The value of hydrogen sulphide as marker of severity and prognosis of in acute pancreatitis

A dissertation submitted in partial fulfillment of the requirements for

DM (Medical Gastroenterology) examination of the

Tamil Nadu Dr. M.G.R. Medical University, Chennai,

to be held in August 2014.

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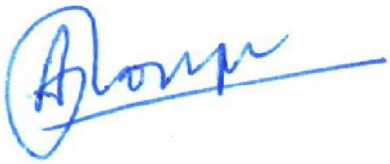

Guide
Dr A J Joseph MD. DM Professor,
Department of Clinical gastroenterology
Date:

Prof. CE.Eapen, MD. DM
Professor and Head,
Department of Clinical Gastroenterology
and Hepatology
Date:

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Dear Mr. Denzil,

Sub: **Fluid Research grant project NEW PROPOSAL:**
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Dr. Iqbal Nabi Qureshi, Senior Registrar, Medical Gastroenterology, Dr. A. J.Joseph, Medical Gastroenterology, Kavitha R, PhD Student
Dr Anup Ramachandran, K. A. Balasubramanian, Gastrointestinal Sciences

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The Institutional Review Board at its meeting held on 4th July, 2012 vide IRB Min. No: ***7914*** accepted the project for *2 years* at a total sanction of *Rs.80, 000/- (Eighty Thousand Only)* Rs. 40,000/- can be sanctioned for 12 months, a subsequent installment of Rs. 40,000/- will be released at the end of the first year following the receipt of the progress report. Kindly arrange to transfer the said amount to a separate account to be operated by Drs. Iqbal Nabi Qureshi & A. J.Joseph.

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CC: Dr. Iqbal Nabi Qureshi, Senior Registrar, Medical Gastroenterology, CMC
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I take this opportunity to express my sincere gratitude to my guide, Dr A J.Joseph, Professor Department of Clinical Gastroenterology, for his guidance, encouragement and constant support in undertaking and completing this project.

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INTRODUCTION

1. INTRODUCTION

1.1.1 Acute pancreatitis - pathophysiology and diagnosis.

Acute pancreatitis (AP) is an inflammatory response to pancreatic insult, which can vary in intensity from a mild self-limiting inflammation, to a severe type with high rate of complications, significant mortality, and high medical cost. Acute pancreatitis is diagnosed in a patient presenting with two of the following three criteria– severe upper abdominal pain which may radiate to the back; a serum lipase or amylase greater than three times normal value; radiologic imaging which is consistent with the diagnosis, usually with imaging modalities like ultrasound abdomen or computed tomography (CT)[1].

1.1.2 Classification – 'acute' vs 'acute on chronic' pancreatitis.

By definition, acute pancreatitis occurs in a previously normal gland and has the potential to resolve over time without residual damage in exocrine and endocrine function. Pancreatitis is classified as 'acute on chronic' if an episode of acute pancreatitis is superimposed on a background of chronic pancreatitis (as evidenced by changes of chronic pancreatitis on imaging studies).

1.1.3 Etiology and pathological classification

In most cases, the etiological agent is either gallstones or alcohol in excess consumption. The critical initiating event is the activation of digestive enzymes in the pancreatic acinar cells. Pancreatic inflammation then leads to two broad categories of changes. One that is limited to inflammation of the pancreas and peripancreatic tissues without necrosis called edematous

interstitial acute pancreatitis. The second, more severe type called necrotising acute pancreatitis is characterised by necrosis of pancreatic and/or peripancreatic tissues.

1.1.4.Disease course and complications

About 80% of patients with acute pancreatitis have mild disease and have a good outcome. The rest have severe pancreatitis with a high rate of mortality (25%) and significant morbidity.

1.1.5.1 Local complications

Local complications of pancreatitis include necrosis and fluid collections.If they persist for more than 4 weeks, they evolve to form pseudocysts and walled off necrosis respectively. Peripancreatic inflammation can also lead to local vascular complications, namely, splanchnic venous thrombosis, bleeding or pseudoaneurysm.

1.1.5.2 Systemic complications

In severe pancreatitis, leukocyte and cytokine mediated distant organ damage leads to the development of multiple organ dysfunction syndrome (MODS). Acute lung injury, that can clinically manifest as acute respiratory distress syndrome and acute kidney injury are the major complications of MODS in acute pancreatitis.

1.2.1 Classification of severity.

Pancreatic damage can leads to a localized and systemic inflammatory response. Acute pancreatitis has been traditionally classified as severe and mild, according to the Atlanta classification[2]based on the absence or presence of local and systemic complications and distant organ failure during the entire course of the illness. The Atlanta classification was recently modified to include a third group of patients, classified as moderately severe acute pancreatitis, who have transient organ failure or have only local complications without persistent organ failure. The majority of the patients who develop acute pancreatitis have mild disease.

1.2.2 Prediction of severity

Severe pancreatitis has to be identified as early as possible to provide more intensive monitoring, appropriate therapy and an accurate prognosis. Thus every clinician who treats pancreatitis is looking for a tool that is easy to use, that can be used early in the course of illness (ideally at admission) and accurately identifies those who have or will develop severe acute pancreatitis.

1.2.3 Predictors of severity

There are many clinical and laboratory parameters which are used to predict severity in patients with acute pancreatitis. These include (a) patient characteristics like age and body mass index (BMI),(b)clinical signs such as shock and tachycardia,(c) bio-chemical parameters such as C-reactive protein (CRP), (d)imaging studies like chest X-ray and CT scan and (e)composite scores such as Ranson's[3] and APACHE-II scoring systems[4].

There are many laboratory markers have been used as predictors of severity. They include hematocrit[5],CRP[6], lactate dehydrogenase,interleukin-6 (IL-6)[7],procalcitonin, polymorphonuclear leukocyte elastase, phospholipase A_2 and urinary trypsinogen activation peptide[8]. Hydrogen sulphide (H_2S), the topic of this study, is a new marker which has been shown in animal models to correlate with severity of acute pancreatitis. Human studies on the usefulness of H_2S in acute pancreatitis are scanty.

1.3.1 Hydrogen sulphide (H_2S)

Hydrogen sulphide has been known for centuries for its distinct odour and toxic nature. Recent studies have established H_2S to be an important 'gasotransmitter' in the body, along with nitric oxide and carbon monoxide[9].

1.3.2 Synthesis and catabolism

H_2S is synthesized in the human body from the amino acids homocysteine, cystathionine and cysteine by the action of at least two distinct enzymes: cystathionine-β-synthase and cystathionine-γ-lyase. In central nervous system, Cystathionine-β-synthase (CBS) and in the cardiovascular system, cystathionine-γ-lyase (CSE) are the key enzymes responsible for the endogenous production of H_2S. Both CBS and CSE enzymes use substrate as L-cysteine and cofactor as pyridoxal-5′-phosphate.[10] As the end product of metabolism, H_2S have a negative feedback effect on the activity of these enzymes [11].In vivo, H_2S is metabolized rapidly by a multiple enzymatic processes. Of all these processes, the mitochondrial oxidation mechanism represents the most important pathway of H_2S catabolism. Finally, H_2S is an endogenous reducing agent which can be easily consumed by a variety of circulating oxidant species in the

vasculature such as hydrogen peroxide, peroxynitrite, superoxide radicals and hypochlorite.[12-15]. Its excretion is mainly by the kidney as free or conjugated sulfate[16].

Pancreatic acinar cell expresses both major H_2S forming enzymes CBS and CSE. Messenger-RNA for CSE is expressed in mouse pancreas and pancreas homogenates can convert L-cysteine to H_2S ex-vivo.

1.3.3 H_2S in animal models of pancreatitis

1.3.3.1 Correlation with severity

Plasma levels of H_2S are increased in mice upon induction of acute pancreatitis. In isolated pancreatic acinar cells, the level of H_2S and CSE messenger RNA were also significantly elevated when stimulated by caerulein[17]. Data from animal studies suggest that there is a good correlation between H_2S levels and the severity of pancreatitis and presence of acute lung injury.

1.3.3.2 Attenuation of inflammation by blockade of H_2S production

The conversion of L-cysteine to H_2S in pancreas homogenates is significantly reduced in mice which were pre-treated with DL-propargylglycine (PAG)[18]. PAG is an inhibitor of the H_2S -synthesizing enzyme cystathionine-gamma-lyase (CSE). In addition, treatment of animals with PAG (either therapeutic or prophylactic) reduced the severity of pancreatitis as shown by a significant reduction of acinar cell injury, necrosis, hyper-amylasemia, myeloperoxidase activity of pancreatic tissue, and by the histological evidence.

As we know, severe acute pancreatitis is associated with lung injury, which is characterized by increased MPO activity in lung tissue, sequestration of neutrophils within the lung and histological evidence of lung injury. When PAG was administered either Prophylactic or therapeutic, it protected mice against lung injury associated with acute pancreatitis as evidenced by a significant reduction in MPO activity and by histologically diminished lung injury [18]. So, there is pro-inflammatory role of H_2S in regulating the severity of pancreatitis and associated lung injury as evidence by these effects of CSE blockade.

Caerulein-induced acute pancreatitis is associated with a significant increase in various chemokines like macrophage inflammatory protein- (MIP-) 1α, monocyte chemoattractant protein (MCP)-1, and MIP-2 in both the lungs and pancreas, suggesting their role as early mediators in both local as well as distant inflammatory response. In caerulein-stimulated pancreatic acinar cells, PAG induced inhibition of H_2S formation reduced CSE messsanger RNA expression and also decreased the levels of MIP 1 α, MIP 2 and MCP 1.[17,19].

1.4 Study rationale

Though many predictors of severity in acute pancreatitis are available, because of low specificities and the fact that severe cases of acute pancreatitis are a smaller proportion, the positive predictive value of the available tests have been disappointing. H_2S seems to correlate well with disease severity in animal models. We set out to evaluate whether H_2S levels correlated with severity in acute pancreatitis and if so how good a predictor of severity it was.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Disease burden

In acute pancreatitis, the annual incidence ranges from 5 to 50 per 10^5 persons [20-23]. Most of the cases of pancreatitis are mild and self-limiting. Mild pancreatitis accounts for about 80 % of all the cases. The overall mortality rate is about 10%[24], with higher mortality rates of 25% - 33% in severe cases.

2.2 Etiology

The most common causes of acute pancreatitis are gallstone disease and alcohol abuse, accounting for 60–80% of the cases[25]. Other etiologic factors are hyperlipidaemia, hypercalcemia, drugs, ampullary and pancreatic ductal obstruction, tumors, pancreas divisum and hereditary pancreatitis[26]

2.3 Severity assessment and prognostication

As discussed earlier, acute pancreatitis without local complications or distant organ dysfunction is classified as mild. In the presence of local complications and/or persistent organ failure, it is classified as severe. Scoring systems such as the Ranson's score[27] and APACHE II [28]have a sensitivity of 65–75% in predicting the course of the illness, but like composite scores measuring multiple pathophysiological parameters, they are complex, need repeated measures and are not specific for acute pancreatitis. In spite of these drawbacks, the above mentioned scores are the best currently available descriptors of the patient's status. They provide a snapshot of the patient's status at index evaluation, and with repeated measurements, provide trends which can

be used for prognostication. They provide the clinician with validated, albeit impractical, tools to assess severity and predict outcome.

2.4 Prediction of severity with biomarkers

Several biomarkers have been studied to try and for predicting the severity of the pancreatitis before the patient develops complications of multiple organ dysfunction. These biomarkers are either enzymes released from the pancreas or mediators induced by the inflammation in the pancreas and distant organs (cytokines and CRP), and have a direct correlation with disease severity.

2.4.1 C-reactive protein

C-reactive protein (CRP) has been well studied and found to be useful. The main drawback of CRP is that peak plasma level is reached only after 2-3 days[29-30], and low values in patients presenting early can be falsely reassuring. CRP values >120 mg/L can detect pancreatic necrosis in 67 to100 % of cases [31].

2.4.2 Hematocrit

In animal models, it was shown that haematocrit rise occurs earlier than systemic inflammatory response or haemodynamic manifestations of acute pancreatitis[32-33]. A reliable predictor of the development of severe acute pancreatitis is admission hematocrit value greater than 47%. Failure of admission haematocrit to normalise after resuscitation or within first 24 hrs predicts the development of local and systemic complications. In patients with no haemoconcentration within first 24 hrs, organ failure is less likely [34].

2.4.3 Procalcitonin

One of the well studied marker of sepsis is Procalcitonin (PCT). It is commonly used as an early marker of systemic inflammatory response induced by sepsis and multi-organ failure [35.] Infected pancreatic necrosis, sepsis and multi-organ failure are the major complications of acute pancreatitis. Studies have shown that increased serum levels of PCT may be a reliable early predictor of a severe outcome such as infected pancreatic necrosis[36-37]

2.4.4 Lactate dehydrogenase

Lactate dehydrogenase (LDH) which is included as one of the criteria in Ranson's score has also been studied independently. In 42 patients with acute pancreatitis studied by Chen et al, serum LDH activity was higher in severe pancreatitis[38].On evaluating the isoenzymes, LDH-4 and LDH-5 were found to be increased, however, LDH-2 and LDH-3 are the predominant pancreatic isoenzymes, which suggest that the pancreas was not the major source of the circulating LDH. In a study done by Uhl and coworkers[39]on 52 patients (mild 29 and severe 23) with pancreatitis, high LDH measurements were noted in 82% of patients with necrotizing pancreatitis.

2.4.5 Interleukin-6 and -8

In response to a tissue injury, macrophages released interleukin 6 (IL-6) which mediates the production of various acute phase reactants including CRP. The peak levels of IL-6 are reached in 24 hours. It was shown that plasma IL-6 concentrations within 24 hours correlated with the mortality rate[40-42]. IL-8 is also increased in patients with severe disease[43-44] It has been suggested

that, IL-6 and IL-8 are more useful than CRP within first 24 hours in predicting the disease severity, because peak levels of both these interleukins are achieved before CRP[45].However, their validity and superiority over standard scores (APACHE II, Ranson's, and Glasgow) have not been fully investigated. Although there are methods that can measure IL-8 and IL-6 rapidly, these facilities are not available in all biochemical laboratories.

2.4.6 Tumour necrosis factor alpha

Tumour necrosis factor (TNF) α, a macrophage-derived cytokine, play a major pathophysiological role in inflammation and sepsis[46].It is a non-specific marker. In large comparative studies, the value of TNF-α in predicting severity has not been assessed. However, there are few studies which suggest a correlation between TNF-α levels and acute severe pancreatitis[47-48].

2.4.7 Trypsinogen activation peptide

In cases of acute pancreatitis, inappropriate trypsinogen activation in the pancreas results in the release of trypsinogen activation peptide (TAP) into the urine, plasma, and peritoneal cavity. Thus, it seems that plasma trypsinogen activation peptide concentration is one of the best and earliest markers of acute pancreatitis [49-50.]TAP has been shown to closely correlate with severity of disease[51] Since it is rapidly excreted in urine, it can be conveniently measured in a urine sample. Elevated urinary TAP (>10 ng/ml) correlates with the severity of disease.[50] This test can be applied to the patients presenting within 12 hours of pancreatitis. The negative predictive value approaches 100% and the positive predictive value of an elevated TAP is 80% [51].

Ransons's and APACHE II scores and CRP are the most effective methods in routine use between 2^{nd} and 3^{rd} day and have not yet been replaced by the newer laboratory markers. Ranson's signs more than or equal to 3 and APACHE-II points of more than 8 are considered as unfavourable early prognostic signs and as severe pancreatitis.

Ranson's Criteria -parameters used:
At admission:

A Age more than 55 years

B White blood cell count more than16 x 10^3 cells/mm^3

C Blood glucose more than 200 mg/dL

D Serum AST more than 250 IU/L

E Serum LDH more than 350 IU/L
Within 48 hours:

1. Serum calcium less than 8.0 mg/dL
2. Drop in Hematocrit of more than 10%
3. Hypoxemia PO2 less than 60 mmHg
4. BUN increased by 5 or more mg/dL after Intravenous fluid hydration
5. Base deficit more than 4 mEq/L
6. Sequestration of fluids more than 6 L

For gallstone pancreatitis, the parameters are:
At admission:

1. Age more than 70 years
2. White blood cell count more than 18 x 10^3 cells/mm^3

3. Blood glucose more than 220 mg/dL
4. Serum AST more than 250 IU/L
5. Serum LDH more than 400 IU/L

Within 48 hours:

1. Serum calcium less than 8.0 mg/dL
2. Drop in Hematocrit more than 10%
3. Hypoxemia PO2 less than60 mmHg)
4. BUN increased by 5 or more mg/dL after Intravenous fluid hydration
5. Base deficit more than 5 mEq/L
6. Sequestration of fluids more than 4 L

The APACHE-II system assigns points for 12 physiologic variables which are heart rate, temperature, respiratory rate, mean arterial blood pressure, serum potassium, sodium, creatinine, arterial pH, oxygenation, white blood cell count, hematocrit and Glasgow Coma Scale.

2.5 Hydrogen sulphide

For the past few years research has been carried out in an attempt to assess the role of hydrogen sulphide in physiology and diseases particularly inflammatory conditions like acute pancreatitis.

2.5.1 Introduction

Hydrogen sulphide has been known since early times. The chemistry of H_2S has been studied since the year1600.Hydrogen sulphide (H_2S) is a water soluble inflammable and colourless gas with the rotten eggs smell. Hydrogen sulphide often results from anaerobic digestion from

the bacterial breakdown of organic matter in sewer and swamps in the absence of oxygen. H_2S also occurs in natural gas, some well waters and volcanoes too. It is also produced in human body in small amount that acts as signalling molecule.

2.5.2 Synthesis, regulation and catabolism

In vivo, its formation is catalysed predominantly by the cystathionine-γ-lyase (CSE) and cystathionine b-synthase (CBS), which uses the amino acids such as L-cysteine, L-homocysteine and L-cystathionine[52]. Endogenous H_2S can be synthesized by the desulfuration of cystine/cysteine by three enzymes; Cystathionine beta synthase, Cystathionine gamma lyase and mercaptopyruvatesulfurtransferase.CBS and CSE are mainly produced in the cytosol[53-58].Both enzymes use cofactor as pyridoxal-5′-phosphate and substrate as L-cysteine[59]. H_2S exerts a negative feedback effect on the activity of these enzymes [60]. H_2S in vivo is metabolized rapidly by multiple enzymatic processes. Finally, H_2S is an endogenous reducing agent which can be easily consumed by a variety of circulating oxidant species in the vasculature such as superoxide or hydrogen peroxide, peroxynitrite and hypochlorite.[12-15]. Its excretion is mainly by the kidney as free or conjugated sulfate.

In mammalian serum, physiological concentration have been reported to be in the 30–100 μM range, whereas it is as high as 160 μM in brain. H_2S can exert toxic effects on some organs at concentration above 250 μM [61-63].Endogenous levels of H_2S have been measured in the circulatory system with rat serum being reported to contain ~46uM H_2S[64].

2.5.3 Role in physiological functions

Hydrogen sulphide has many important roles in human body. As a gaso-transmitter, H_2S passes through cell membranes without using specific transporters and exerts many biological effects such as cytotoxic effects or cytoprotective actions[65-71]CBS expression has been found in all parts of the brain, liver and pancreas. In mouse pancreas, CBS is ubiquitously distributed, but CSE was found mostly in the exocrine and in very small amounts in the freshly prepared islets. However, high glucose increased the CSE expression in the beta-cells [72].These enzymes are constitutively expressed in many tissues and their expression can be upregulated at the site of injury. In some tissues of the body, both CBS and CSE are required for H_2S biosynthesis, whereas in others, only one of them is sufficient.[73]

The potent vasodilatory effect of H_2S in large blood vessels and portal vein has been proven in studies on rats[74].Its relaxation effect on resistance arterioles has also been reported[75].H2S can relax vascular and non-vascular smooth muscle.

In hippocampus of brain, it might have a role in the induction of long-term potentiation[76] and the hypothalamic release of corticotrophin releasing hormone[77]. H_2S facilitates long term potentiation in active synapses suggesting a role in neuromodulation and active learning. H_2S can also have significant effects on mitochondrial respiration, and can even serve as a metabolic fuel for tissues like colon and liver [78].

2.5.4 Role in inflammation

H_2S is also is an endogenous anti-inflammatory agent. It is reflected through various studies.H_2S seems to play an important role as a modulator of leukocyte adherence to the vascular

endothelium. Administration of β-cyanoalanine, an inhibitor of CSE causes a rapid increase in the adherence of white blood cells (WBC) to the vascular endothelium in mesenteric venules of rats[79]. In studies, H_2S donors have been shown to suppress WBC adherence to the vascular endothelium induced by superfusion of veins with a pro-inflammatory peptide[79].These inhibitory effects were reversed by glibenclamide, suggesting the role of ATP-sensitive K^+ (K^+_{ATP}) channels. The up-regulation of adhesion molecule expression on leukocyte and endothelium that was observed following aspirin use was suppressed by pre-treatment with an H_2S donor[80.] H_2S donors also attenuated pain, which is also one of the hallmark feature of inflammation, and this effect was mediated through activation of K^+_{ATP} channels[81]. In rats, H_2S donors have been shown to decrease the visceral pain.[82-83].

On the other hand, H_2S may have a pro-inflammatory role. H_2S via its vasodilatory properties causes fall in blood pressure and cardiac output. The levels of H_2S have been found to have increased in endotoxic and septic shock[84].

H_2S has been shown to regulate number of physiological functions like inflammation, nociception, blood pressure, insulin secretion, learning and memory power in part via the modulation of KATP channel activity[85-88]. As such, modulation of endogenous H_2S through either H_2S-donor molecules or H_2S synthesis inhibitors represent potentially therapeutic targets for a variety of diseases where H_2S synthesis is involved, such as chronic inflammation, endotoxic or hemorrhagic shock, hypertension, diabetes, obesity and diseases such as arthritis and inflammatory bowel disease[89-94]

2.5.5 Role in gastrointestinal inflammation

One of the mechanism by which NSAIDs induce gastrointestinal toxicity is the modulation of endogenous gastric H_2Ssynthesis. Both H_2S synthesizing enzymes namely CBS and CSE are variably expressed in gastrointestinal tract. NSAIDs reduced H_2S production by reducing the expression of CSE in the gastric mucosa and aggravating acid-induced gastric ulcer formation[95]. Gastroprotective effects mediated by NaSH (an H_2S donor) act by reducing neutrophil infiltration in the gastric mucosa and by reducing adherence of WBC to the vascular endothelium. Reduced H_2S synthesis may also play a role in the development of the ulcerative colitis. In an experiment done by Wallace et al on a rat model, with the development of colitis, the colonic H_2S synthesis initially increased in response to trinitrobenzenesulfonic acid (TNBS), and then later decreased with colonic damage, whereas inhibition of CBS enzyme aggravated the colitis[96].

2.5.6 Role in inflammatory joint disease-

In patients of rheumatoid arthritis, synovial fluid contained up to four-fold concentrations of H_2S than in paired plasma samples and more than two-fold-higher H_2S levels than synovial fluid aspirates from patients with non-inflammatory arthritides [97].Synovial fluid H_2S concentrations was negatively correlated with Synovial total WBC Counts. It could represent a novel index of disease activity in the inflamed joint.

2.5.7 Role in renal ischemic-perfusion injury

In porcine model of non-heart beating donor kidney, H_2S has been shown to improve the outcome of ischemia-reperfusion injury. In a study by Hosgood SA et al, kidneys were subjected

to warm ischemia followed by cold storage and then H_2S donor sodium hydrogen sulphide was given before and after reperfusion with autologous blood, it was found that H_2Ssignificantly improved the markers of renal function. Renal blood flow improves because of vasodilatory effect of H_2S. There may be a possible role of H_2S in renal transplant recipients[98].

2.5.8 Role of Hydrogen sulphide in pancreas

There are many studies that have suggested that H_2S has both pro and anti-inflammatory properties in pancreatitis, depending largely on the experimental conditions. It has been proposed that low concentrations (NaHS- 5 and 10 µM) of H_2S, have a protective effect[99].Jenab N Sidhapuriwala et al also studied the pre-treatment with NaHS in mice with caerulein induced pancreatitis and found that it was associated with decreased inflammation in both pancreas and lung[100].On the other hand higher concentrations of NaHS (100 µM) induced inflammation in the acini[101-102]. In experimental pancreatitis, there was upregulation of CSE expression and H2S production whereas inhibition of endogenous H2S formation with DLpropargylglycine (PAG), a CSE inhibitor, reduces the severity of pancreatitis and affords protection against the lung injury in mice[103].

H_2S has been reported to induce apoptosis in both exocrine and endocrine cells of the pancreas[101]. The mechanism behind it is the activation of effector caspase -3 and initiator caspase- 8 and 9 which leads to induction of apoptotic cascade, upregulation of pro-apoptotic proteins and down regulation of anti-apoptotic proteins. H_2S has also been shown to have anti-apoptotic effects on pancreatic endocrine cells which was evident when high glucose induced mouse islet cells apoptosis, which was suppressed by 100 uM of NaHS and 3 mM L-Cysteine[104]

H_2S has been shown to modulate pancreatic nociception. Nishimura et al showed that pancreatic H_2S most probably targets T-type Ca(2+) channels, and that endogenous H_2S produced by CSE acting on these Ca(2+)channels are involved in pancreatitis-related pain[105].

2.5.9 Role in pancreatitis associated lung injury

There are studies which demonstrated the role of H_2S in pancreatitis associated lung injury. Madhav Bhatia et al demonstrated that administration of PAG protected animal against acute pancreatitis associated lung injury which was evidenced in their study by a significant reduction of lung myeloperoxidase activity and by histological evidence [106].The exact molecular mechanisms by which H_2S modulates inflammatory signalling in the lung are unknown. H_2S has potential role in acute lung injury and ARDS through different mechanism like regulation of leucocyte activity and neurogenic inflammation. It has both proinflammatory and anti-inflammatory actions which were demonstrated in various studies.PAG, a CSE inhibitor which inhibits H_2S synthesis suppresses the overproduction of pro-inflammatory cytokines and chemokines in sepsis, which is a key feature of systemic inflammation and also attenuates acute lung injury caused by sepsis or endotoxemia. Its anti-inflammatory action has been shown in murine model in which lung injury was induced by smoke fumes, intraperitoneal administration of NaHS an H_2S donor alleviates lung injury, improved survival and histological condition of lung[107].However in Oleic acid induced lung injury model, administration of H_2S donors in rats down-regulated the production of pro-inflammatory IL-6 and IL-8 in lung but increased the level of anti-inflammatory IL-10 in plasma and lung, thus alleviating lung edema, PMNs infiltration in

lung and severity of lung injury[108].The protective effect of H_2S in acute lung injury has been reported in some models of lung injury caused by smoke inhalation whereas the opposite findings are usually obtained in lung injury caused by systemic insults such as sepsis and pancreatitis. High dose of H_2S donor tends to aggravate inflammation in lungs and while the low dose (or slow release) of H_2Sdecreased inflammation. The discrepancy in the role of H2S in lung injury may be due to the different doses of H_2Sdonors used and different animal models.

2.5.10 Role in sepsis

Lipopolysaccharide (LPS), a bacterial toxin which has been shown to induce activation and upregulation of vascular K-ATP channels resulting in hypotension[109-110]. H_2S act as an endogenous ligand for KATP channels and induces vasorelaxation in vascular endothelium, reflecting its role in endotoxic shock[111]. In septic shock, plasma H_2S levels were also reported to be elevated. It was found that administration of PAG, a CSE inhibitor to LPS treated animals, decreases plasma H_2S levels and increased survival suggesting that increased plasma H_2S concentration in blood was playing a role in hemodynamic instability observed in septic shock.[112]

2.5.11 H_2S donors

Various H_2S salts are used for the study purpose in animal models. They are either fast or slow H2S donors. The fast H_2S donors, Na_2S and NaSH, result in an instantaneous release of H_2S when added to aqueous solutions[113]. It is very unlikely that under normal physiological condition, the tissues are ever exposed to such a rapid local concentration of H_2S, since

endogenously produced H_2S through action of CBS and CSE is relatively slow and sustained[114-115]. Injection of these salts (fast H_2S donors) into animal models results in a small increase in plasma H_2S concentrations that too over a very short period of time. To overcome the above problem, slow releasing H_2S donors came into existence. ADT-OH {5-(4-hydroxyphenyl)-3H-1, 2-dithiole-3-thione} a derivative of NSAID molecules is a slow releasing H2S donor[116]. The problem with this molecule is that biological effects associated may be due to the dithiolethione moiety itself, rather than released H_2S. Therefore, care should be taken to interpret the data. GYY4137 is another a very slow-releasing H_2S donor compound that releases two molecules of H_2S and has been shown to induce vasodilator activity *in vivo* via KATP channel-dependent mechanisms in hypertensive rats[117].Its decomposition product are inactive so allowing its use to study the physiological effects of slow releasing H_2S in inflammatory condition.

2.5.12 Is H_2S a pro-inflammatory or an anti-inflammatory mediator?

Its role in inflammation is still a debate. Now, it is becoming apparent that H_2Shas a dual role in inflammation and current studies vary in opinion, largely based on rate of release, dose and type of H_2S donor used The pro-inflammatory role of $H_2S$was shown in animal models such as LPS induced endotoxemia, pancreatitis, hemorrhagic shock[118] and burns injury[119]. In all of these studies pretreatment with PAG, an inhibitor of H_2S synthesis, protected animal against injury and administration of fast H_2Sdonors like NaHS aggravated the injury. On the other hand, its anti-inflammatory role was shown in animals gastric and colonic epithelium where Lawesson reagent(a sulfide donor) or NaHS at low dose have been studied[120]. The slow release H_2Sdonor GYY4137 has also been shown to protect mice against LPS induced endotoxemia. There are also

conflicting reports on the role of H_2S on leukocyte rolling and adhesions. Previous studies have shown that H_2S both positively and negatively regulate leukocyte activation and migration[121].

2.5.13 Human Studies

Most of the studies on the role of H_2S in pancreatitis were done on rats. The role of H_2S in humans is not well studied. To the best of our knowledge, the first human study on the role of H_2S in acute pancreatitis was done by Eric WL Wee et al in which they have shown that H2S and substance P were involved in inflammatory response in acute pancreatitis and their levels correlated with the severity in early hours of pancreatitis[122].

2.5.14 Future trends

Pharmacological research related to H_2S is an emerging field, which is likely to produce a number of therapeutic possibilities. There are drug candidates which are in early stages of development. H_2S-related future therapeutic avenues might also include genetic approaches and drugs using overexpression of H_2S-producing enzymes as a therapeutic modality might be explored.

The present study is first of its kind to study the role of H_2S in patients with acute pancreatitis in routine clinical practice who seek medical care several days after onset of pain. Our hypothesis is that H_2Sbiosynthesis is increased in pancreatic inflammation and that elevation of H_2S levels in blood would correlate with the severity of pancreatitis, providing us with a tool that will help clinicians by improving prognostic accuracy and by early identification of patients who will need more intensive monitoring.

AIMS AND OBJECTIVES

3. AIM

To establish the value of hydrogen sulphide as new marker for severity and prognosis in acute pancreatitis.

4. OBJECTIVES

1. To prospectively enrol adult patients admitted with acute pancreatitis and assess severity of illness to classify them as mild or severe pancreatitis
2. To investigate the correlation between hydrogen sulphide levels and severity of illness. (Primary objective)
3. To investigate the changes in hydrogen sulphide levels during the course of the illness and correlate with the course of the disease, by estimating it at admission, after initial resuscitation (48 hrs later) and at discharge.
4. To investigated whether H2S levels correlate with specific organ dysfunction namely, acute lung injury, haemodynamic compromise, renal dysfunction or infections

MATERIALS AND METHODS

5.MATERIALS AND METHODS

5.1 Recruitment

5.1.1 Site and duration

The study was a prospective single centre study, conducted at the Christian Medical College Hospital, Vellore, Tamil Nadu, India, from July 2012 to September 2013 in the Department of GI sciences. The study protocol was approved by Institutional Review Board of the institution and the registration number was 7914 dated 4-7-12.

5.1.2Participants

All patients who were admitted with the diagnosis of acute pancreatitis were enrolled. The diagnosis was made if the patient fulfilled two out of the following three criteria.

1. Pancreatic type abdominal pain
2. Serum lipase or amylase more than 3 times normal
3. Imaging study (USG or CT) suggestive of acute pancreatitis

Patients of either sex between the ages of 12 and 80 were included. Written and informed consent was taken from either patients or their relatives prior to enrolling in the study.

5.1.3 Exclusions

Pregnant females were excluded. Patients with a diagnosis of chronic pancreatitis, pseudocyst or pancreatic tumours were also excluded.

5.2 Management

All patients diagnosed to have acute pancreatitis were admitted without delay. Every attempt was made at admission to define the etiology of pancreatitis. The patients were asked about history of alcohol use, drugs or trauma.

The patient were resuscitated with intravenous fluids and provided adequate analgesia. Vital parameters were monitored closely and patients were triaged to a high dependency unit if there were features of local complications or multi organ dysfunction, either at admission or at any time during the course of the hospital stay. Intensity of patient monitoring varied from twice daily if the patient was stable, to continuous monitoring in the ICU if critically ill. Naso-jejunal feeding was initiated if the patient was unable to tolerate oral feeding 4 days after admission. Antibiotics were initiated if there was clinical suspicion of sepsis but continued only if proven by cultures.

5.3 Laboratory tests

Liver function tests, calcium, lipid profile and ultrasound of the abdomen were done to determine the etiology .Other standard tests at admission included complete blood count, renal function, electrolytes, glucose, C-reactive protein, lactate dehydrogenase, chest x-ray and arterial blood gas. A CT scan was done selectively after 48 hours of onset of symptoms if it was deemed to contribute to treatment decisions.

5.4 H_2S estimation

5.4.1 H_2S estimation time points

Within 24 hrs after admission, 2-3 ml of blood was collected in EDTA tubes and sent to the laboratory for estimation of H_2S levels. After 48hours a second blood sample was collected for analysis. A third blood sample was collected at the time of discharge.

5.4.2 Procedure for H_2S estimation

Due to its volatile nature, H2S estimation was carried out immediately after sample collection. Hydrogen sulphide level in plasma was measured spectrophotometrically. The blood sample was aliquoted and 100µl mixed with 50µl of distilled water in micro-centrifuge tubes which also contained 300µl of zinc acetate (1% w/v) to trap H2S. The reaction was stopped after 5 min by addition of 200µl of N, N-2dimethyl-p-phenylenediamine sulphate (20mM in 7.2 M HCl), followed immediately by addition of 200µl of FeCl3 (30mM in 1.2 M HCl). For about 20 minutes, mixture was kept in dark. In order to precipitate protein from the samples, 150µl of trichloroacetic acid (10% w/v) was added. It was then centrifuged for 10 minutes at 10,000 rpm. The absorbance of the resulting supernatant was measured at 670 nm using a 96-well plate reader .All samples were assayed in duplicates. Mean of two values will be taken for final calculation. ±0.005 – 0.01 O.D (optical density) variations is considered normal. If it exceeds this variation, experiment will be repeated. Since, the assay was standardized beforehand regarding amount of starting material we did not have to repeat any assays.H2S concentration in the plasma was calculated against the calibration curve of standard H2S solutions (NaHS: 3.125-100 µM)[123].

5.5 Definitions

5.5.1 Severity of pancreatitis

In 1992, the Atlanta symposium classified acute pancreatitis as ‘mild’ and ‘severe’ on the basis of certain criteria, which have come to be known as the Atlanta criteria (Table-1). This classification (and not the revision of 2012) was used in this study.

I Organ Failure
1. Systolic blood pressure less than 90 mm Hg
2. Serum creatinine more than 2 mg/dl
3. Hypoxemia i.e PaO_2 equal or less than 60 mm Hg
4. Gastrointestinal bleeding more than 500 mL/24 hr

II Local Complications either in the form of necrosis, abscess or pseudocyst

III Unfavourable early prognostic signs
A. APACHE II points more than 8
B. Ranson’s signs equal to or more than 8

Table-1 Atlanta criteria for severe acute pancreatitis.

5.5.2 Alcohol related acute pancreatitis

Alcohol was accepted as the etiological factor when gall stones was ruled out by ultrasound abdomen and there was a history of heavy alcohol intake within three to four days of admission or a history of prolonged alcohol abuse, greater than 80 g/ day, with further intake in the four days prior to admission.

5.5.3 Hypercalcemia induced pancreatitis

Acute pancreatitis was attributed to hypercalcemia if the serum calcium was more than 12 mg/dl (normal range 9-10.5 mg/dl) in the absence of gallstones or alcohol abuse.

5.5.4 Gallstone-induced pancreatitis

Gallstones were accepted as etiological factor if imaging, either USG abdomen or CECT abdomen, was suggestive of gall bladder stones or common bile duct stone and /or blood tests showed deranged liver function test (high bilirubin > 2 mg/dl or transaminitis i.e ALT > 2 times normal)in a patient with acute pancreatitis.

5.5.5 Acute fluid collection

Acute fluid collection was defined as fluid located near or in the pancreas, and lacking a definite wall, typically occurring early in course of acute pancreatitis.

5.5.6 Sepsis syndrome

Sepsis-It is the systemic response to infection and is defined as the presence of SIRS in addition to a presumed or documented infection

Systemic inflammatory response syndrome (SIRS) is defined by presence of two or more of the following:

1. Fever of more than 100.4°F or less than 96.8°F.
2. Heart rate more than 90 per minute
3. Respiratory rate of more than 20 per minute or arterial carbon dioxide tension ($PaCO_2$) of <32mm Hg

4. Total leukocyte count i.e.>12 x $10^3/\mu L$ or < 4 x $10^3/\mu L$ or >10% band forms)

5.5.7 Mechanical ventilation

Patients requiring endotracheal intubation and invasive ventilatory support.

5.5.8 Acute lung injury (ALI)

Acute lung injury was defined as hypoxemia (PaO_2<60 mmHg or PaO_2/FiO_2<300 mm Hg) with bilateral pulmonary infiltrates on chest X-ray.

5.5.9 Acute kidney injury (AKI)

Acute kidney injury was defined as rapid deterioration in kidney function within 48 hours of admission, with an absolute increase in serum creatinine > 0.3 mg/dl or percentage increase in serum creatinine more than 50 %, and reduction in urine output to < 0.5 ml/kg/hr for more than 6 hours.

5.6 Statistical methods

5.6.1 Sample size

Sample size was calculated on the basis of a previous human study. In the study by Eric WL Wee et al, 35 patients were studied.Only patients who presented within 72 hours of onset of symptoms were included. Acute pancreatitis was classified as severe in 19 and mild in 16 by the Atlanta criteria. The H_2S level measured at 0-24 hours after admission, was significantly different between patients who had mild and patients who had severe pancreatitis; mild34.3 (±21.1) vs. severe 189.4(±158.9), (p=0.004). To replicate such a large difference, a sample size

of 1 in each group is sufficient for a study with a power of 0.80 and a confidence level of 0.95. Since the majority patients presenting to our hospital have variable duration of symptoms, often more than 3 days prior to admission, we decided to recruit 20 patients in each arm. Based on the previous year's admission data, 40% of acute pancreatitis was predicted to be of severe type. Thus, a sample size of 50 was calculated including both mild and severe cases. We also expected instances in which H_2S estimation would be not possible due to poor sample quality and delays in sample transport and analysis. It was decided to continue recruitment till a minimum of 50 satisfactory samples was collected for 1^{st} time point of H_2S estimation (i.e. within 24 hours of admission).

5.6.2 Planned data analysis

Results will be expressed as mean and median with appropriate measures of dispersion.If the data does not satisfy the assumption of normality a non-parametric approach will be used. To calculate paired differences Wilcoxon signed rank test will be used. Mann-Whitney u-test will be used to compare groups.

RESULTS

6. RESULTS

6.1 Patient recruitment

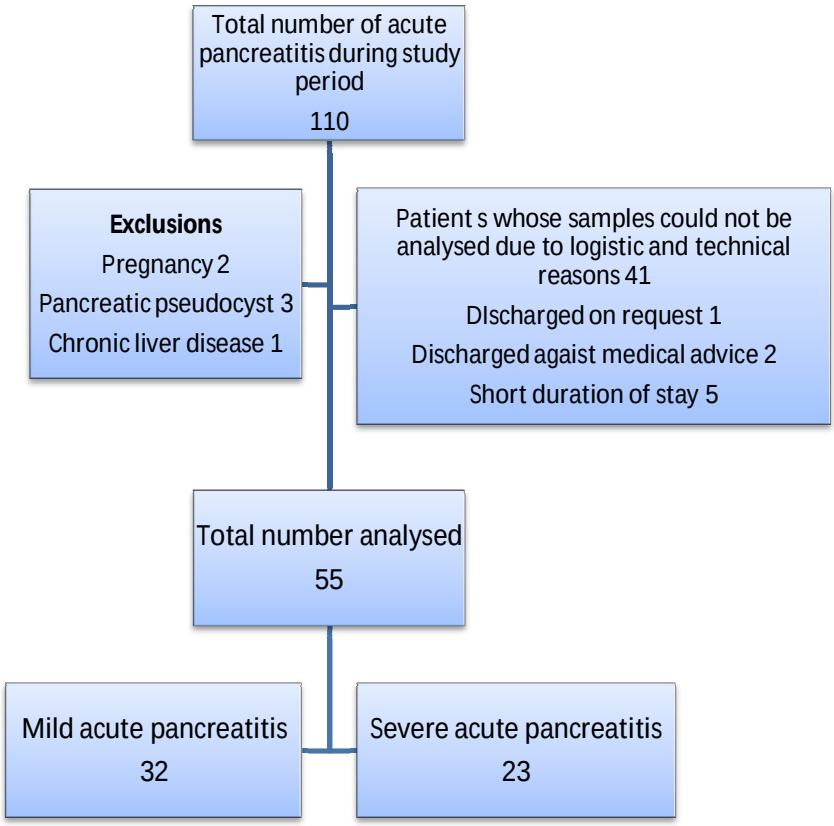

Figure:1 Flow chart showing recruitment of cases of acute pancreatitis.

A total of 110 patients were admitted with a diagnosis of acute pancreatitis during the study period. Recruitment of cases is shown in figure1. In 37% of the patients the H_2S estimation could not be carried out due to various logistic and technical reasons. Round the clock laboratory staffing was not available to conduct the H_2S estimation procedure. Since the estimation could not be done on stored samples, the test was not carried out on patients who were admitted during weekends or on national holidays. H_2S estimation was not carried out in samples which either did not reach the laboratory within 1 hour of collection or had evidence of hemolysis.

Other reasons for non-inclusion included patients with mild pancreatitis who were well and were discharged within 24 hours, and patients who were discharged at the request of the family even though they were advised to have in-hospital care. Two pregnant women,3 patients with pseudocysts and 1 patient with acute on chronic liver failure were also excluded. Of the 55 patients who were included, three H_2S estimates (i.e. at admission,48hrs and at discharge) were available for 34 patients and two (i.e. at admission and 48 hrs) for 50 patients.

6.2 Patient characteristics

DEMOGRAPHICS	PATIENTS (N=55)
Age (years)	41.6(Mean)(Range-18-80 yrs)
Sex (male/female)	43/12
BMI(Kg/m^2)	24.63 (Mean)(Range-18.2-36.8)

Table-2 Patient characteristics (age, sex and body mass index)

DAY OF PRESENTATION AFTER THE ONSET OF PANCREATITIS	CASES (N=55)
Day 1	2
Day 2	14
Day 3	20
Day 4	6
Day 5	8
Day 6	5

Table: 3 Day of onset of pancreatitis and presentation in the study patients.

A total of 55 patients of acute pancreatitis were enrolled in the study, of which forty three were male and 12 were female. Age of patients included ranged between 18-80 yrs,the mean age was 41.6 yrs. Mean body mass index(BMI) was 24.63 kg/m^2 (range=18.2-36.8 kg/m^2).In mild pancreatitis cases,20 patients(62.5%) had normal BMI (18.5-24.9 kg/m^2) and 12 patients(37.5%) were overweight (BMI > 25 kg/m^2).Among severe cases,11 patients(47.8%) had normal BMI and 12 patients (52.17%) were overweight. Figure 2 & 3.Table 2 .

In our study, most of the patients i.e 36.3 % (n=20) presented to us on day 3rd of pancreatitis.14 patients presented on day 2nd of pancreatitis. Only 2 patients were presented to us on day one of pancreatitis. Out of all 55 patients, 42 patients presented on or before 4^{rth} day of pancreatitis. Rest 13 patients presented after day 4.None of the patient included in the study presented after day 6. Table 3

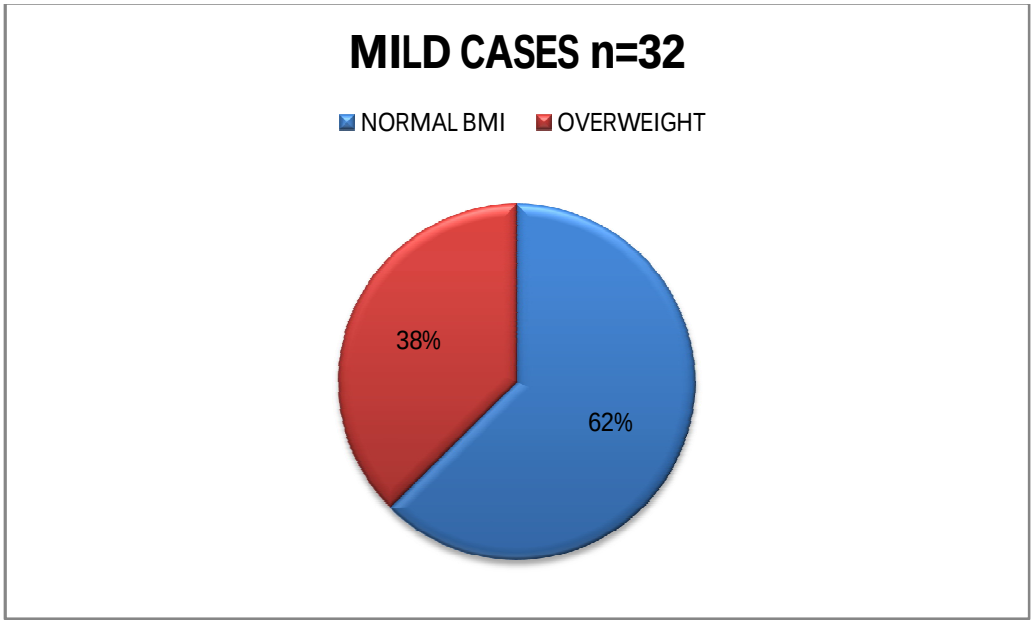

Figure :2Mild acute pancreatitis. Normal BMI n=20. Overweight n=12

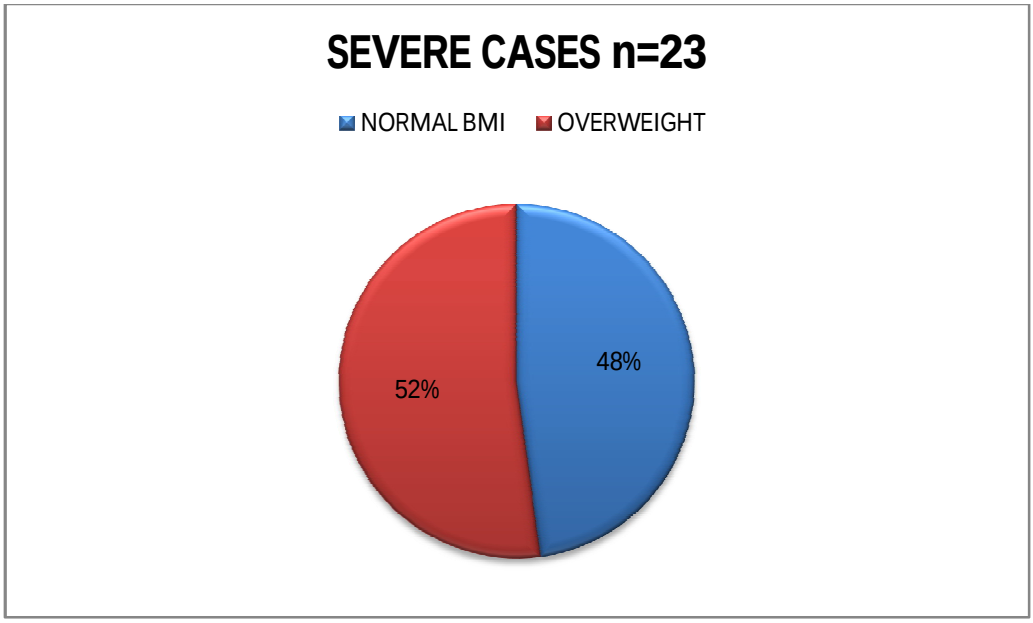

Figure:3 Severe acute pancreatitis. Normal BMI n=11. Overweight n=12.

6.3 Etiology of pancreatitis

ETIOLOGY	**NUMBER OF CASES(%)**
Alcohol	25(45.5%)
Gallstones	15(27.3%)
Post ERCP	1(1.8%)
Hypercalcemia	1(1.8%)
Idiopathic/unknown	13(23.6%)

Table-4 Etiology of acute pancreatitis

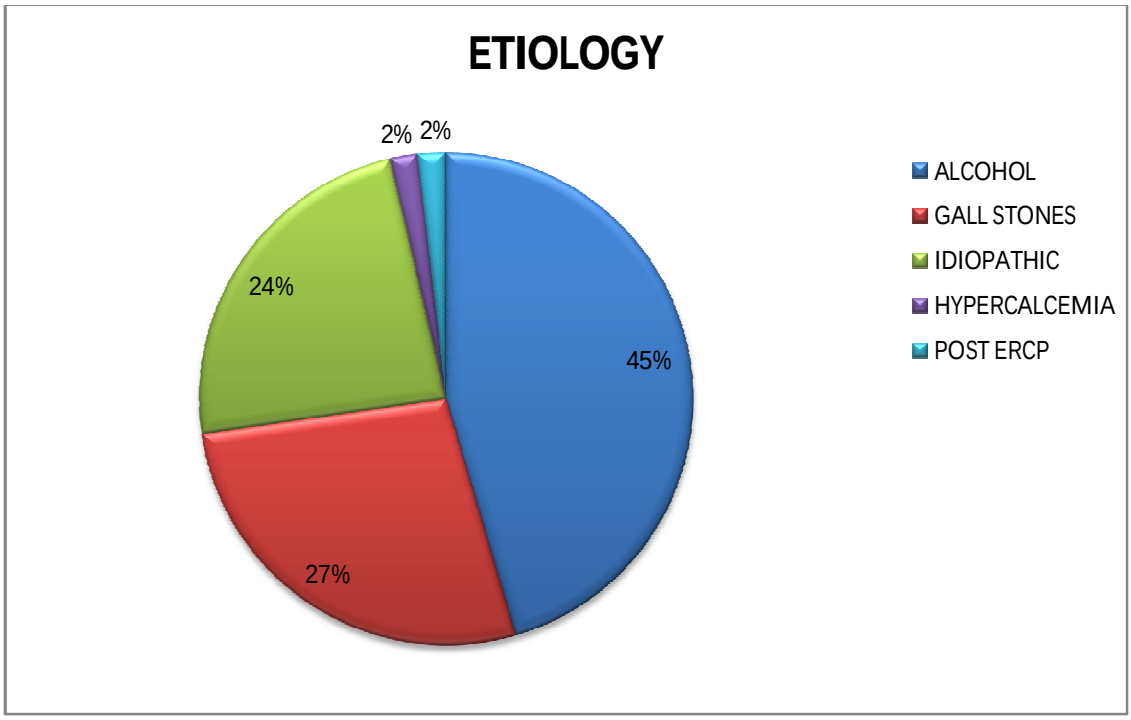

Figure 4 :Pie chart showing various etiologies of acute pancreatitis.

The most common aetiologywas alcohol in 25 patients (45.5%) and gall stones in 15 (27.3%).The cause could not be ascertained in 13 patients, one patient had post-ERCP pancreatitis and one had hypercalcemia related to a parathyroid adenoma.(Table 4 and figure 4)

6.4 Severity of pancreatitis

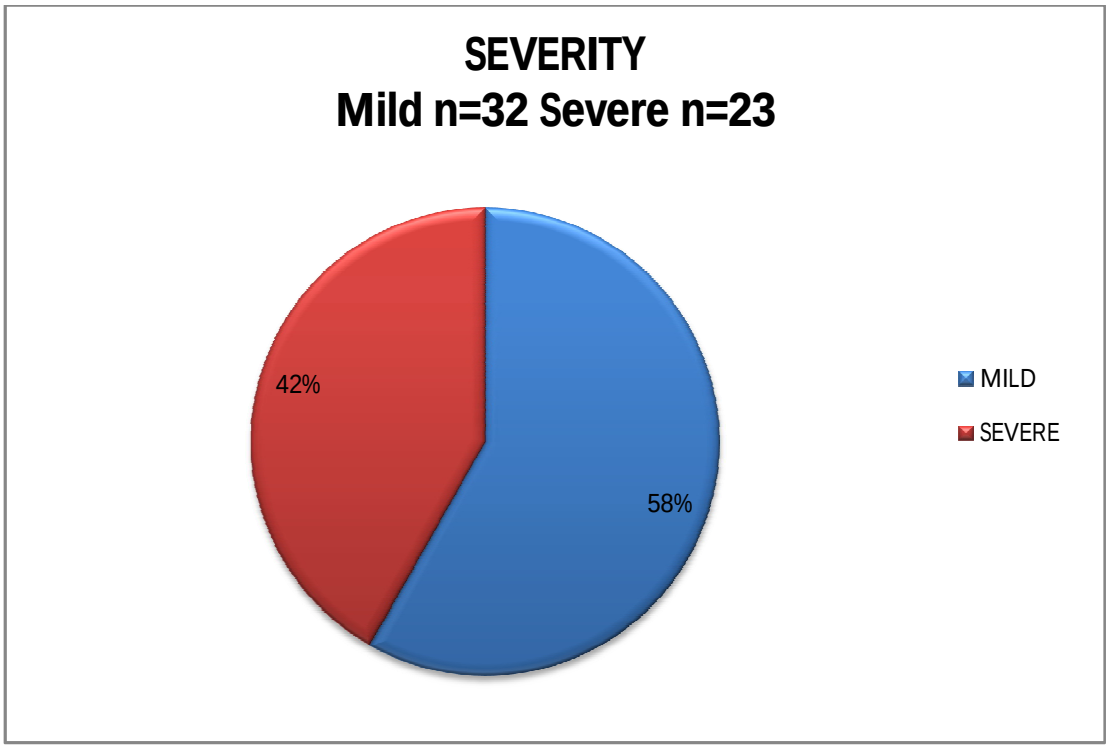

Figure 5 :Pie chart showing percentages of mild and severe cases of acute pancreatitis.

Among the 55 patients, 32 (58.2%) had mild pancreatitis and 23 (41.8%) had severe pancreatitis, as defined by the 1992 Atlanta classification. (figure 5). In our study, the proportion of severe cases was more than that described in literature. This was probably due to a referral bias. The hospital is a tertiary care centre and severe cases are usually referred to us for management.

6.5 Course and complications

ORGAN FAILURE-	CASES (%)
Acute lung injury	19(34.5%)
Acute kidney injury	5 (9.1%)
LOCAL COMPLICATION-	
Fluid collection	13(23.6%)
Vascular complication	2(3.6%)
SYSTEMIC COMPLICATION	
Sepsis	22(40.7%)
MORBIDITY	
Duration of hospital stay	6(Mean)(Range 1-22)
Mechanical ventilation requirement	3 (5.5%)
MORTALITY	
Death	2 (3.6%)

Table 5–Complications of pancreatitis

6.5.1 Local complications

Fifteen patients developed local complications. Acute fluid collection, the one most commonly noted, was seen in 13. Two patients developed splanchnic venous thrombosis.(Table 5)

6.5.2 Organ failure

Nineteen patients(34.5%) had acute lung injury and five (9.1%)had acute kidney injury. Three patients (5.5%) required mechanical ventilation. None of the patients underwent dialysis. However, two of the study patients who died, hemodialysis was indicated, but was not done. In one patient, the reason was shorter duration of stay. He died before dialysis could be initiated and for the other patients,the relatives had not given consent for dialysis.

6.5.3 Morbidity and mortality

Twenty-two patients (40.7%) had sepsis and SIRS, either at presentation or at some time during course of hospital stay. Mean duration of hospital stay was 6 days (range-1-22).In 14 patients, infectious organism was identified and rest 8 patients, infection was presumed on clinical basis (i.e cholangitis, pneumonia, fever pattern and requirement of antibiotics).Most common site of infection was urinary tract (n=8) (Enterococcus was isolated in 3 patients, E.coli in 2, yeast in 2 and morganella in 1 patient). Blood culture had grown organism in five patients (E coli in 1,staphylococcus sp in 2,enterococcus in 1 and proteus in 1 patient). Bile culture was positive in three patients (E coli in 2 and NFGNB in 1 patient).Sputum culture had grown NFGNB in one patient. Three different organisms from three different sites (proteus in blood, enterococcus in urine and NFGNB in sputum) were identified in one patient who died and one other patients had two sites of infection including blood and urine. Twenty patients required ICU admission, with duration of ICU stays ranging from 1-22 days. Two patients died due to multi organ dysfunction.

6.6 H_2S estimation – distribution of values

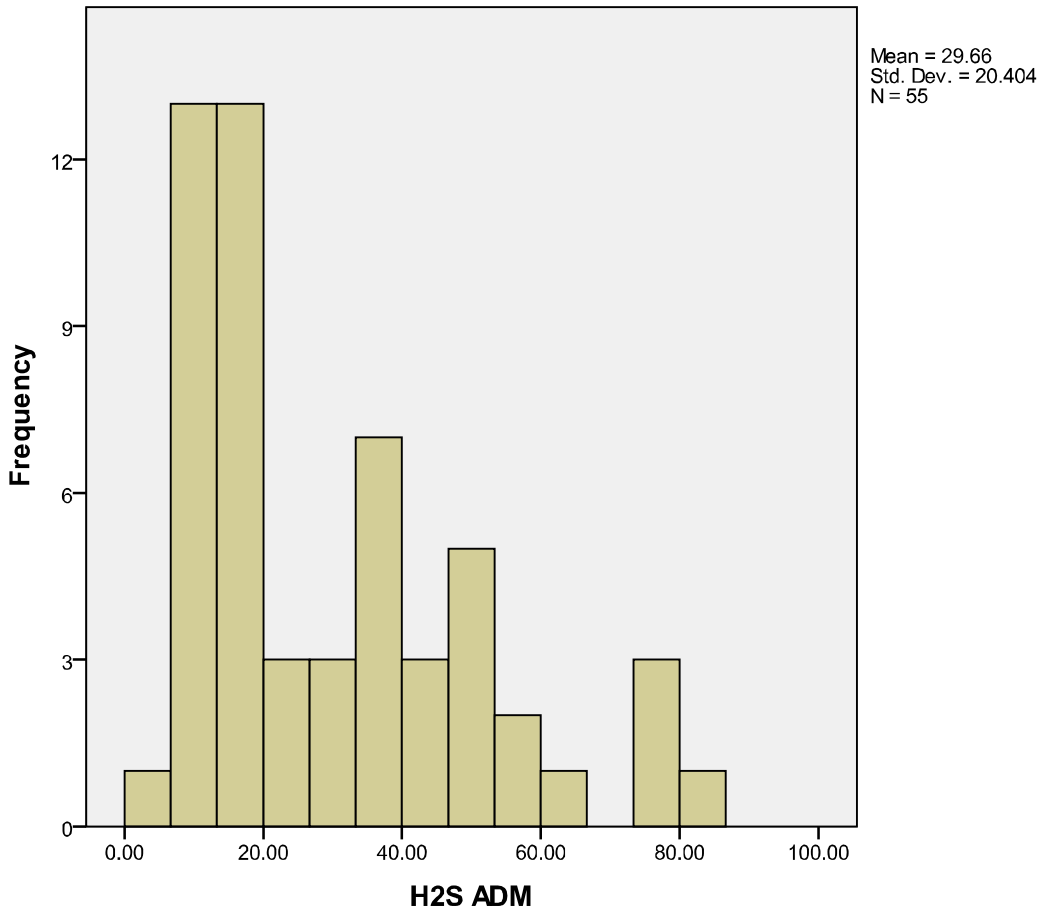

Figure:6 Histogram showing admission level of H2S(μmol/L), all cases

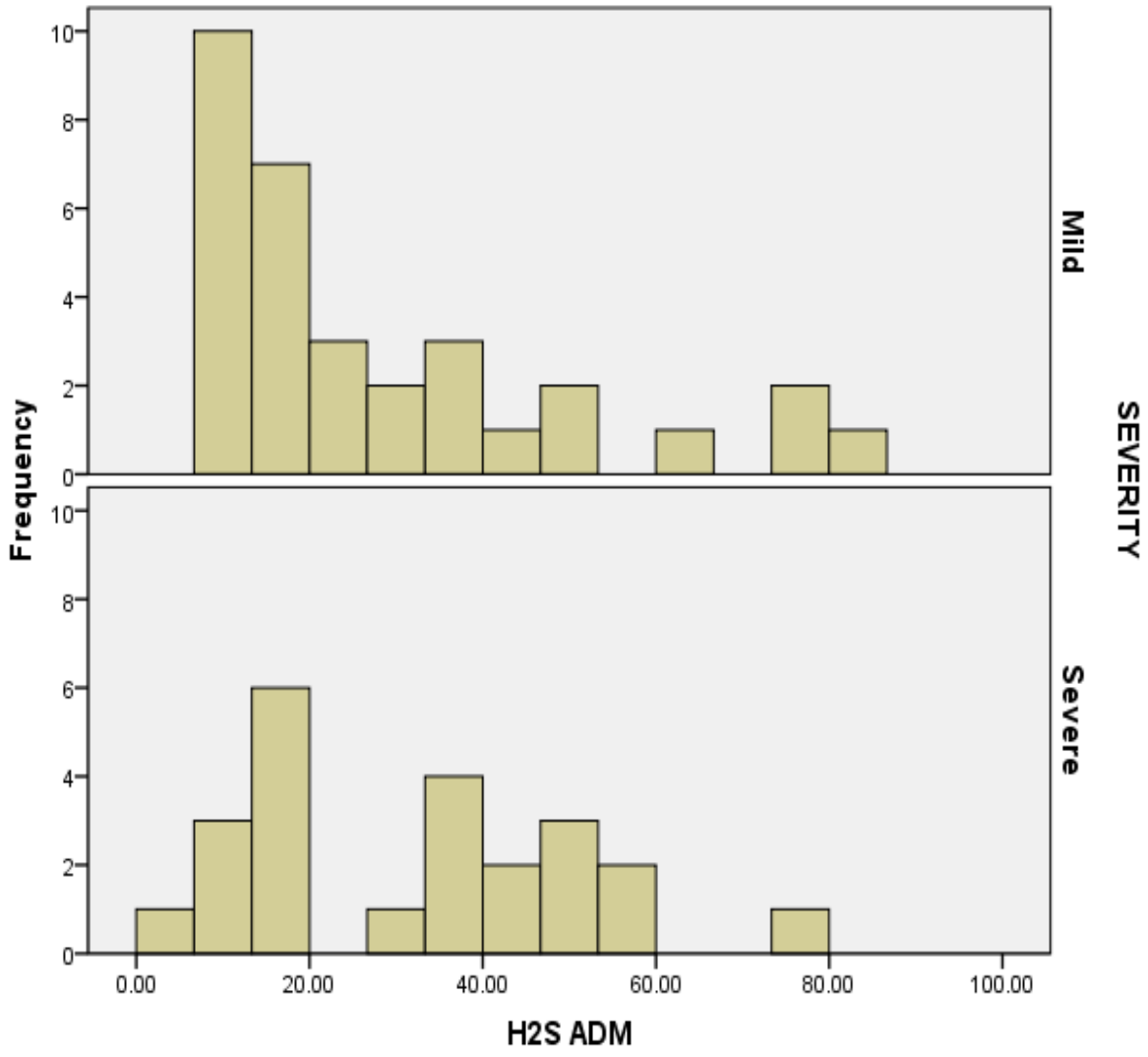

Figure:7 Histogram showing levels of H2S(µmol/L) at admission, of mild and severe cases separately.

The values of H2S at admission did not follow a normal distribution, either for the whole group or within mild and severe subgroups. Overall H2S values ranged from 6.07 to 81.43 (µmol/L)(Figure 6).For severe cases range of H2S values was 6.07 to 74.43(µmol/L). For mild cases the range of H2S was 7.14- 81.43(µmol/L). Figure7. As data did not follow a normal distribution, non-parametric methods were used in statistical analysis.

6.7 Hydrogen sulphide levels at admission

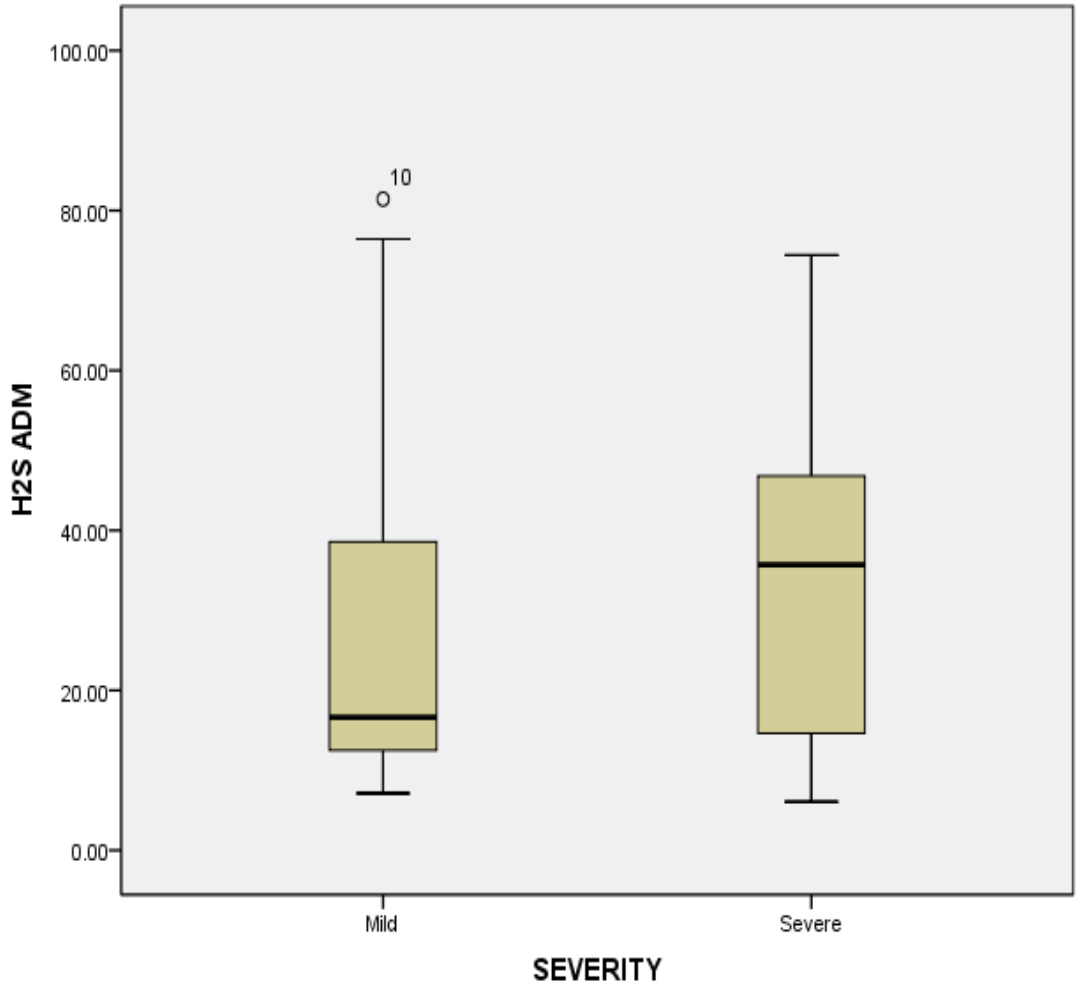

Figure 8: H2S levels (µmol/L) at admission in mild (n=32) and severe cases (n=23)of acute pancreatitis.

The value of H_2S estimated within 24 hours of admission was compared between the groups of patients with mild and severe pancreatitis. H_2S levels at admission were lower in patients with mild pancreatitis (median=16.64µmol/L, range= 7.14-81.43µmol/L)compared to patients with severe pancreatitis (median=35.7 µmol/L, range=6.07-74.43µmol/L),but the difference was not statistically significant(p value=0.339).Figure 8

6.8 Hydrogen sulphide levels at 48 hours

6.8.1 Comparison of values at 48 hours

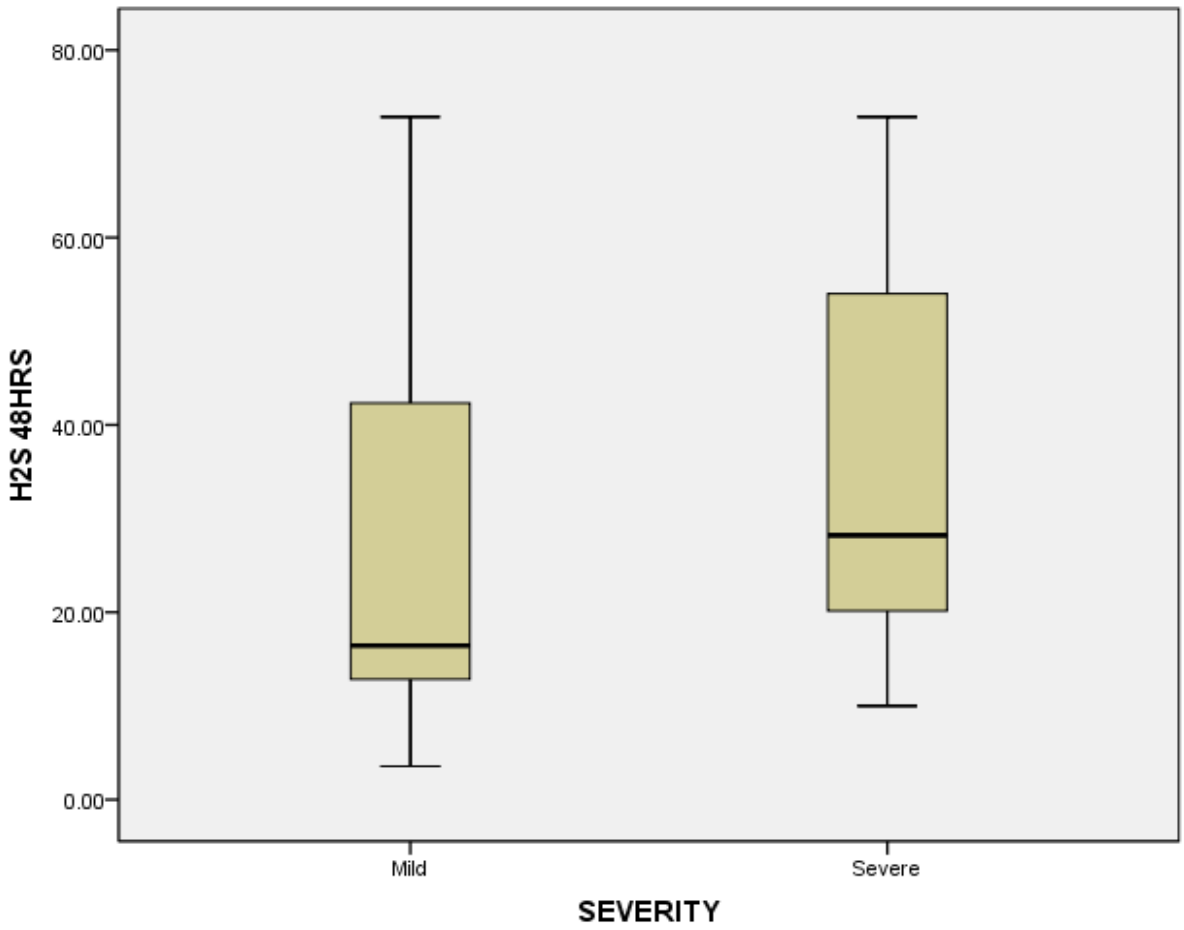

Figure 9:H2S levels(µmol/L) at 48 hrs in mild (n=32) and severe (n=23) cases of acute pancreatitis

We also evaluated the level of hydrogen sulphide 48 hours after admission. This was based on the fact that in many cases the patient's condition is better assessed after providing adequate analgesia and fluid resuscitation. Plasma H2S levels at 48 hours after admission were higher in the severe group (median=28.21µmol/L, range=10-72.86 µmol/L) than the mild pancreatitis group

(median=16.34 μmol/L, range=3.57-72.86μmol/L). It was not statistically significant (p value=0.127). Figure9.

6.8.2 Temporal trends in H_2S

We also analysed the change in H_2S after fluid resuscitation by comparing the H_2S level at 48 hrs to that at admission. Our premisewas that the direction and degree of change in H_2S would correlate with improvement or deterioration, and provide a clue to the hospital course of the patient. Twenty five patients (16 mild cases and 9 severe cases) had decrease in levels at 48hrs, while 23 patients (12 mild cases and 11 severe cases) had an increment in levels. The changes were found to be bidirectional and did not accurately reflect the patient's prognosis.

6.9 Correlation of H_2S and duration of symptoms

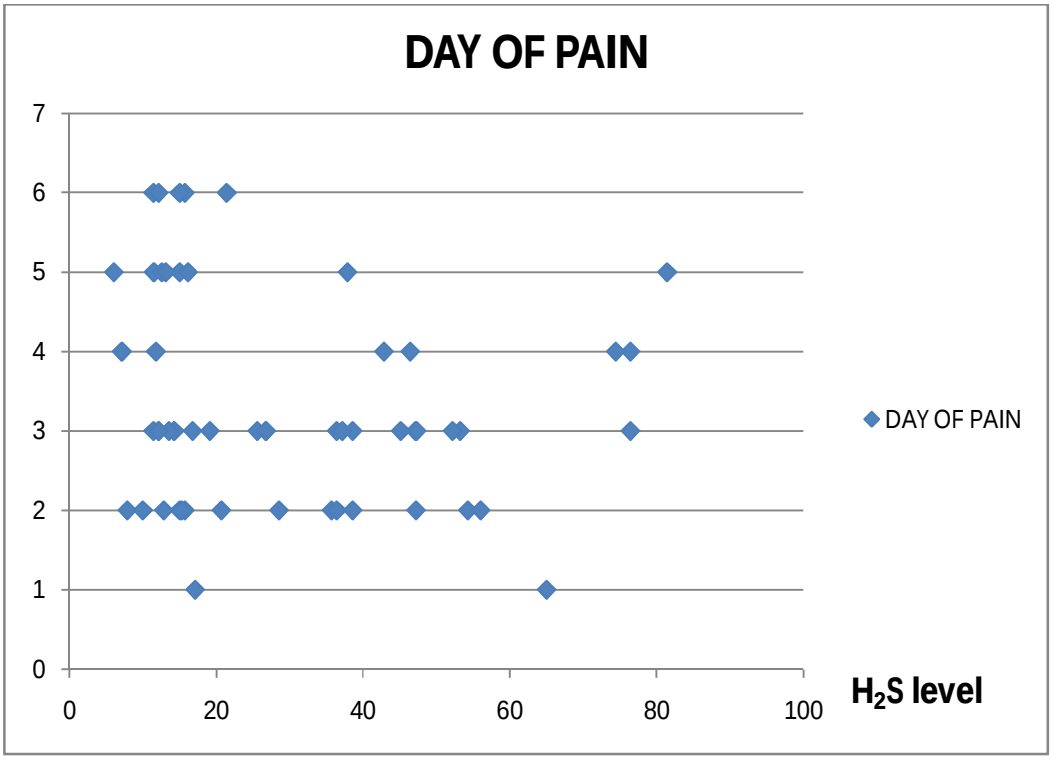

Figure: 10. Scatter plot of admission H_2S values according to duration of pain

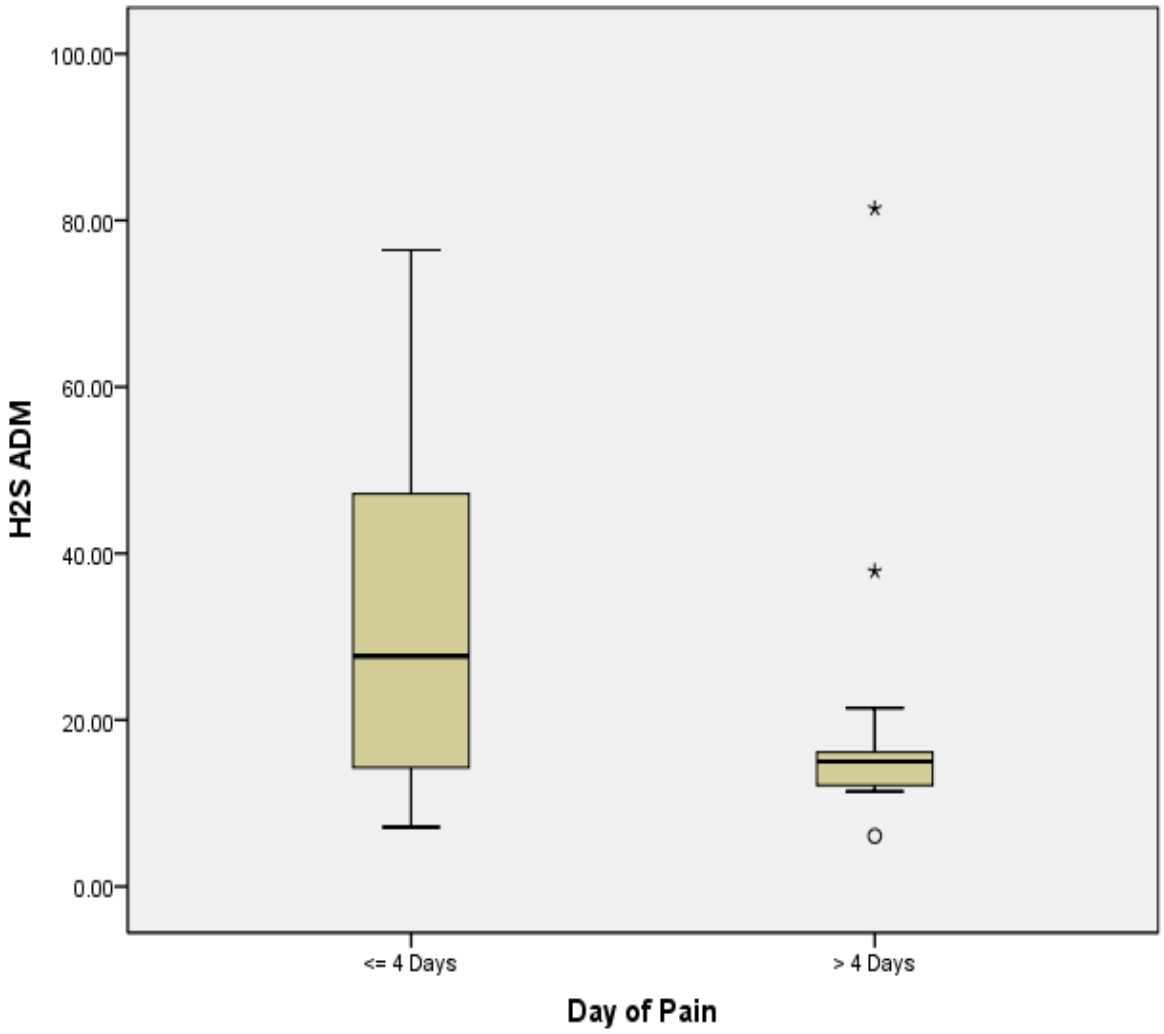

Figure 11: Admission H2S levels (µmol/L) in cases presenting within 4 days of onset of pain (n=42)and after 4 days of onset of pain (n=13)

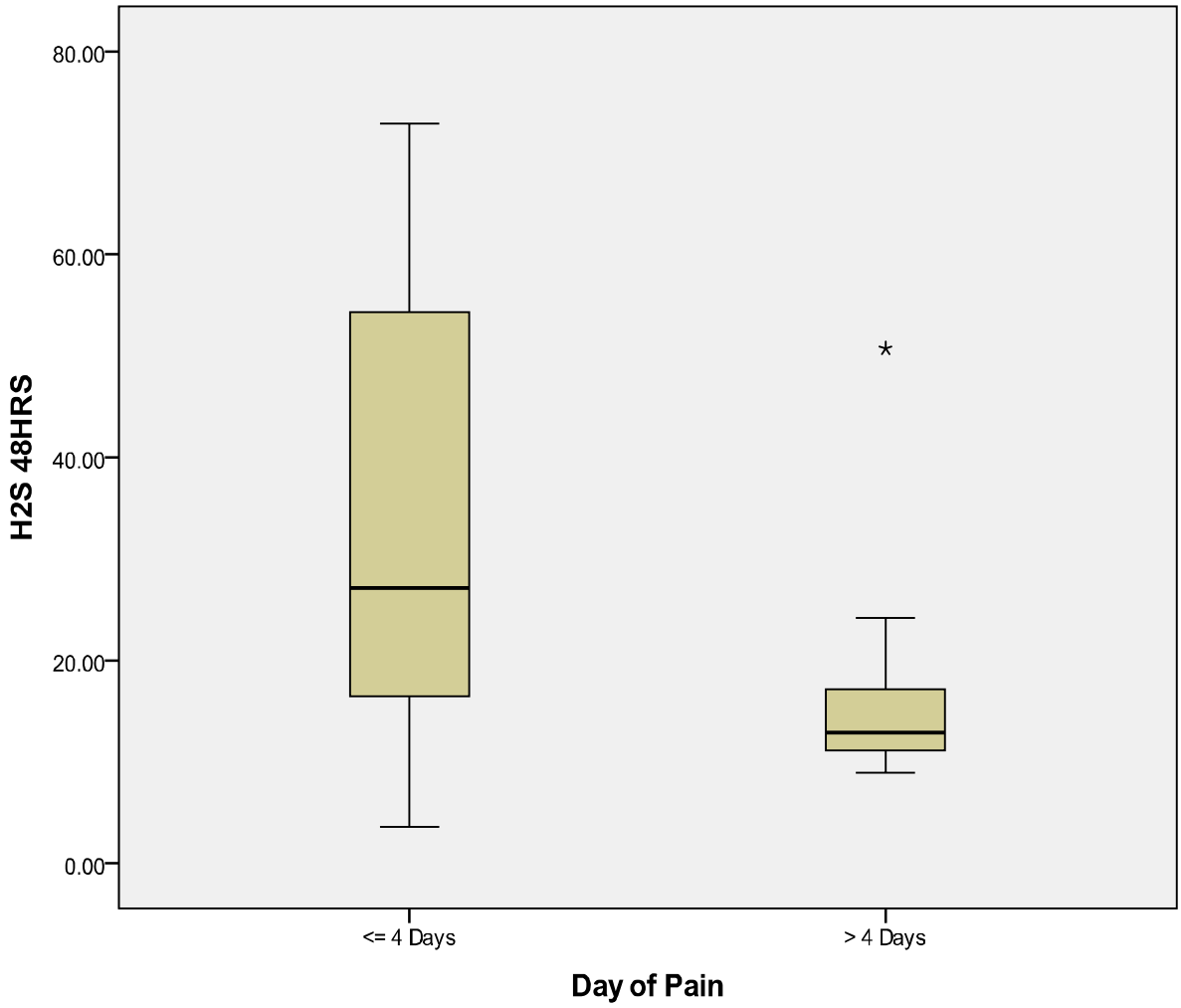

Figure 12 : H2S levels (µmol/L) at 48 hrs in cases presenting within 4 days of onset of pain (n=37) and after 4 days (n=11) of onset of pain.

In the present study, we found that hydrogen sulphide levels were higher in those who presented earlier after the onset of pancreatitis.(n=42). Patients who presented earlier i.e. on or before 4days of onset of pain had higher values of H2S at admission (n=42, median=27.67 Vs n=13, 15 µmol/l, p-value=0.032) and at 48 hrs(n=37,median =27.14 µmol/L Vs n=11, median=12.86 µmol/l, p-

value=0.004) compared to those who presented later i.e. after 4 days of onset of pain. Figure11 & 12.

6.10 Value of H_2S estimation in early presenters

In the study by Eric WL Wee, an extremely clear difference in H_2S values was noted between mild and severe groups. The patients recruited for that study were all symptomatic for less than 72 hours. We investigated whether H_2S would be a useful discriminator, if we limited its use to those presenting early in the course of illness.

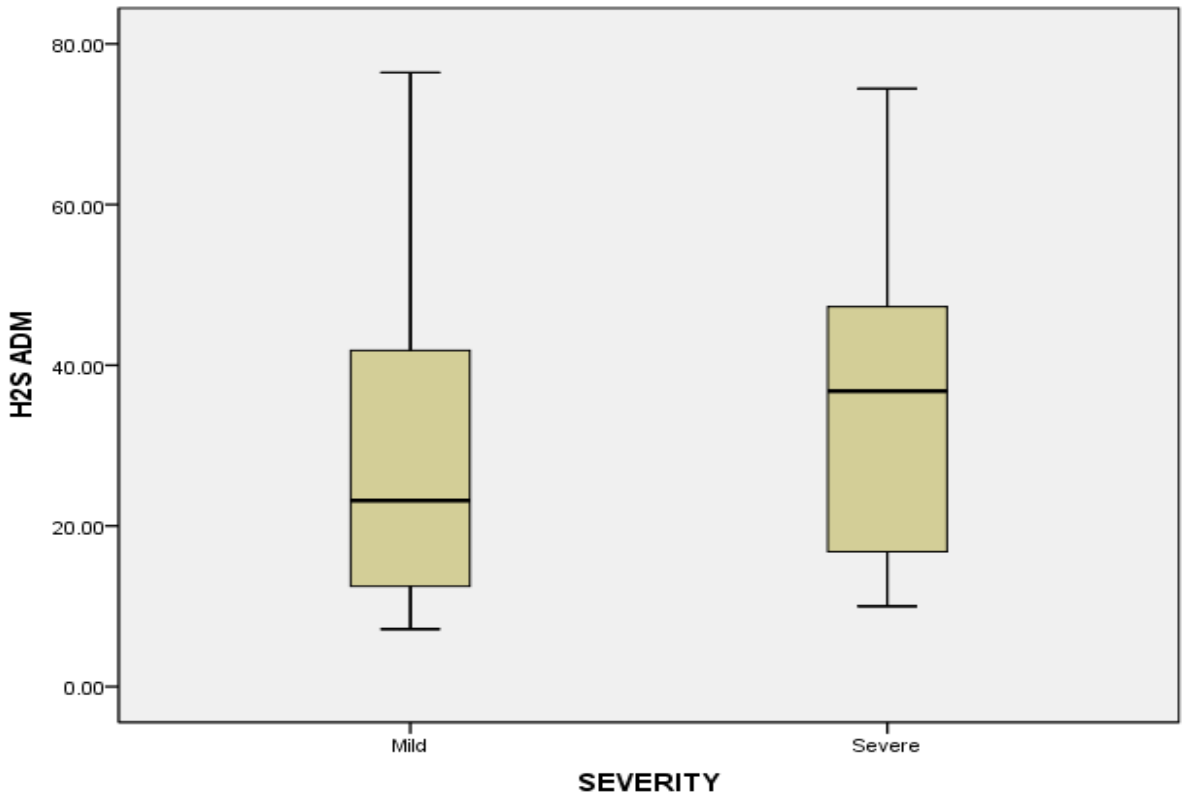

Figure: 13 Comparison of H2S levels (µmol/L) at admission in mild(n=24) and severe cases(n=18) presenting within 4 days of onset of pain

In patients who presented within 4 days of onset of pain H2S levels at admission were not significantly different between mild and severe cases.(Mild cases, n=24,median= 23.14 µmol/L ,R=7.14 – 76.43 µmol/L Vs Severe cases n= 18, median=36.78 µmol/L,R=10 – 74.43 µmol/L.P value=0.186).Figure: 13

We also analyzed the H_2S levels in patients who presented to us within 72 hrs of onset of pancreatitis, as previous human study had shown significant difference between mild and severe cases in patients within 72 hrs of onset of abdominal pain. However we could not find any significant difference between H_2S levels in mild and severe cases within 3 days of onset of abdominal pain(Mild cases, n= 20,Mean=30.12,Median=26.17 Vs Severe cases, n=15,Mean=32.20,Median=35.71)(p value=0.74).

6.11 Correlation of H_2S with complications of pancreatitis

6.11.1 Correlation with acute lung injury

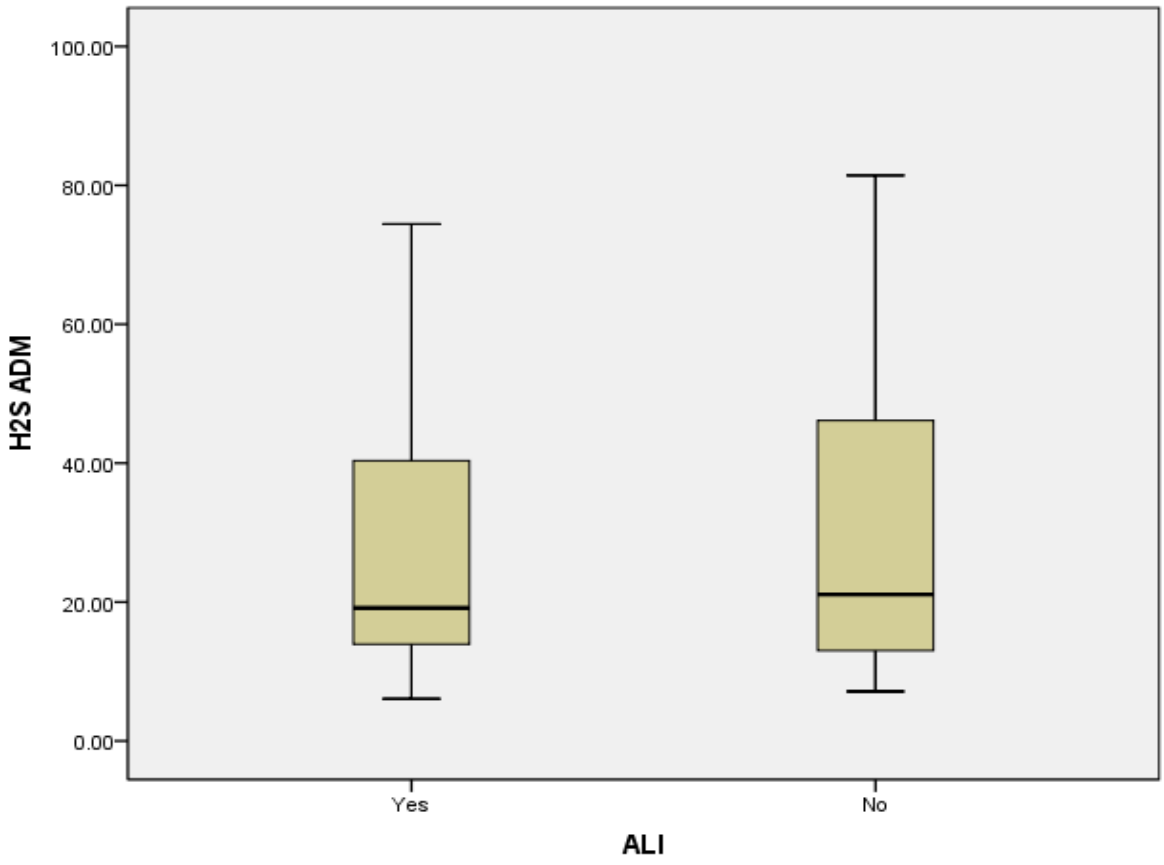

Figure 14: H2S levels (µmol/L) at admission in patients with (n=19) and without acute lung injury(ALI)(n=36)

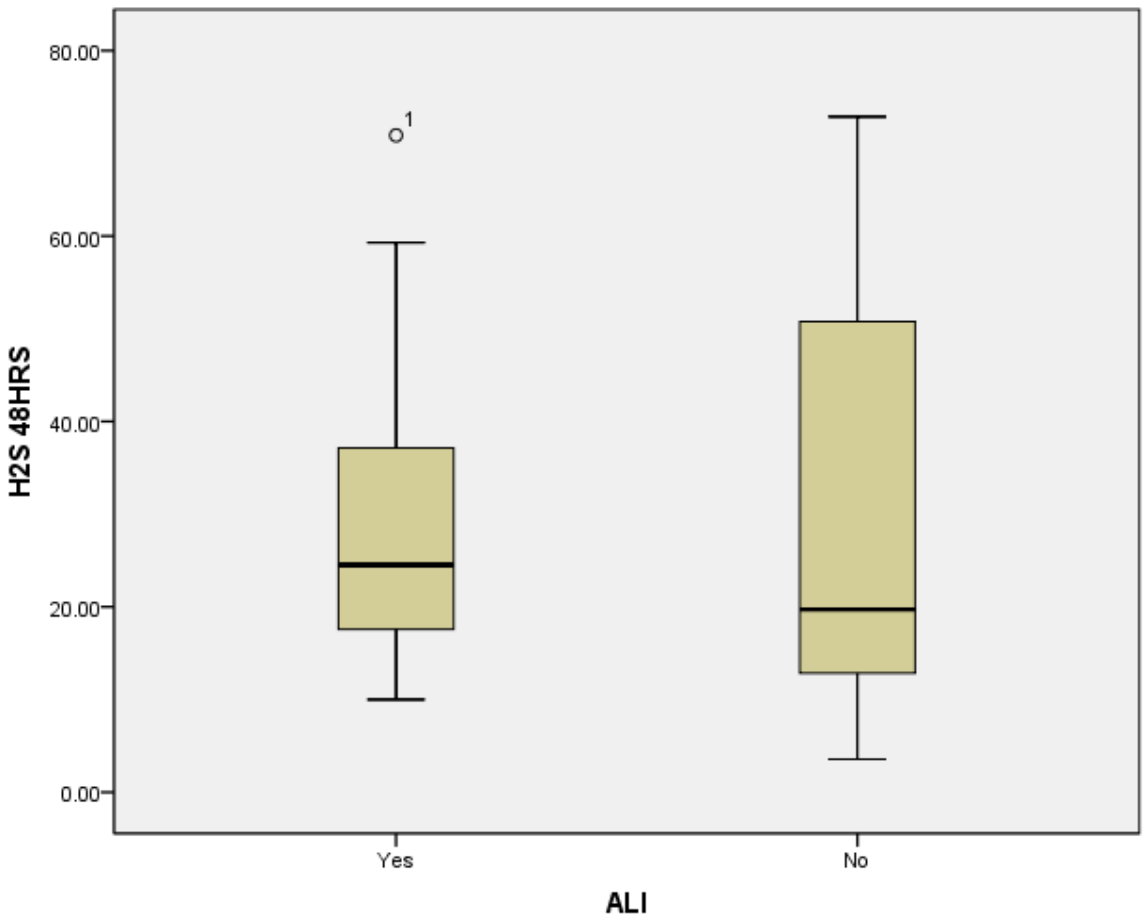

Figure 15: H2S levels (µmol/L) at 48 hrs in patients with (n=16) and without acute lung injury(ALI) (n=32)

Plasma H2S at admission in patients who later developed acute lung injury was not higher than those who did not develop lung injury (n=19, median=19.15 µmol/L, r = 6.07 - 74.43µmol/L Vs n= 36, median=21.07 µmol/L, r= 7.14 – 81.43µmol/L), and therefore did not predict acute lung injury (p value=0.915). Even at 48 hrs of fluid resuscitation, H2S levels were not significantly different between these two groups (n=16,median=24.51µmol/L, r = 10 – 70.86µmol/L Vs n=32,median =19.7µmol/L, r = 3.5 – 72.86µmol/L, p value=0.72).Figure 14 and 15.

We also did not find any correlation between development of lung injury and change in H_2S value in the first 48 hrs. In patients who developed lung injury an equal number (n=8) showed increase and decrease in plasma H_2S.

6.11.2 Correlation with acute kidney injury (AKI).

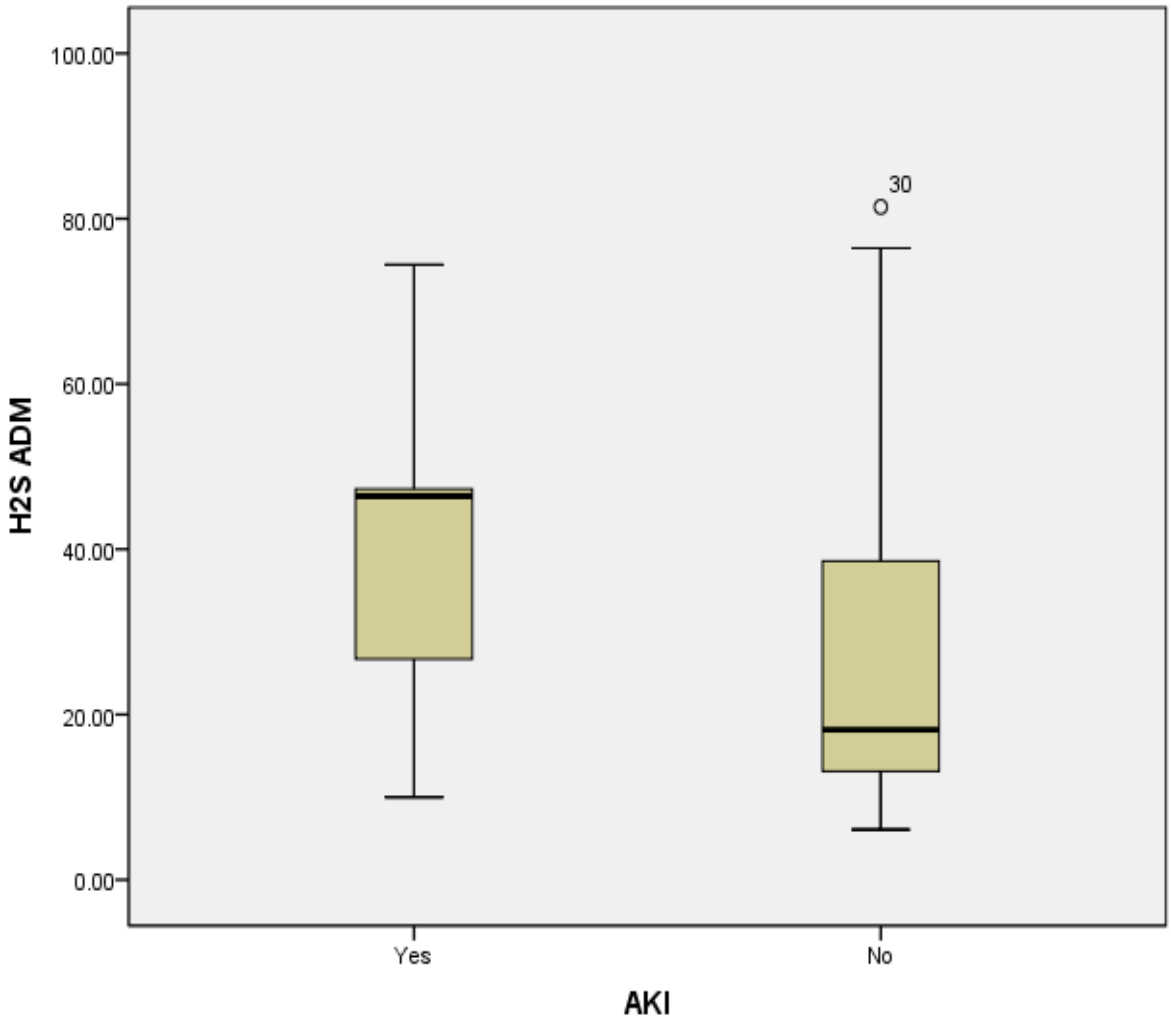

Figure 16: H2S levels (µmol/L) at admission in patients with acute kidney injury(AKI) (n=5) and without acute kidney injury (n=50) in acute pancreatitis

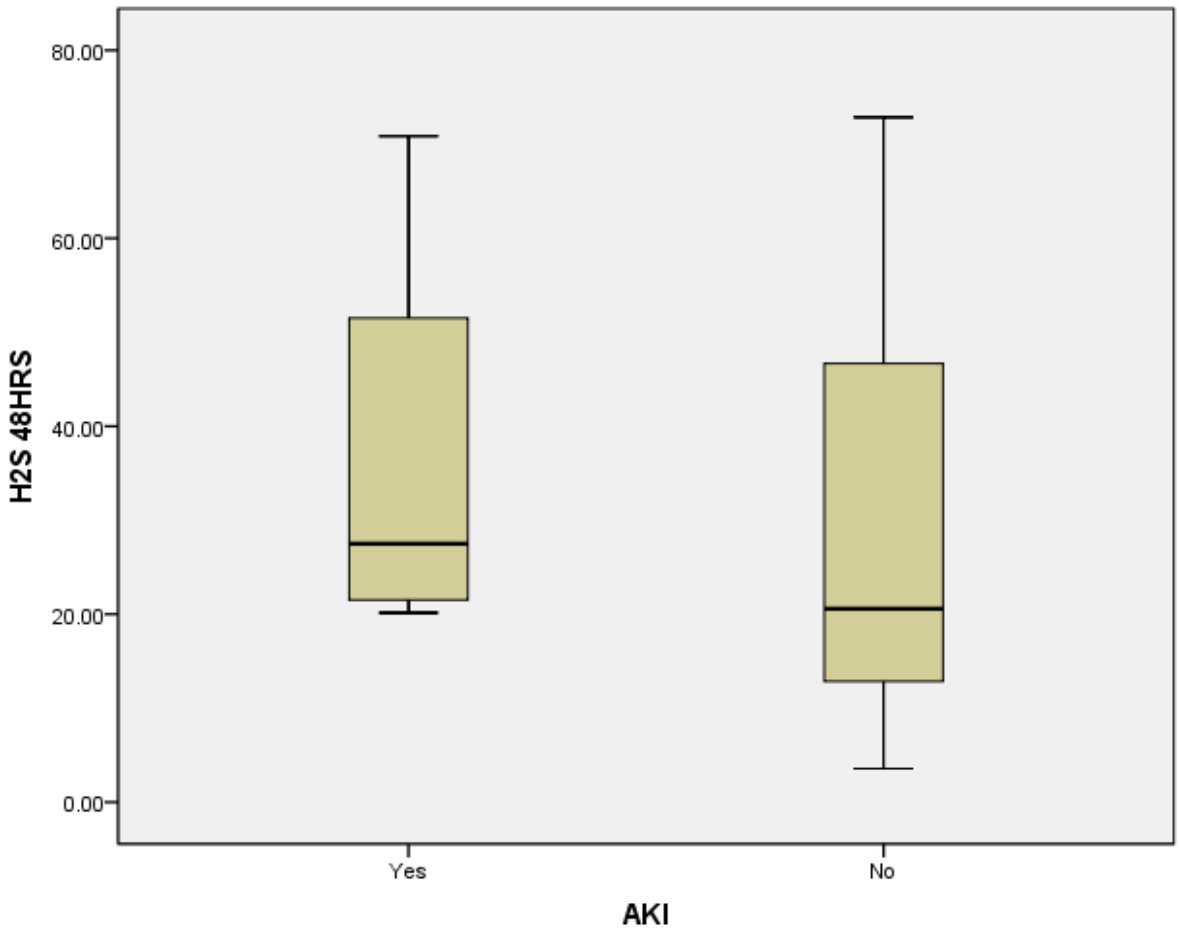

Figure 17: H2S levels (µmol/L) at 48 hrs in patients with acute kidney injury(AKI)(n=4)and without AKI(n=44) in acute pancreatitis

In five patients who had acute kidney injury, admission plasma H2S was higher than in patients who did not have kidney injury, but the difference was not statistically significant (median 46.43µmol/L, r= 10- 74.43 µmol/L Vs 18.14 µmol/L, r= 6.07-81.43µmol/L, p value=0.30)(Figure 16). Similarly, at 48hours there was no difference in H2S levels between the two groups(n=4,median=27.5µmol/L, r = 20.16 -70.86µmol/L Vs n=44,median=20.57µmol/L, r =

3.57 -72.86µmol/L, p value=0.33)(Figure 17).In four out of five patients who had acute kidney injury, the H_2S levels decreased in the first 48 hours. This was not statistically significant.

6.11.3 Correlation with sepsis

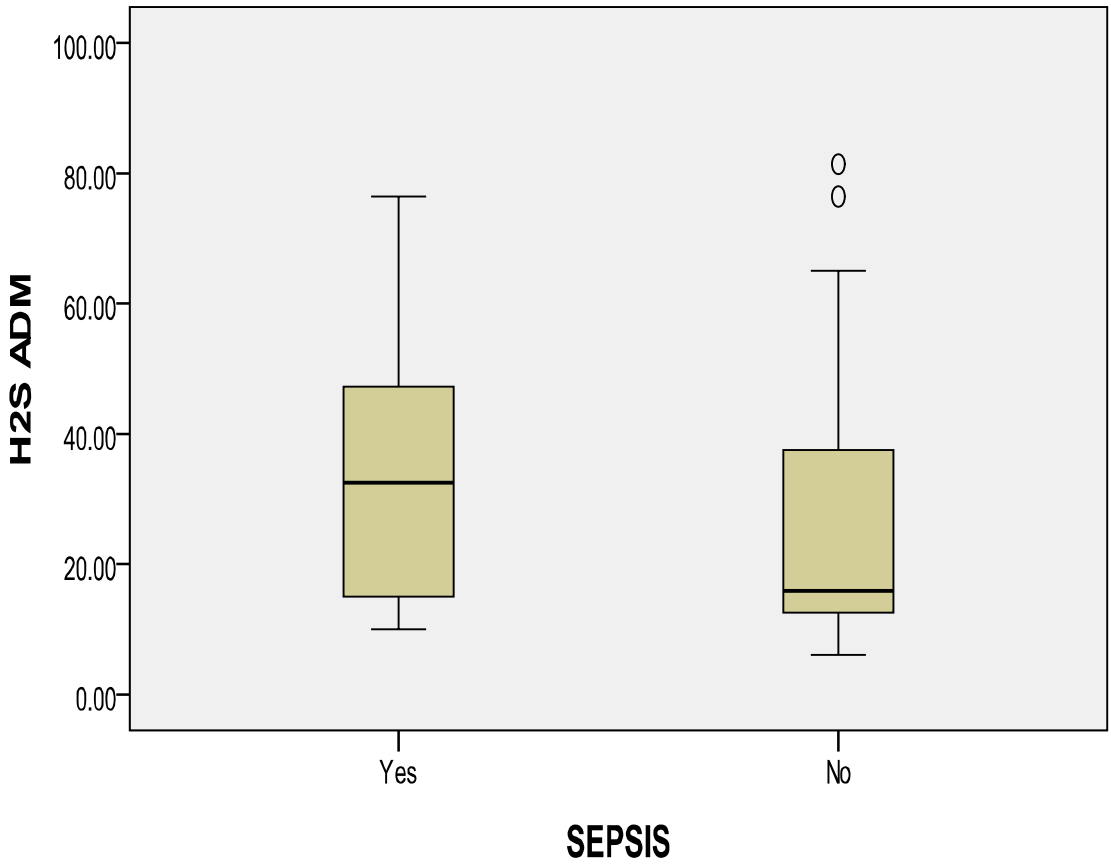

Figure 18: H2S levels(µmol/L) at admission in acute pancreatitis patients with sepsis (n=22)and without sepsis (n=32)

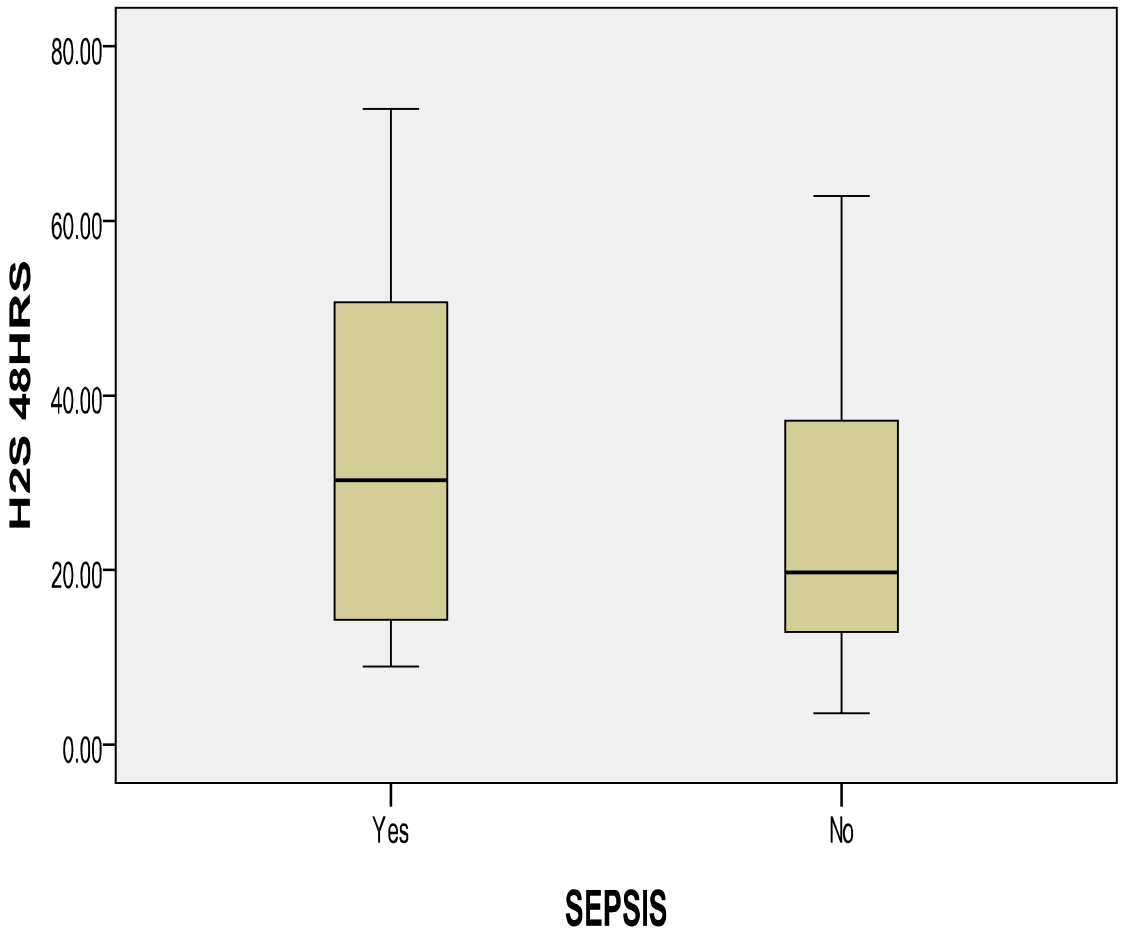

Figure 19: H2S levels (µmol/L) at48 hrs in acute pancreatitis patientswith sepsis (n=21)and without sepsis (n=26)

H_2S levels of all patients with sepsis and SIRS were compared with those without. Patients with sepsis had slightly higher H2S values at admission (n= 22,median=32.5µmol/L, r= 10-76.43µmol/L Vs n=32, median=15.9µmol/L, r =6.07-81.43µmol/L) and at 48 hours(n=21,median=30.2µmol/L, r =8.93-72.86µmol/L Vs n=26, median=19.7µmol/L, r =3.5 – 62.86µmol/L) when compared to those without. However, the difference was not statistically significant (p value=0.18 and 0.32 respectively).Figure 18 & 19

On serial measurements, no trend which could be used to predict the development of sepsis was noted in the first 48 hours. Among patients who developed sepsis, a reduction in H_2S was noted in 12 out of 22 patients, the remaining 10 had increase in levels compared to baseline.

Duration of hospital stay was in the range of 1 to 22 days. Mean duration of hospital stay was 6 days. Majority of patients (n=34) had duration of stay of less than or equal to 1 week and 15 patients had duration of stay of less than 1 week. Out of all 55 patients, twenty patients had required intensive monitoring in ICU

6.11.4 Correlation with mortality

Plasma H2S level at admission in the two patients who expired of multi-organ failure was also not different significantly from the patients who survived(Median =28.64 µmol/L Vs 20.7 µmol/L)

6.12 Other biomarkers

6.12.1 C-reactive protein

CRP was done for 43 patients, 25 mild and 18 severe cases. Median CRP values were significantly higher in severe cases as compared to mild cases (195 mg/LVs 63.4 mg/L, p value <0.001).(Figure 20) In our study, CRP was well correlated with the severity of disease. However, CRP levels were not correlated with the H_2S values.(Figure:21)

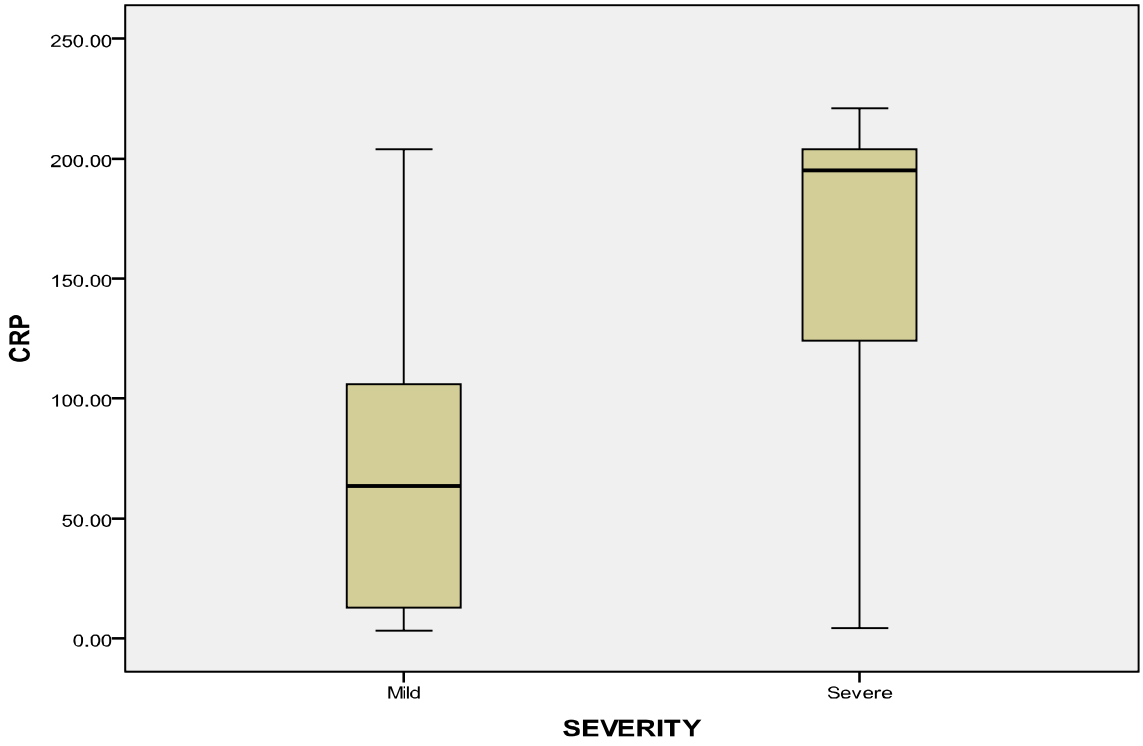

Figure: 20 Comparison of CRP levels (mg/L) in mild and severe cases of pancreatitis.

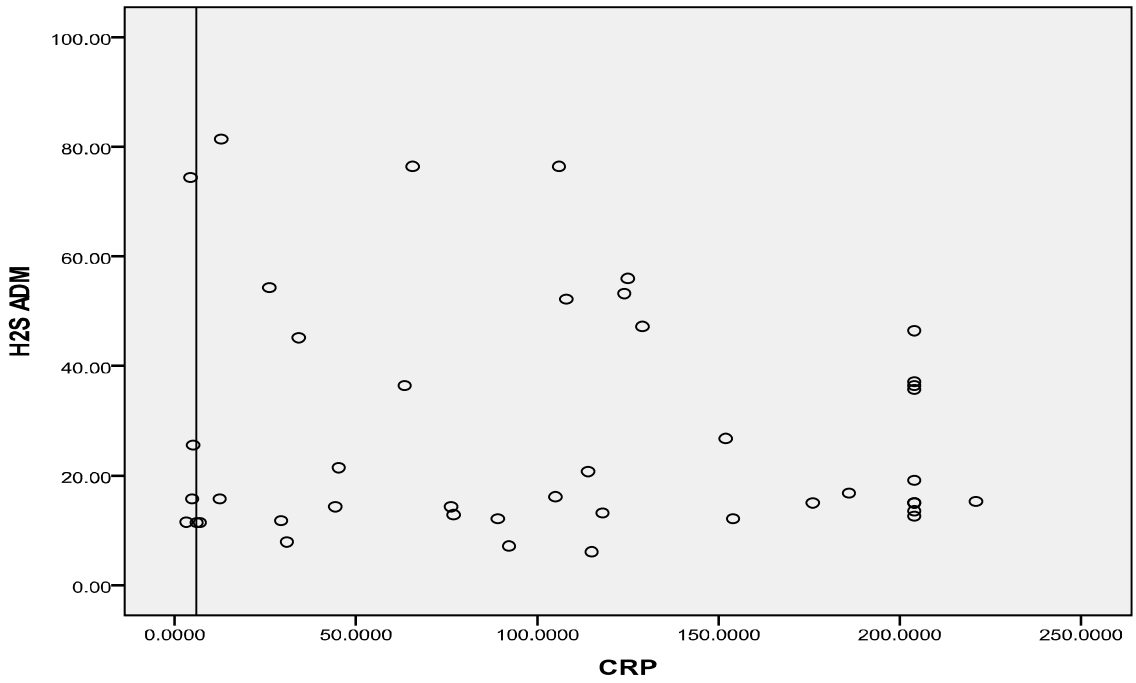

Figure 21:Scatter diagram showing comparison of H2S (µmol/L) and CRP levels

6.12.2 LDH

Lactate dehydrogenase (LDH) was done in 51 patients, mild 30 cases,severe 21cases. Median LDH values were higher in severe cases than in mild cases(778 U/L Vs 498.5 U/L) ,but the difference was not statistically significant(p value=0.1).(figure:22).In this study, the levels of H_2S were also not correlated with LDH values.(figure:23)

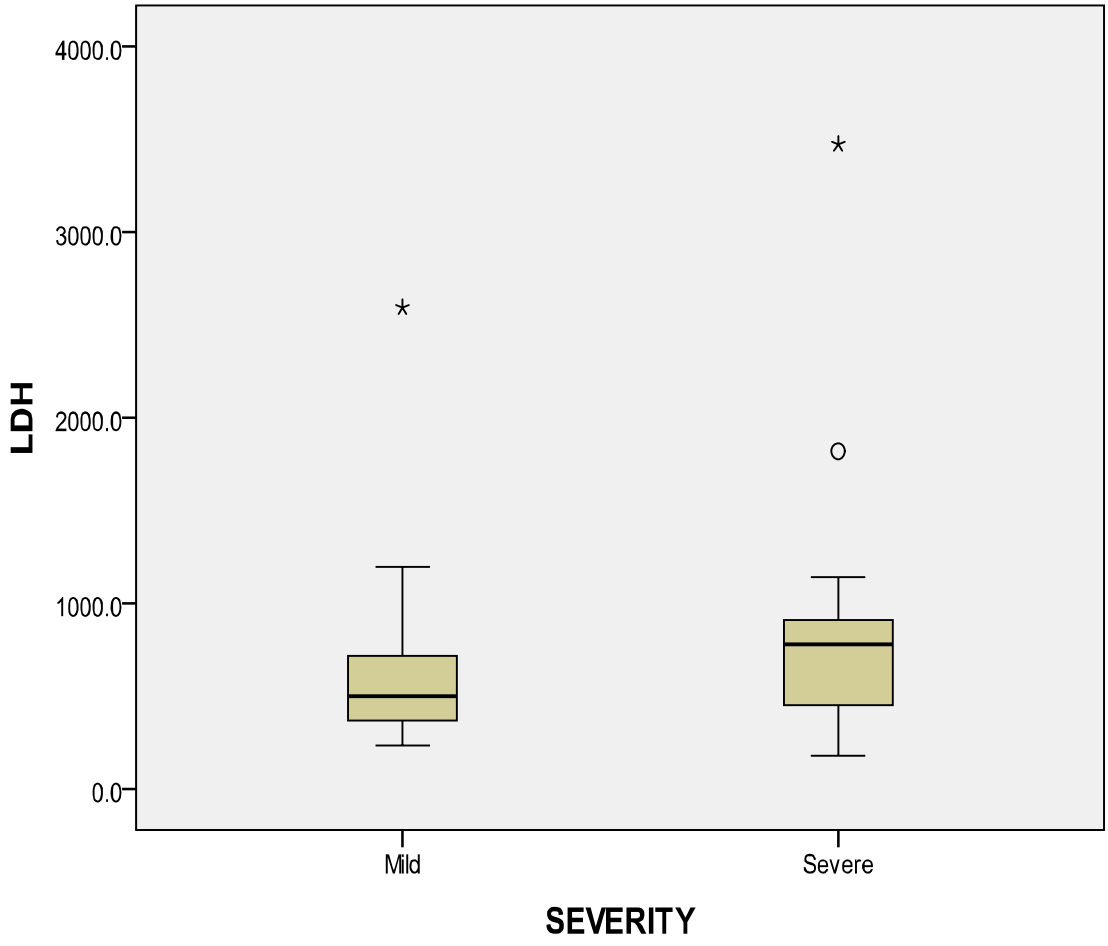

Figure:22 Comparison of LDH levels (U/L) in mild and severe cases.

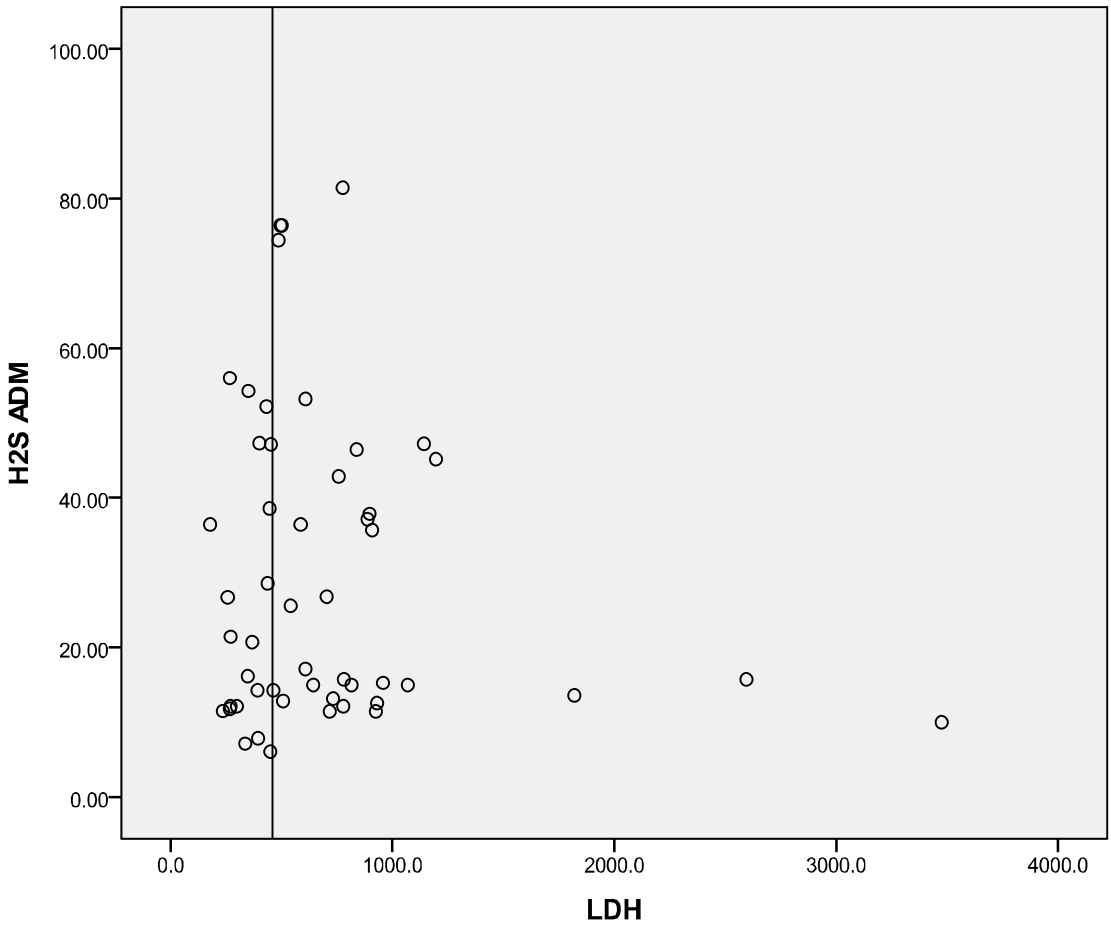

Figure 23: Scatter diagram showing comparison of H2S (µmol/L) and LDH levels .

DISCUSSION

7. DISCUSSSION

7.1 Characteristics of the patient cohort

The most common etiology for acute pancreatitis in our study was alcohol (~45%), followed by gall stones (~27%).The most common systemic complication observed was acute lung injury (~ 35 %), followed by acute kidney injury (~ 9 %). Fluid collections were the most common local complication though none of the patients developed a pseudoaneurysm. In this study, sepsis was noted to be present in about 40 % of patients, and mortality was 3.6%. Severe cases usually account for 20-25 % of all cases of pancreatitis, but in our study, the number of cases of severe pancreatitis was more (41.8% of the group). This can be attributed to referral bias.

7.2 Prediction of severity in acute pancreatitis

Even though the initiating cause in acute pancreatitis is varied, the cascade of events that follows is quite similar, irrespective of etiology. The disease can vary from a mild self-limited illness with an extremely good prognosis to one in which there is multi organ dysfunction, high morbidity and mortality. In India, where resources in terms of ICU beds and intensive care physicians are scarce, predicting severity and course of pancreatitis is all the more relevant to allocate existing resources prudently

7.3 Currently available predictors

Pancreatitis is classified as severe when there are local or systemic complications with persistent multi organ dysfunction. In practice, the Ranson’s and APACHE II scores are used to assess the severity of illness and predict prognosis. The other commonly used parameters that correlate with patient outcome are CRP measured at 48-72 hours and CT scan done after 48 hours, from onset of

pain. Many other biomarkers have been studied as predictors of severity. Many have good sensitivity but suffer from poor specificity. With only a minority of acute pancreatitis being severe (20%), the predictive value of a positive test is low. Thus, none of the biomarkers have been shown to robustly predict the course of illness in pancreatitis. Often close and repeated monitoring for any change in the patient's vital parameters is the best clue to the course of illness.

7.4 Hydrogen sulphide

H_2S has been found in recent years to be an important gasotransmitter, along with nitric oxide and carbon monoxide. The measurement of hydrogen sulphide has to be carried out immediately after collection due to its volatile nature. The colorimetric method used in this study measures by spectrophotometry, the amount of methylene blue produced. Methylene blue can form dimers and trimers in a concentration dependent manner which affects the estimation of H_2S. Therefore when this method is used, it has to be validated by measuring the monomer to dimer equilibrium in patient and control samples. Some samples could not be analysed in the study due to the inability to carry out the test on stored samples and due to the presence of sample hemolysis.

7.5 Animal studies

Animal studies have shown that hydrogen sulphide levels correlate with severity of pancreatitis and acute lung injury, and that attenuation of the H2S production led to a less severe forms of pancreatitis and lung injury. In animal studies, the uniform age of the animal, the controlled degree of artificial pancreatic insult, the ability to estimate H_2S early in the illness and the uniformity of sampling times gave reproducible results. This study was undertaken in order to

assess the role of hydrogen sulphide in acute pancreatitis and to test its applicability in a routine clinical setting.

7.6 Human studies

Contrary to findings in animal studies, our study did not validate the usefulness of H_2S as a marker of severity in acute pancreatitis in the clinical setting. There was also no correlation found between H_2S and CRP levels.

The differences in etiology, severity of insult, duration of illness, development of complications, genetic variations in inflammatory response and other unmeasured variables produced a wide range of H_2S values, rendering the test unhelpful. It is likely that similar problems exist in other biomarker studies. Tests which perform well in animal studies often do poorly in human studies, leading to negative studies and non-publication. This can also explain the lack of published human studies in the usefulness of H_2S as a predictor of severity in acute pancreatitis. Aside from a single published abstract (Eric WL Wee et al AGA Gastroenterology Volume 136,Issue 5,supplement 1,May 2009), this study is the first to study the role of hydrogen sulphide in cases of acute pancreatitis in humans in a routine clinical scenario where patients seek medical care after at varied time points following the onset of disease.

7.7 Post-hoc analysis of early presenters

In the study conducted by Eric WL Wee et al, which showed that H_2S was a very good predictor of severity, only patients who presented within 72 hrs of onset of symptoms were included. Serum H2S levels may not correlate with the severity of pancreatitis in patients who present

several days after onset of pain, as seen in routine clinical practice. The majority of the patients in the current study presented more than 3 days after onset of pain. We looked at the subgroup of patients who presented early in course of illness i.e. within 3 and 4 days after onset of pain. We did not find a significant difference in this subgroup of patients, with mild and severe acute pancreatitis.

7.8 Assessment after 48 hours

The purpose of estimating the values of H_2S at 48 hrs was to assess the patient after fluid resuscitation and providing adequate analgesia. H_2S levels were not significantly different between cases of mild and severe pancreatitis, at 48hours after admission. We also analysed whether temporal trends in H_2S could provide prognostic data. We predicted that patients with a mild disease course would show a decrease in levels. This was not borne out on analysis. On the contrary, many patients had increase in H_2S levels despite clinical improvement.

7.9 Therapeutic blocking of hydrogen sulphide production

Previously, Madhav Bhatia et al demonstrated that administration of PAG (propargylglycine), a CSE inhibitor, protected mice against severe pancreatitis and lung injury. The therapeutic potential of H_2S modulation may be realised in the prevention of post-ERCP pancreatitis, where the initiating event occurs in the hospital and treatment before onset or early in the illness can be given.

7.10 Limitations of this study

The intrinsic instability of H_2S and the lack of fully automated assays resulted in problems with estimation. The stringency of the sample requirements resulted in a loss of recruitment - due to delay in sample collection and transport, suboptimal sample quality due to hemolysis, weekend admissions and non-availability of 24 hour laboratory facilities.We excluded samples which could not be processed immediately and did not study the effect that delay in sample testing would have on H_2S estimates.

Our hospital is a tertiary referral centre, and a higher proportion of cases had severe acute pancreatitis. Often, patients had received treatment prior to admission to our hospital. Though the results may not have been different, it should be kept in mind that it was a single centre study with a high proportion of severe cases.

7.11 Conclusions

There was no difference in admission and 48 H_2S levels between mild and severe pancreatitis. Levels of H2S did not correlate with systemic complications (ALI, AKI and sepsis), or mortality. Temporal trends of H_2S in the initial days of admission were also not found to be helpful.

The present study could not establish hydrogen sulphide as a marker of severity of acute pancreatitis. Futures studies grouping patients according to time of presentation may clarify its usefulness in patient subgroups.

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APPENDIX 1

PROFORMA

NAME HOSPITAL NO.

AGE ADDRESS-

SEX OCCUPATION-

D.O.A - D.O.D -

CHIEF COMPLAINTS DURATION

1.
2.
3.
4.

HISTORY OF PRESENT ILLNESS

PAST HISTORY

PERSONAL HISTORY

GENERAL EXAMINATION-

Wt- Ht- BMI

VITALS-

PR- BP- RR- SPO2-

P/A- RESP CNS CVS

INVESTIGATION- AT ADMISSION

CBC -Hb-

TC - DC -

Hct-

LFT

CREATININE

UREA

NA/K

BBVS

CXR-

CULTURES

ABG-

USG ABDOMEN-

CRP

LDH

Ca

FASTING LIPID PROFILE

TG CH

LDL

AMYLASE-

LIPASE-

AFTER 48 Hrs

CBC Hb-

TC- DC-

Hct_

CREATININE

ABG

ICU STAY(DAYS) -

MECHANICAL VENTILATOR REQUIREMENT-

CECT ABDOMEN-

BALTHAZAR STAGING-

ORGAN FAILURE

BISAP SCORE-

RANSONS SCORE-

DIAGNOSIS-

SEVERITY OF ACUTE PANCREATITIS—

HYDROGEN SULFIDE LEVELS

AT ADMISSION AFTER 48 HOURS AT DISCHARG

APPENDIX-2

MASTER CHART

S.NO.	HNUM	AGE(yrs)	SEX	SEVERITY	H2S ADM	H2S 48HRS	H2S AT D	ALI	AKI
1	291368 F	31	1	2	37.86	50.71	38.57	1	2
2	281093 F	19	2	1	38.57	27.14		2	2
3	194104 F	43	1	1	36.43	38.57	36.43	2	2
4	546009 C	73	1	1	28.57	46.07		2	2
5	290269 F	38	1	1	76.43	57.86		2	2
6	329012 F	29	2	1	7.14	3.57	7.14	2	2
7	309641 F	29	1	2	6.07			1	2
8	327517 F	27	1	1	7.86			2	2
9	342730 F	44	2	1	11.43	8.93	9.28	2	2
10	373384 A	65	1	1	15	10.71	10.71	2	2
11	554522 B	50	1	2	46.43	22.86	17.86	1	1
12	374333 F	29	1	2	10			1	1
13	367979 F	31	1	1	12.14	16.43	11.43	2	2
14	374526 F	36	1	2	12.14	15	9.64	1	2
15	373239 F	66	2	1	15.71	19.28	16.43	2	2
16	361242 F	40	1	1	21.43	14.28		2	2
17	246773 F	26	1	1	20.71	16.43		2	2
18	389154 F	41	1	2	35.71	37.14	22.14	1	2
19	384699 F	43	1	2	15	10	5	1	2
20	389094 F	36	1	1	14.28			2	2
21	315187 F	49	1	1	11.43	12.86	11.43	2	2
22	013331 F	52	2	2	14.28	10.58	12.14	1	2
23	401398 F	66	1	1	12.86	13.93		2	2
24	401388 F	49	1	1	15	12.86		2	2
25	406258 F	46	1	2	42.86	21.43	10.71	1	2
26	379541 F	21	2	1	11.78	12.86	12.14	2	2
27	415599 F	32	1	1	15.71	14.28	12.14	2	2
28	566953 D	64	1	1	17.14	15	16.43	2	2
29	422515 F	32	1	2	36.43	37.14	34.28	1	2
30	434377 F	29	2	1	11.5	12.75		2	2
31	422613 F	28	1	2	13.57		11.78	1	2
32	434625 F	34	1	1	81.43			2	2
33	434120 F	63	1	2	54.28	59.28	87.86	1	2
34	441047 F	39	1	1	76.43	72.86	91.43	2	2
35	028262 D	28	2	1	65	62.86		2	2
36	448324 F	59	1	1	47.14	54.28	54.28	2	2
37	448492 F	71	1	2	53.21	72.86		2	2

38	471690 F	38	1	2	56	67	65	2	2
39	455700 F	33	1	2	47.21	57.28	54.28	2	2
40	455941 F	34	1	2	37.14	30.25	42.14	1	2
41	374526 F	35	1	2	19.15	20.16	19.28	1	2
42	072576 F	24	2	1	25.57	20.57	23.12	2	2
43	474374 F	27	1	2	12.56	10	21.86	1	2
44	703980 A	38	1	1	45.14	47.28	47.14	2	2
45	474434 F	27	1	1	13.14	12.28	11.54	2	2
46	664561 F	39	1	1	26.78		20.12	2	2
47	604983	58	2	2	74.43	70.86	50.28	1	1
48	611960 F	55	2	1	16.14	24.15	20.86	2	2
49	701361 A	80	1	2	16.78	20.58	17.26	1	2
50	657780 F	18	2	1	12.14	11.21	11.86	2	2
51	582566 D	22	1	1	52.21	54.28	47.81	2	2
52	479777 F	65	2	1	38.57	35.56		2	2
53	657667 F	80	1	2	26.71	20.16		2	1
54	651775 F	27	1	2	15.26	26.17		1	2
55	639560 F	35	1	2	47.28	32.14		1	1

S.NO.	HNUM	ETIOLOGY	DAY OF PAIN AND IST SAMPLE	CRP	LDH	DURATION	ICU STAY	VENTILATION	BMI
1	291368 F	A	5		897	19	3	0	24.1
2	281093 F	O	3		446	6	0	0	22.4
3	194104 F	O	3	63.4	586	6	0	0	20.9
4	546009 C	B	2		437	8	0	0	22.9
5	290269 F	A	4	65.6	495	10	0	0	26.8
6	329012 F	O	4	92.2	335	10	8	0	33.2
7	309641 F	O	5	115	450	12	0	0	26.4
8	327517 F	A	2	30.9	394	4	0	0	28.5
9	342730 F	B	6	6.94	716	9	0	0	20.3
10	373384 A	A	2	176	643	5	0	0	20.8
11	554522 B	A	4	204	838	7	6	0	33.2

		A	2		3475	1	1	1	30.8
12	374333 F	A	3	89.1	270	5	4	0	33.5
13	367979 F	A	6	154	778	4	3	0	24.8
14	374526 F	B	6	12.4	2595	7	0	0	36.8
15	373239 F	A	6	45.2	271	3	0	0	21.3
16	361242 F	A	2	114	367	4	0	0	21.5
17	246773 F	A	2	204	909	7	2	0	21
18	389154 F	A	6	204	815	10	3	0	25.9
19	384699 F	A	3	44.3	392	4	0	0	24
20	389094 F	B	3	6.03	925	11	0	0	23.2
21	315187 F	O	3	76.2	462	5	2	0	27.9
22	013331 F	O	2	76.9	507	4	0	0	23
23	401398 F	B	5	204	1068	6	0	0	27.1
24	401388 F	B	4		757	13	0	0	22
25	406258 F	B	4	29.3	267	7	0	0	30.8
26	379541 F	B	2	4.78	781	11	0	0	25.8
27	415599 F	B	1		607	6	0	0	18.9
28	566953 D	A	2	204	178	11	10	0	21.4
29	422515 F	O	5	3.19	235	2	0	0	19
30	434377 F	A	3	204	1820	20	19	1	25.2
31	422613 F	A	5	12.8	776	3	0	0	21
32	434625 F	O	2	26.1	350	10	6	0	23.2
33	434120 F	A	3	106	502	6	0	0	26.3
34	441047 F	O	1			6	2	0	21
35	028262 D	B	3		452	6	0	0	27
36	448324 F	A	3	124	608	6	0	0	22.6
37	448492 F	O	2	125	266	8	0	0	25
38	471690 F	A	2	129	1142	4	0	0	22
39	455700 F	A	3	204	887	12	3	0	26
40	455941 F	A	3	204		5	4	0	24.8
41	374526 F	B	3	5.07	540	3	0	0	20
42	072576 F								

		A	5	204	930	10	9	0	26.2
43	474374 F								
		A	3	34.2	1195	5	0	0	25
44	703980 A								
		B	5	118	733	4	0	0	25.4
45	474434 F								
		A	3	152	703.9	3	0	0	23.6
46	664561 F								
		O	4	4.32	486	8	7	0	27.2
47	604983								
		B	5	105	347	9	0	0	23.4
48	611960 F								
		O	3	186		15	7	0	24.4
49	701361 A								
		O	3		298	4	0	0	18.2
50	657780 F								
		B	3	108	432	3	0	0	22
51	582566 D								
		B	2			11	0	0	23
52	479777 F								
		O	3		257	4	0	0	22.4
53	657667 F								
		O	2	221	958	7	4	0	25.4
54	651775 F								
		A	3		400	22	22	1	26.2
55	639560 F								

S.NO.	HNUM	SEPSIS	LOCAL COLLECTION	VASCULAR	DEATH
1	291368 F				
		1	1	2	0
2	281093 F				
		0	2	2	0
3	194104 F				
		0	2	2	0
4	546009 C				
		1	2	2	0
5	290269 F				
		0	2	2	0
6	329012 F				
		0	2	2	0
7	309641 F				
		0	2	2	0
8	327517 F				
		0	2	2	0
9	342730 F				
		1	2	2	0
10	373384 A				
		0	2	2	0
11	554522 B				
		1	1	2	0
12	374333 F				
		1	2	2	1
13	367979 F				
		0	2	2	0
14	374526 F				
		0	1	2	0
15	373239 F				
		0	2	2	0
16	361242 F				

		0	2	2	0
17	246773 F				
		0	2	2	0
18	389154 F				
		0	2	1	0
19	384699 F				
		1	2	2	0
20	389094 F				
		0	2	2	0
21	315187 F				
		1	2	2	0
22	013331 F				
		1	2	2	0
23	401398 F				
		0	2	2	0
24	401388 F				
		0	2	2	0
25	406258 F				
		0	1	2	0
26	379541 F				
		0	2	2	0
27	415599 F				
		1	2	2	0
28	566953 D				
		1	2	2	0
29	422515 F				
		1	1	2	0
30	434377 F				
		0	2	2	0
31	422613 F				
		0	1	2	0
32	434625 F				
		0	2	2	0
33	434120 F				
		1	1	2	0
34	441047 F				
		1	2	2	0
35	028262 D				
		0	2	2	0
36	448324 F				
		0	2	2	0
37	448492 F				
			2	1	0
38	471690 F				
		1	1	2	0
39	455700 F				
		1	1	2	0
40	455941 F				
		1	1	2	0
41	374526 F				
		1	2	2	0
42	072576 F				
		0	2	2	0
43	474374 F				
		1	1	2	0
44	703980 A				
		0	2	2	0
45	474434 F				
		0	2	2	0
46	664561 F				
		0	2	2	0
47	604983				

		1	2	2	0
48	611960 F	0	2	2	0
49	701361 A	1	1	2	0
50	657780 F	0	2	2	0
51	582566 D	0	2	2	0
52	479777 F	1	2	2	0
53	657667 F	0	2	2	0
54	651775 F	0	2	2	0
55	639560 F	1	1	2	1

Male-1
female-2

Mild-1
Severe-2

ALI-Acute lung injury

Yes-1
No-2

Acute kidney injury

Yes-1
No-2

Etiology

A -alcohol
B-Biliary
O-Others

Sepsis

Yes-1
No-0

Vascular complication

Yes-1
No-2

Local complication

Yes-1
No-2

Death

yes-1
No-0