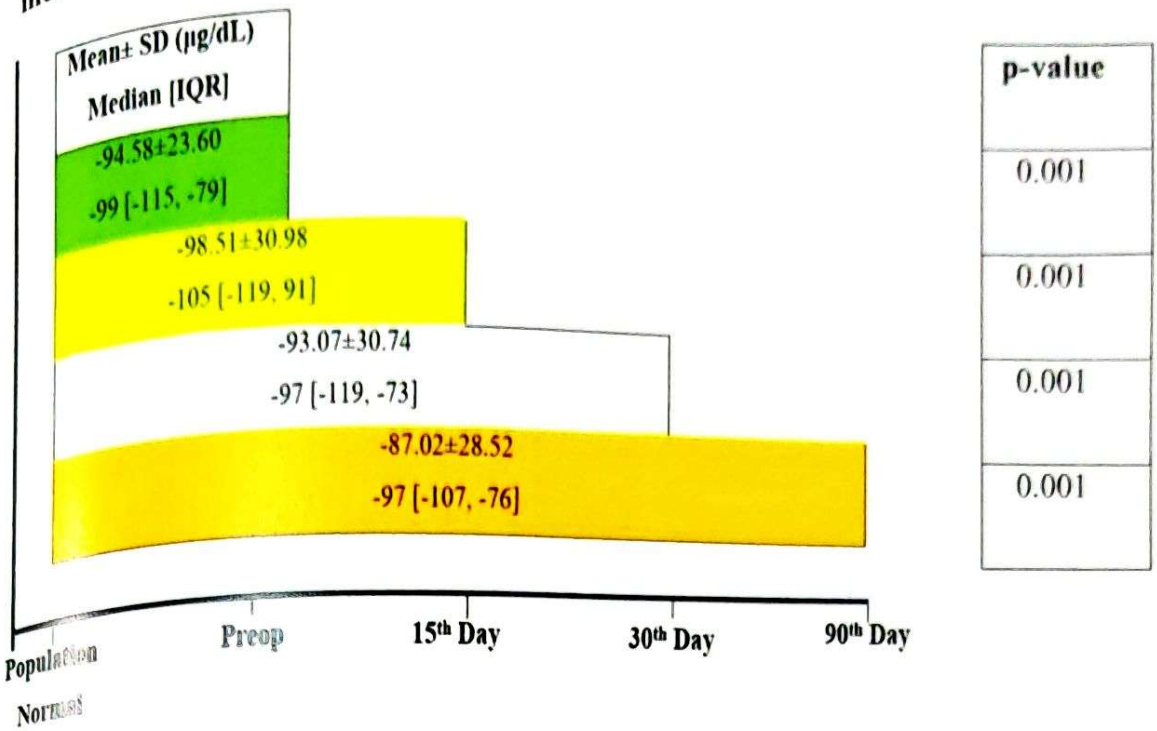
The above table descriptively presents the difference in serum iron levels between different time intervals in mean ± Standard deviation, and median [Interquartile range].

TABLE 7: The difference in values of Serum zinc levels collected at different time intervals among mucormycosis patients. (N = 41)



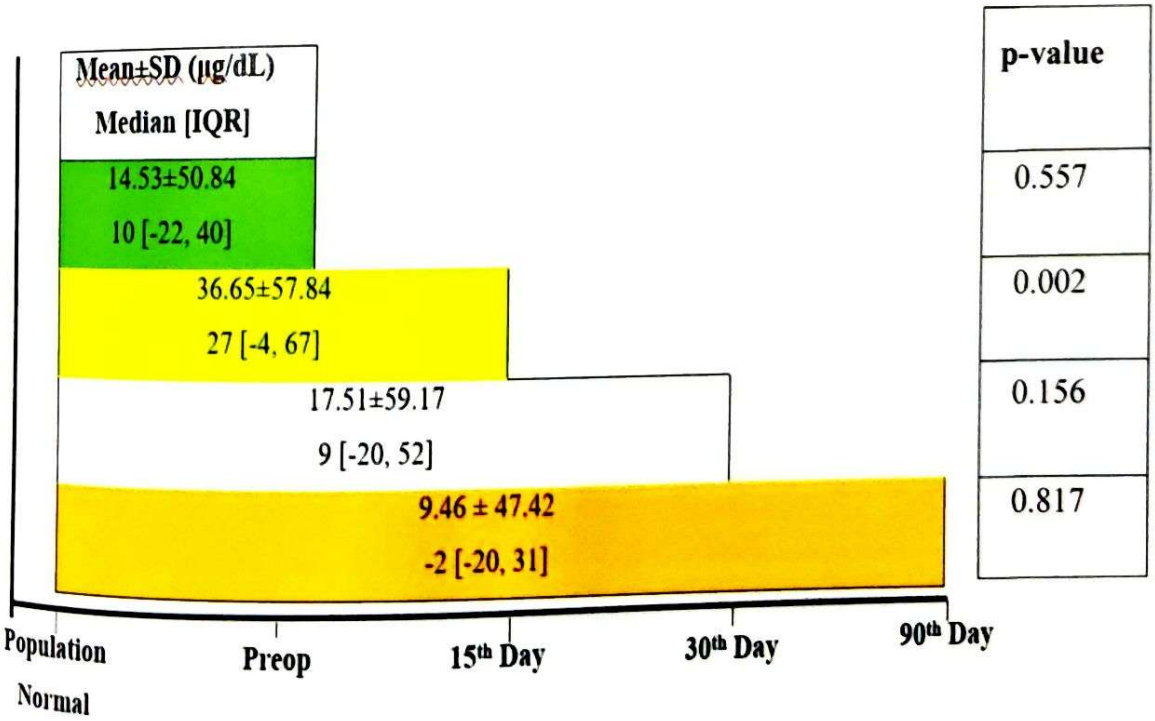
The above table descriptively presents the difference in serum zinc levels between different time intervals in mean ± Standard deviation, and median [Interquartile range].

TABLE 8: The difference in values of Serum iron levels from the normal population mean collected at different time intervals among mucormycosis patients. (N = 41)



The difference in values of serum iron levels from the normal population mean of 120 µg/dL collected at different time intervals has been analyzed and documented as in the above table. Wilcoxon signed rank test comparing the patient median serum iron levels and population normal iron levels was done and found to be significant in all four comparisons. ($p < 0.001$)

TABLE 9: The difference in values of Serum zinc levels from the normal population mean collected at different time intervals among mucormycosis patients. (N = 41)



The difference in values of serum zinc levels from normal population mean of 90 µg/dL collected at different time intervals has been analyzed and documented as in above table. Wilcoxon signed rank test comparing the patient median serum zinc levels and population normal zinc levels was done and found to be not significant. In the group 15th day postop comparison to normal serum zinc levels was significant (p = 0.002)

TABLE 10: Comparison of preoperative disease with disease progression by radiological findings.

Pre-op disease	Disease progression				Total	
	Improved		Worsened			
	N	%	N	%	N	%
15th day	32	78	9	22	41	100%
30th day	29	74.35	10	25.65	39	100%
90th day	31	79.5	8	20.5	39	100%

The above table communicated that on the 15th day follow-up period, out of 41 patients, the majority 32(78%) had improvement in the disease, while 9 (22%) patients had worsened disease.

The present table also revealed that on the 30th day follow-up period, out of the 39 patients, 29 had improvement in the disease, while 10 patients had worsened disease.

The table expressed that on the 90th day follow-up period, out of 39 patients 31 had improvement in the disease, and 8 patients had worsened disease.

TABLE 11: Levels of serum iron trend (µg/dL) with disease progression

Disease status	Iron levels trend (µg/dL)			
	Preop	15th Day	30th Day	90th Day
Improved	27 [11, 51]	19 [10, 33]	23 [12, 55.5]	33 [23, 46.5]
Worsened	20[9.75, 29]	11.5 [10.25, 17.75]	11[6.5, 42.5]	27.5 [24.5, 34.75]
Wilcoxon signed rank test (p-value)	0.256	0.374	0.406	0.169

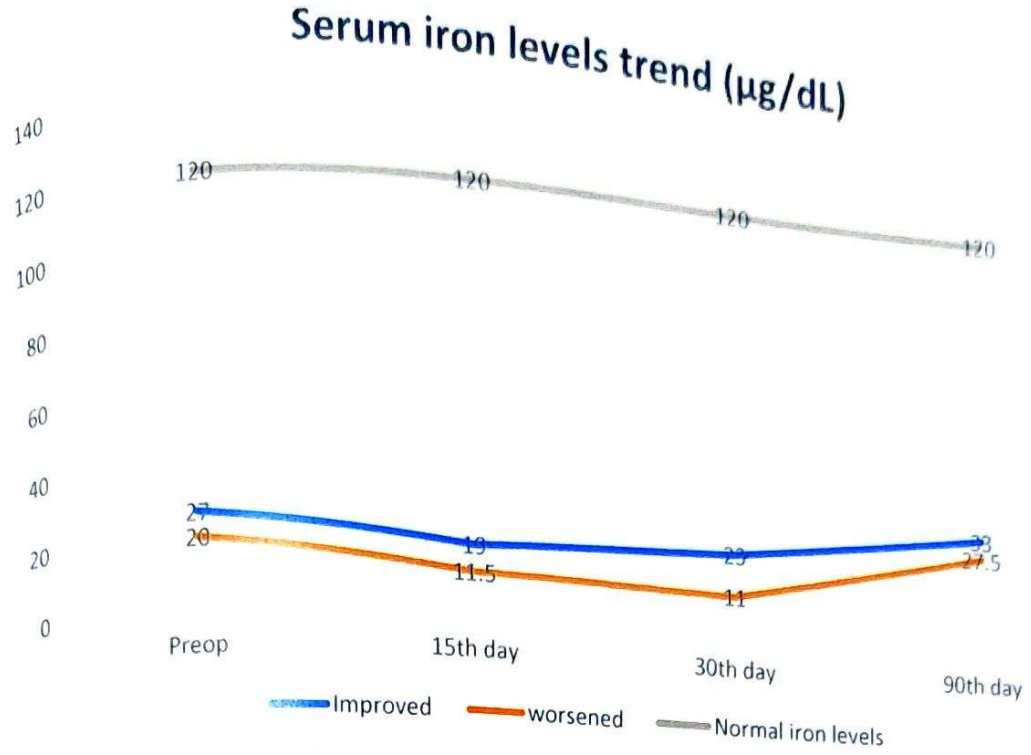


FIGURE 13: Serum iron levels trend (µg/dL) with disease progression

The table highlighted that the median serum iron values in patients with improved disease condition were 27 µg/dL preoperatively, 19 µg/dL on the 15th day, 23 µg/dL on the 30th day and 33 µg/dL on the 90th day. And the median serum iron values in patients with Worsened disease condition was 20 µg/dL preoperatively, 11.5 µg/dL on the 15th day, 11 µg/dL on the 30th day and 27.5 µg/dL on the 90th day. **Friedman's test predicts no significant association between the iron level trend and the disease progression.**

TABLE 12: Levels of serum zinc trend (µg/dL) with disease progression

Disease status	Zinc levels trend (µg/dL)			
	Preop (Median [IQR])	15th Day	30th Day	90th Day
Improved	90 [63, 134]	107 [78, 147]	109 [79.5, 143.5]	79 [63.5, 107.5]
Worsened	99 [59.25, 105.75]	105.5 [77.75, 143.25]	65 [45, 90.5]	85.5 [61.5, 123.5]
Wilcoxon signed rank test (p-value)	0.040	0.177	0.083	0.233

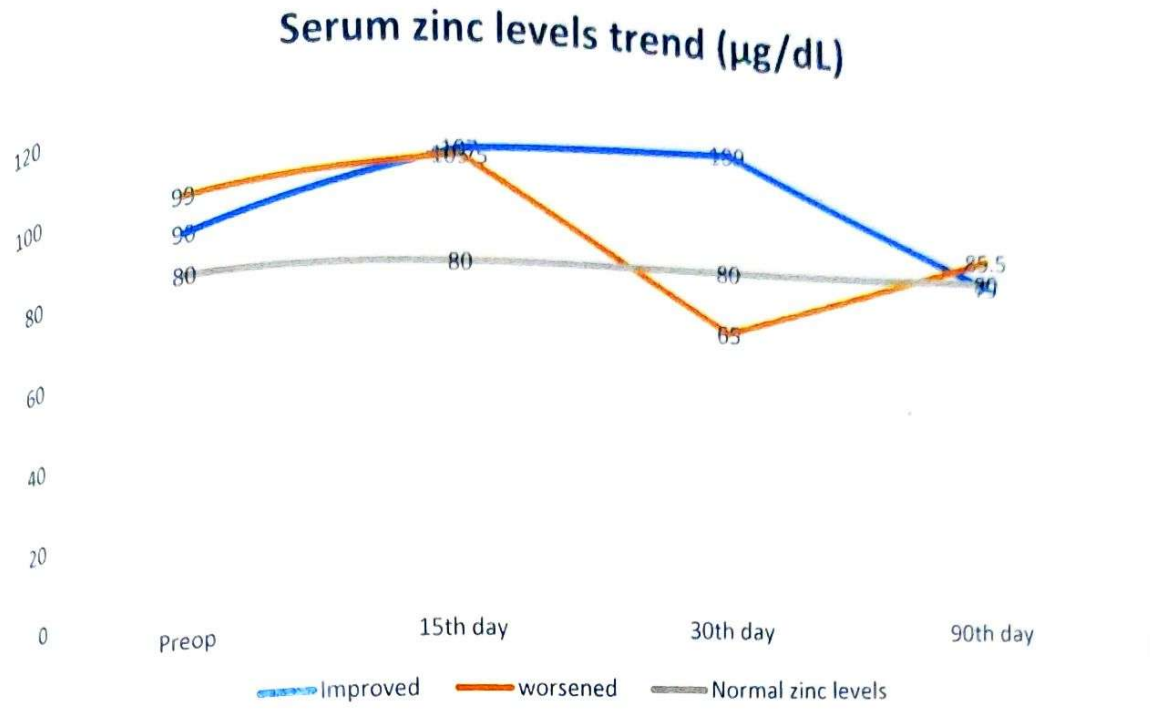


FIGURE 14: Serum zinc levels trend (µg/dL) in with disease progression

The table highlighted that the median serum zinc values in patients with improved disease condition were 90 µg/dL preoperatively, 107 µg/dL on the 15th day, 109 µg/dL on the 30th day and 79 µg/dL on the 90th day. And the median serum zinc values in patients with Worsened disease condition was 99 µg/dL preoperatively, 105.5 µg/dL on the 15th day, 65 µg/dL on the 30th day and 85.5 µg/dL on the 90th day. **Friedman's test predicts a moderate association between the zinc level trend and the disease progression (p=0.04).**

TABLE 13: Correlation of serum iron levels (µg/dL) with disease progression (Spearman's rank correlation)

Time interval	Correlation of serum iron levels (µg/dL) with disease progression	Significance (p-value)
Preop	-0.169	0.303
15th day	-0.082	0.620
30th day	-0.385	**0.015**
90th day	-0.223	0.172

The above table suggests that there is an irregular negative correlation between serum iron levels and disease progression but is not significant ($p > 0.05$) for preop, 15th day and 90th day. This implies worsening disease condition was related to decreasing serum iron levels, but generalization cannot be done because significance was not met. The serum iron level for 30th day post-op correlates moderately and negatively with disease progression with spearman's correlation of -0.385 ($p = 0.015$).

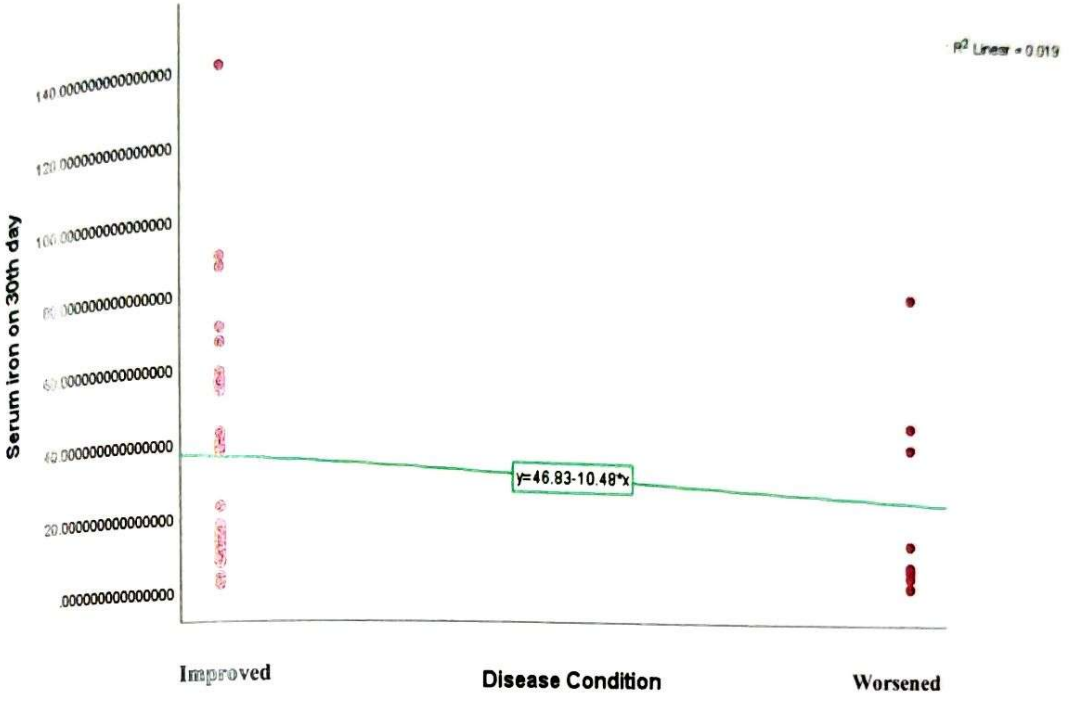


FIGURE 15: Scatter plot representing serum iron values on 30th -day post-op with disease progression

The figure shows negative correlation between serum iron levels on 30th day post-op plotted for patients who had improved and worsened disease outcome.

TABLE 14: Correlation of serum zinc levels (µg/dL) with disease progression (Spearman's rank correlation)

Time interval	Correlation of serum zinc levels (µg/dL) with disease progression	Significance (p-value)
Preop	-0.56	0.733
15th day	-0.011	0.946
30th day	-0.319	**0.048**
90th day	-0.195	0.234

The above table suggests that there is an irregular negative correlation between serum iron levels and disease progression but is not significant ($p > 0.05$) for 15th day and 90th day. This implies worsening disease condition was related to decreasing serum zinc levels, but generalization cannot be done because significance was not met. The serum iron level for 30th day correlates moderately and negatively with disease progression with spearman's correlation of -0.319 ($p = 0.048$).

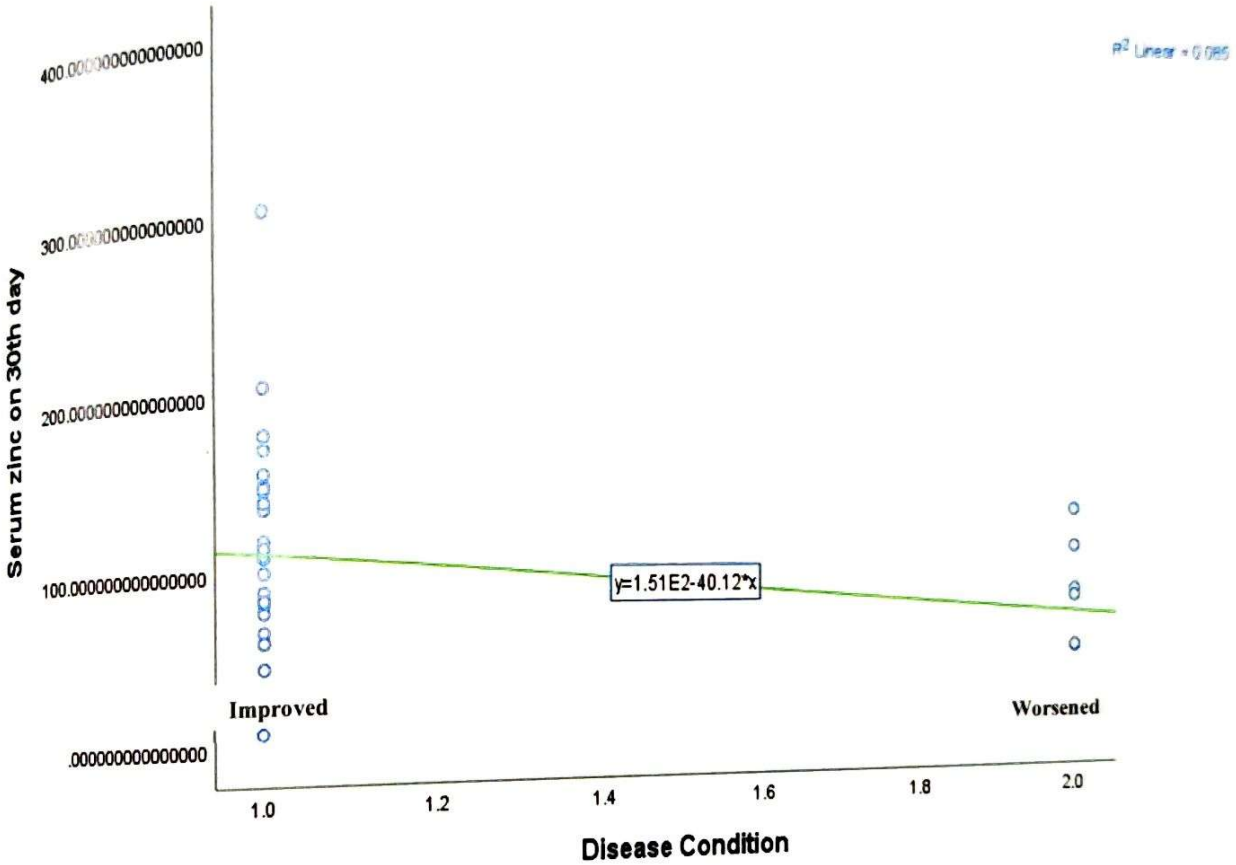


FIGURE 16: Scatterplot representing serum zinc values on 30th day post-op with disease progression

The figure shows the negative correlation between serum zinc levels on 30th day post-op plotted for patients who had improved and worsened disease outcome.

TABLE 15: Correlation of difference of serum iron from normal serum iron level and disease progression

Time interval	Correlation of difference of serum iron from normal with disease progression	Significance (p-value)
Preop	-0.152	0.376
15th day	-0.090	0.589
30th day	-0.155	0.345
90th day	-0.042	0.067

The above table suggests that there is a negative correlation between the difference of serum iron levels from mean of normal serum iron levels in the population (110 µg/dL in females and 130 µg/dL in males) and disease progression but is not significant ($p > 0.05$) for preop, 15th day and 90th day with disease progression with Spearman's rank correlation.

TABLE 16: Correlation of difference of serum zinc from normal serum zinc level and disease progression

Time interval	Correlation of difference of serum zinc from normal with disease progression	Significance (p-value)
Preop	-0.056	0.202
15th day	-0.166	0.298
30th day	-0.319	**0.048**
90th day	-0.125	0.345

The above table suggests that there is a negative correlation between the difference of serum zinc levels from the mean of normal serum zinc levels in the population (80 µg/dL) and disease progression but is not significant ($p > 0.05$) for preop, 15th day and 90th day post-op. The serum zinc level for the 30th day post-op correlates moderately with disease progression with spearman's rank correlation of -0.319 ($p = 0.048$).

TABLE 17: Area under the ROC curve and determined cut-off levels for serum iron levels.

Time	Area under curve	Cut-off point (µg/dL)	Sensitivity	Specificity
15th day	0.558	18.5	54.8%	75%
30th day	0.649	14.5	64.5%	62.5%
90th day	0.560	31	58.1%	62.5%

The Area under the Receiver Operating Curve (ROC) signifies how well the serum iron levels used above predicts the worsening of disease over improvement. For 15th day, 30th day and 90th day the values were 0.558, 0.649 and 0.560 respectively. The cut-off point of serum iron level has been calculated using Youden's index taking the best of sensitivity and specificity for the above test. It implies the area under the ROC is maximum for 30th day serum iron levels with cut off point of 14.5 mcg/dL, below which the patients shall have disease worsening with sensitivity and specificity of 64.5% and 62.5% respectively.

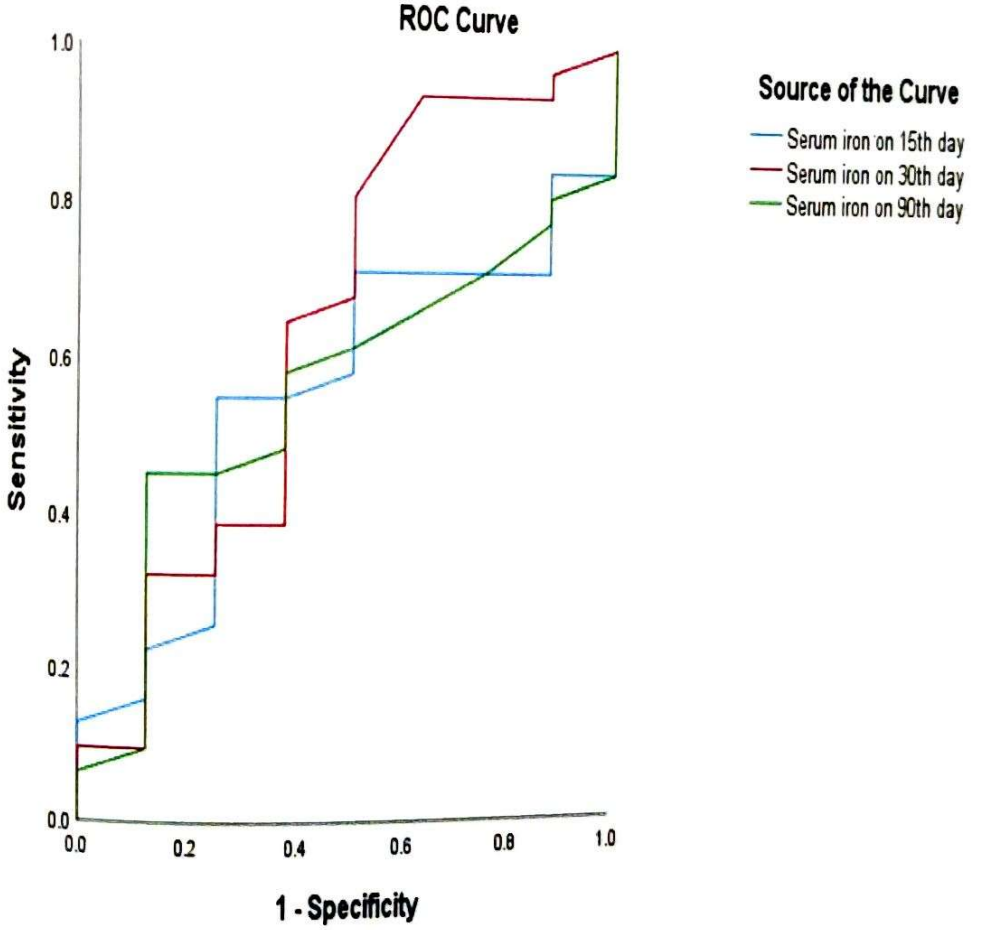


FIGURE 17: ROC curve for serum iron levels

TABLE 18: Area under the ROC curve and determined cut-off levels for serum zinc levels.

Time	Area under curve	Cut-off point (µg/dL)	Sensitivity	Specificity
15th day	0.508	87.5	64.5%	50%
30th day	0.728	84.5	64.5%	75%
90th day	0.482	64.5	74.2%	37.5%

The Area under the Receiver Operating Curve (ROC) signifies how well the serum zinc levels used above predict the worsening of disease over improvement. For the 15th day, 30th day and 90th day the values were 0.508, 0.782 and 0.482 respectively. The cut-off point of serum zinc level has been calculated using Youden's index taking the best of sensitivity and specificity for the above test. It implies the area under the ROC is maximum for 30th day serum zinc levels with a cut-off point of 84.5 µg/dL, below which the patients shall have disease worsening with sensitivity and specificity of 64.5% and 75% respectively.

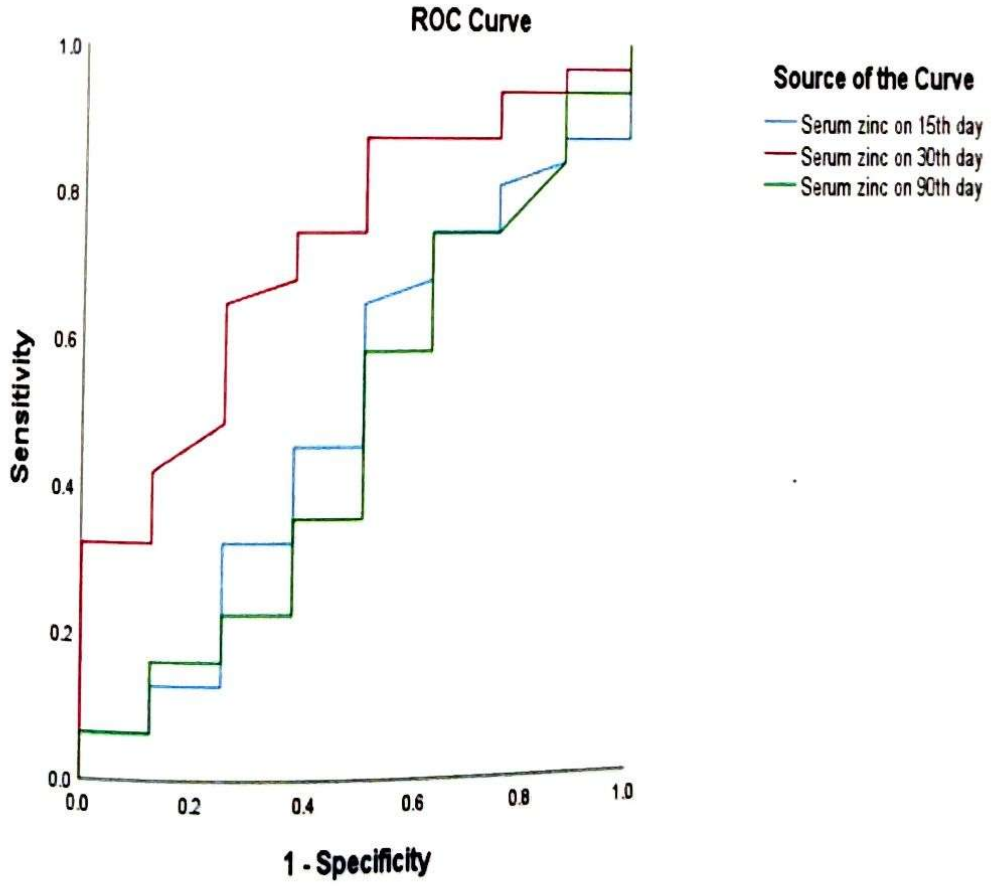


FIGURE 18: ROC curve for serum zinc levels

DISCUSSION

We conducted a prospective observational study where we enrolled 41 patients of Mucormycosis admitted at All India Institute of Medical Sciences Jodhpur, out of which two patients were lost to follow due to death in between the follow-up period from December 2021 to August 2022 (duration of 9 months). The present study was conducted with the aim to evaluate the fluctuations in serum iron and zinc levels in mucormycosis patients in connection to the disease activity and also to investigate whether levels of iron and zinc can be utilized to forecast the activity of the disease.

Although mucormycosis is not gender-dependent, the infection has been reported more in males. In this study, 78.05% of patients were male and the remaining 21.95% were female. In this context, similar findings were reported by Patel A et al(103) and Kamat M et al (35). In a descriptive study, Farghly YS et al stated that mucormycosis was predominantly seen in males with Diabetes mellitus(104).

Bhatt K et al cited that Covid-19 is mainly responsible for the recent mucormycosis (105). Whereas our research finding highlighted that only 34.15% of patients had a history of COVID infection in the past and the majority i.e 65.85% of patients had no such history.

Depending on varying factors, mucormycosis infection is classified into various types: rhino-orbital-cerebral/rhino-orbital/sino nasal/cutaneous and other disseminated types of mucormycosis. Several previous works of literature highlighted that the most common form of mucormycosis is rhino-orbital-cerebral (44-49%). In contrast, Moorthy A. et al explored in his study that 48.98% of patients were having Sino nasal mucormycosis followed by 26.83% having Sino orbital mucormycosis (106). In this context, Deepankar P et al also reported that the most common subtypes were rhino-orbital (61.4%), followed by sino-nasal (16.8%), rhino- orbito- cerebral (16.8%) and orbital (3.0%)(107). In a meta-analysis, Patel et al. also described rhino-orbital mucormycosis as a major type (67.7%) of mucormycosis. Our study communicated that out of 41 patients, the majority i.e 20 (48.78%) patients had Sino nasal mucormycosis followed by 11 (26.83%) Rhino orbital mucormycosis patients and 9 (21.95%) patients were having Rhino-orbital-cerebral mucormycosis.

Furthermore, comorbidities have a vital role in mucormycosis infection. In terms of comorbidities, our findings communicated that out of 41 patients, 12 (29.27%) were diabetic

and 14 (34.15%) patients had diabetes along with some other comorbidities (like CAD, HTN, Thyroid disease etc) and 9 (21.95%) patients had no known comorbidities. In reference to our research findings, Deepankar P et al revealed that diabetes mellitus was present in 71% of cases of mucormycosis. A systematic review also highlighted that a total of 224 (85.8%) patients were diabetic (107).

Bhadania S et al reported a statistically significant difference in levels of serum ferritin (p = 0.008) between the radiologically judged two groups of the mild extent of invasion of mucormycosis (rhinosinusitis) and severe extent of invasion (rhino-orbital/cerebral mucormycosis), with a severe extent group having higher levels of serum ferritin(108). In contrast, a cross-sectional study by Rao C et al (2022) highlighted that the mean serum iron (50.37) and TIBC (255.37) were significantly higher in Stage 4 CAM cases compared with less invasive stage 2 CAM cases (109).

Iron has a significant role in the disease progression of mucormycosis. In this context, a cross-sectional study by Khan I et al (2022) reported that most of the patients with Covid-associated Mucormycosis have an iron overload state defined by elevated serum iron and serum ferritin levels(110). Additionally, Rao C et al (2022) revealed a significant correlation between the clinical severity of CAM and higher mortality, and increased serum iron levels and inflammatory markers in this population of patients(109).

Singh A et al highlighted that Serum Ferritin levels were above the normal range in 87.5% (140 out of 160) of the patients tested, and the median value noted as being considerably raised at 968.6 ng/ml (111).

Moreover, Umar H et al (2022) revealed that the mean serum iron values (33 vs. 45 µg/dl, p = .03) were significantly lower in COVID-19-associated mucormycosis cases than in controls (96).

In context to these findings, the present study communicated that the level of serum Iron in mucormycosis was 31.92±21.88 in the pre-operative phase. On day 15th, the level was 28.13±31.22 (decreased) followed by 34.21±31.29 on the 30th day (a little increase). But the serum Iron level increased on day 90th and was 40.56±26.02. Whereas, the median level of serum Iron among mucormycosis was 24 [11, 50] µg/dl in the pre-operative phase. On day 15th, the iron level was 17 [10, 33] µg/dl followed by 18 [8, 55] µg/dl on the 30th day. The

level of serum Iron was increased on day 90th and it was 33 [23, 45] µg/dl. The Friedman test applied for four interval iron levels has been found significant (p = 0.007).

Furthermore, the present study also explored the role of serum Zinc among patients with mucormycosis. It is possible that the excess availability of zinc in the body owing to its incessant use by COVID-19 patients might be adding to the risk of fungal infections like mucormycosis in the patients (92).

An observational study reported that the level of serum Zinc was 97±11 among patients with mucormycosis (97). In the context of this study, the levels of serum Zinc were assessed in the preoperative and postoperative phases in present study. The level of serum Zinc was 94.54±50.84 in the pre-operative phase. The median level of serum Zinc among mucormycosis was 90 [57, 127] µg/dl in the pre-operative phase. On day 15th, the level was 107 [70, 149] µg/dl followed by 100 [60, 136] µg/dl on the 30th day. The level of serum Zinc on day 90th was 79 [60, 119] µg/dl. The Friedman test applied for four interval zinc levels has been found significant (p = 0.01).

In a case-control study, Kumar et.al highlighted that intake of zinc by either self-administration by patients to acquire innate immunity or by over-prescription of multivitamins containing zinc was found to be associated significantly with COVID-19 associated mucormycosis giving the result of 89.1% of cases having a history of zinc intake(96). Whereas in our study none of the patients had history of oral zinc supplementation.

A study by Bano Tanveer et al also highlighted that the association between zinc intake and severity at the time of presentation was found to be statistically insignificant (112).

Mansour et al (2022) reported a statistically significant difference among study groups regarding the serum zinc level (p=0.039), where lower mean serum zinc levels were noted in the patients and it was associated with the severe outcome of COVID-19 infection (93).

Our research communicated that on the 15th day follow-up period, out of 41 patients, the majority 23(56%) had no changes in the disease activity, while 4 (9.76%) patients had decreased disease activity and 14 (34.15%) patients had an increase in the disease activity. The majority were of the 2d stage followed by 3b and 3c stages. On the 30th day follow-up

period, out of the 41 patients, 22 had no change in the disease activity, 8 patients had decreased disease activity and 9 patients had worsened disease.

On the 90th day follow-up period, out of 41 patients, 29 had no changes in the disease activity, 9 patients had decreased disease activity and 1 patient had an increase in the disease activity. In support of our findings, Muthu V et al reported that zinc level did not contribute to the pathogenesis of mucormycosis(95).

PVSN et al (2022) conducted a cross-sectional comparative study to analyse Serum Zinc in association with the severity and mortality in SARS-CoV-2 patients (94). The study found a significant difference in the levels of zinc ($p < 0.0014$) when compared between the controls, discharged and deceased individuals.

In a similar study, Maares M et al revealed that depending on the severity of COVID-19, free serum zinc showed significantly decreased concentrations (113).

The study showed that lower serum iron and zinc levels are found with worsening disease outcomes and there is an irregular negative correlation between serum iron as well as zinc levels and disease progression. It may be because both iron and zinc as micronutrients are essential for the growth and survival of the fungi and thus they consume them from the host cells for their own survival.

CONCLUSION

Mucormycosis is a life-threatening and devastating invasive fungal infection that emerged amidst the COVID-19 Pandemic and affected millions of people especially those with known comorbidities (commonly Diabetes mellitus) and some with a history of COVID-19 infection in the past.

In an attempt to establish an association and thereafter predictability of disease progression with serum iron and zinc levels, we have found a significant change in the serum iron and zinc levels over four different temporal points (pre-operatively, postoperative days 15^{th}, 30^{th} and 90^{th}) which had a significant negative correlation between both serum iron and zinc levels and the disease progression.

Disease severity on the 30^{th} day had a sensitivity and specificity of 64.5% and 62.5% respectively, with a cut-off of 14.5µg/dL for iron. The sensitivity and specificity for Zinc were 64.5% and 75% respectively, with a cut-off of 84.5µg/dL. This was despite the median levels at the initial presentation being less than normal for iron and normal for zinc in most patients.

Hence, the study concluded that the serum iron and zinc at the postoperative 30^{th} day can be used as a serum marker for predicting the disease progression with sensitivity and specificity of 64.5% and 62.5% respectively for iron and 64.5% and 75% respectively for zinc.

STRENGTHS AND LIMITATIONS

Strengths of the study:

1. Biochemical and radiological analyses were done for all patients uniformly at 4 different points with regular follow-up.
2. All the biochemical analyses were done with single standard re-agents individually for iron and zinc.
3. The age of the patient was evenly distributed, removing the age-related influence on serum iron and zinc levels.

Limitations of the study:

1. Small sample size – By the end of 2nd wave of COVID-19 Pandemic, mucormycosis cases were reducing in trend.
2. Single-centre study.
3. Pre-analytical variables can never be completely accounted for hence test reports may be skewed.
4. All radiological assessments were done by different individuals.

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APPENDICES

APPENDIX-A

Department of Otorhinolaryngology

All India Institute of Medical Sciences, Jodhpur

INSTITUIONAL ETHICAL CLEARANCE CERTIFICATE

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
All India Institute of Medical Sciences, Jodhpur
संस्थागत नैतिकता समिति
Institutional Ethics Committee

Chairman

Dr. F.S.K. Barar
Basic Medical Scientist

Members

Justice N N Mathur
Legal Expert

Dr. Varsha Sharma
Social Scientist

Mr. B.S. Yadav
Lay Person

Dr. K.R. Haldiya
Clinician

Dr. Arvind Mathur
Clinician

Dr. Kuldeep Singh
Clinician

Dr. Pradeep Kumar Bhatia
Clinician

Dr. Pankaj Bhardwaj
Clinician

Dr. Surajit Ghatak
Basic Medical Scientist

Dr. Vijaya Lakshmi Nag
Basic Medical Scientist

Dr. Sneha Ambwani
Basic Medical Scientist

Dr. Abhinav Dixit
Basic Medical Scientist

Dr. Tanuj Kanchan
Basic Medical Scientist

Dr. Praveen Sharma
Member Secretary

No. AIIMS/IEC/2021/3860 Date:11/9/2021

Certificate Reference Number: AIIMS/IEC/2021/3724

To,
Dr. Bikram Choudhury
Additional Professor
Department of Otorhinolaryngology
AIIMS, Jodhpur

Subject: Estimation of pre & post operative levels of serum iron and zinc in patients with mucormycosis and to explore the possibility of these being used to predict the disease activity.

Dear Dr. Choudhury,

This has reference to your above mentioned **MS Thesis Portocol of Dr. Hage Duniya**. The project was discussed in the Ethics Committee online meeting held on 31.8.2021 and the following members of the Ethics Committee attended the meeting:

1.	Dr. F.S.K Barar, Former Professor & Head, Pharmacology, SMS Medical College	Chairman
2.	Justice N.N. Mathur, Former Vice Chancellor, National Law University, Jodhpur	Member
3.	Dr. Varsha Sharma, Former Principal, Konoria Girls PG College, Jaipur	Member
4.	Mr. B.S. Yadav, Principal, Delhi Public School, Jodhpur	Member
5.	Dr. K.R. Haldiya, Former Scientist F, DMRC, Jodhpur	Member
6.	Dr. Arvind Mathur, Former Principal, Dr. S.N. Medical College, Jodhpur	Member
7.	Dr. Kuldeep Singh, Professor, Dept. of Paediatrics, AIIMS, Jodhpur	Member
8.	Dr. Abhinav Dixit, Professor, Dept. of Physiology, AIIMS, Jodhpur	Member
9.	Dr. Tanuj Kanchan, Professor, Dept. of Forensic Medicine, AIIMS, Jodhpur	Member
10.	Dr. Pankaj Bhardwaj, Additional Professor, Dept. Of CM & FM, AIIMS, Jodhpur	Member
11.	Dr. Praveen Sharma, Professor, Dept. of Biochemistry, AIIMS, Jodhpur	Member Secretary

The Project has been approved with effect from 31.8.2021 subject to the following conditions:

- The approval is valid for the period of the conduct of study according to this protocol under the responsibility of Principal Investigator: Dr. Bikram Choudhury, Co-investigators: Dr. Mithu Banerjee, Dr. Amit Goyal, Dr Kapil Soni, Dr. Vidhu Sharma, Dr. Sarbesh Tiwari and Dr. Vidhi Jain.
- No significant changes to the research protocol could be made and implemented without prior consent of the IEC and any changes/deviations from the protocol which increase the risk for the subjects should be submitted to the IEC and approved by it prior to implementation.
- It is confirmed that the Ethics Committee of AIIMS, Jodhpur is composed of and functions as per ICH GCP and other applications regulatory guidelines.
- It is hereby confirmed that neither you nor any of the study team members have participated in the voting/decision making procedures of the committee.
- The study progress report should be made available to the IEC for review annually.

With Warm regards

Yours Sincerely,

Dr. Praveen Sharma
Member Secretary

Member Secretary
Institutional Ethics Committee
AIIMS, Jodhpur

Basni Phase-2, Jodhpur, Rajasthan-342005; **Website:** www.aiimsjodhpur.edu.in; **Phone:** 0291-2740741 Extn. 3109
E-mail : ethicscommittee@aiimsjodhpur.edu.in; ethicscommitteeaiimsjdh@gmail.com

APPENDIX-B

Department of Otorhinolaryngology

All India Institute of Medical Sciences, Jodhpur

Serial no._____

INFORMED CONSENT FORM

Subject: Consent for participation in study

Participant's registration number: _____.

I..... of..... declare that on date .....all the details of this information sheet given to me has been explained in my language. I am told that in this process, my blood samples will be taken for measuring level of zinc and iron before the surgery and will also be taken after the surgery to compare the variation in levels. This research is being done for studies and treatment. I understand that all information related to me in this research will be kept by the responsible person of AIIMS Jodhpur. I allow them to see all the information related to me. I have been told that all the information related to me will be kept confidential. I have also been told that the results of this research can be published in any book or journal and can be displayed in any conference. I have also been told that my name or any other identity will not be used without my consent. I know that I am participating in this research with my consent and I can refuse to participate in this research at any time without any reason.

I agree to participate in this research.

(Participant's Signature)

Date: Place:

Name of the Participant: _____

Son / Daughter / Spouse of: _____

Complete postal address: _____

This is to certify that the above consent has been obtained in my presence.

1) Witness – 1

Name:

Address:

2) Witness – 2

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Name:

Address:

Principle investigator's signature: Dr. Hage Duniya

Date: Place:

APPENDIX-C

Department of Otorhinolaryngology

All India Institute of Medical Sciences, Jodhpur

प्रतिभागी सुचना पत्र

□वषय: अध्ययन में भागीदारी के □लए सहमति

प्रतिभागी का पंजीकरण नंबर: _____

मै ..... यह घोषणा करता हूं □क दिनांक ..... इस सूचना पत्र के सभी □ववरण मुझे मेरी भाषा में दिए गए हैं। मुझे बताया गया है □क इस प्र□क्रया में, जिंक और आयरन लेवल की तुलना करने के □लए ऑपरेशन से पहले और उसके बाद भी मेरी खून के नमूने □लए जाएंगे I यह शोध अध्ययन और उपचार के □लए □क्रया जा रहा है। मैं समझता हूं □क इस शोध में मुझसे संबं□धत सभी जानकारी एम्स जोधपुर के जिम्मेदार व्यक्ति द्वारा रखी जाएगी। मैं उन्हें अपने संबं□धत सभी जानकारी देखने की अनुमति देता हूं। मुझे बताया गया है □क मेरे संबं□धत सभी जानकारी गोपनीय रखी जाएगी। मुझे यह भी बताया गया है □क इस शोध के परिणाम □कसी भी पुस्तक या पत्रिका में प्रका□शत □कए जा सकते हैं और □कसी भी सम्मेलन में प्रद□र्शत □कए जा सकते हैं। मुझे यह भी बताया गया है □क मेरा नाम या □कसी अन्य पहचान का उपयोग मेरी सहमति के बिना नहीं □क्रया जाएगा। मुझे पता है □क मैं अपनी सहमति से इस शोध में भाग ले रहा हूं और मैं बिना □कसी कारण के □कसी भी समय इस शोध में भाग लेने से इनकार कर सकता हूं।

मैं इस शोध में भाग लेने के □लए सहमत हूं।

(हस्ताक्षर): दिनांक:

स्थान:

प्रतिभागी का नाम: _____

पुत्र / पुत्री / पति / पत्नी: _____

पूरा डाक पता: _____

यह प्रमा□णत करना है □क मेरी उपस्थिति में उपरोक्त सहमति प्राप्त हुई है।

1) गवाह - 1 2) गवाह - 2

नाम - नाम-

पता - पता -

प्रमुख अन्वेषक के हस्ताक्षर: डॉ हागे दुनिया

स्थान: दिनांक

APPENDIX-D

Department of Otorhinolaryngology

All India Institute of Medical Sciences, Jodhpur

PATIENT INFORMATION SHEET

TITLE: Estimation of pre & post-operative levels of serum iron and zinc in patients with mucormycosis and to explore the possibility of these being used to predict the disease activity.

This study requires your detailed information and recall of previous clinical history for the assessment to be done to proceed in this study. You will be subjected to detailed clinical assessment and routine workup and will be followed up till the biochemical values after surgery is known. This study would require the details of preoperative and postoperative values ofiron and zinc values. You are expected to attend to all the questions put in front ofyou depending on the mutual comfort of you and the investigator. There are noobvious, expected or known adverse effects on the patient due to this study. You havebeen invited to take part in a study, which will help us in better understanding of thedisease. You are free to withdraw from the study at any time and this will not haveany negative implication on you/your future treatment in the hospital.

Contact Person for further queries:

Dr. Hage Duniya

8302659712

APPENDIX-E

Department of Otorhinolaryngology

All India Institute of Medical Sciences, Jodhpur

रोगी सूचना पत्र

टाइटल: एस्टिमेशन ऑफ़ प्री एंड पोस्ट ऑपरेटिव लेवल्स ऑफ़ सीरम आयरन एंड जिंक इन पेशेंट्स □वथ मुकर मैक्सिस एंड तू एक्स्प्लोर दी पॉ□सबि□लटी ऑफ़ दीस बीइंग युसेड तू प्रे□क्टडा □जीज एक्टि□वटी

इस अध्ययन में आगे बढ़ने के □लए मूल्यांकन के □लए इस अध्ययन के □लए आपकी □वस्तृत जानकारी की आवश्यकता है। आपको □वस्तृत नैदानिक मूल्यांकन और रूटीन वर्क अपके अधीन □कया जाएगा और सर्जरी के बाद बायोके□मस्ट्री रिपोर्ट तक इस का पालन □कया जाएगा। इस अध्ययन के □लए प्रीऑपरेटिव और पोस्टऑपरेटिव सीरम जिंक और आयरन □वचरण की आवश्यकता होगी। आपको और जांचकर्ता के आपसी आराम के आधार पर आपके सामने रखे गए सभी प्रश्नों में भाग लेने की उम्मीद है। इस अध्ययन केकारण रोगी पर कोई स्पष्ट, अपे□क्षत या ज्ञात प्रतिकूल प्रभाव नहीं हैं। आपको एक अध्ययन में भाग लेने के □लए आमंत्रित □कया गया है, जो हमें आपकी बीमारी को बेहतर समझने में मदद करेगा। आप □कसी भी समय अध्ययन से वापस लेने के □लए स्वतंत्र हैं और अस्पताल में आपके भ□वष्य के उपचार पर इसका कोई नकारात्मक प्रभाव नहीं होगा।

आगे के प्रश्नों के □लए संपर्क व्यक्ति:

डॉ हागे दुनिया

8302659712

APPENDIX-F

Department of Otorhinolaryngology

All India Institute of Medical Sciences, Jodhpur

INITIAL EVALUATIONS

NAME:

AGE/Gender:

ADDRESS:

PRESENTING SYMPTOMS:

PAST H/o COVID INFECTION

H/o COMORBIDITIES

KOH Mount: Proven Not proven

EXAMINATION

Vitals

FACE EXAMINATION

ORAL CAVITY EXAMINATION

NOSE EXAMINATION

EAR EXAMIANTION

EYE EXAMINATION

OTHER FINDINGS:

INVESTIGATION CHART

ROUTINE BASIC INVESTIGATIOS

CBC

PT/INR- s/

KFT (Urea/Creatinine) - / mg/dL

Na/K/Cl- / / mEq/ L

HbA1c- g%

Ferritin -

INVESTIGATIONS PREVALENT TO THE STUDY

Serum Iron -

Serum Zinc -

OTHER INVESTIGATIONS -

APPENDIX-G

Department of Otorhinolaryngology

All India Institute of Medical Sciences, Jodhpur

PRE-OPERATIVE ASSESSMENT

Clinical and radiological status of the patient will be assessed.

Assessment of levels of serum iron and zinc preoperatively using Beckman Coulter method for iron and colorimetric method for zinc will be done.

APPENDIX-H

Department of Otorhinolaryngology

All India Institute of Medical Sciences, Jodhpur

POST- OPERATIVE ASSESSMENT

Assessment of level of serum iron and zinc post operatively on 15 days, 30 days and 90 days using Beckman Coulter method for iron and colorimetric method for zinc and assessment done.