

**To study the safety and efficacy of Mesenchymal  
Stromal Cells (MSC) in the treatment of Recurrent  
High Fistula-in-ano  
A pilot study**



A dissertation submitted to the M.G.R Medical University, Tamil Nadu, in partial fulfillment of the requirement for the M.S. branch I (General Surgery) examination to be held in April 2015

# Certificate

This is to certify that the topic entitled “To study the safety and efficacy of Mesenchymal Stromal Cells (MSC) in the treatment of recurrent high fistula-in-ano- A pilot study” is a bonafide work done by Dr. Zeeshan Rahman, post graduate in General Surgery, of Christian Medical College, Vellore.

This work has been carried under my guidance and supervision in partial fulfillment of the regulation of Dr. M.G.R. Medical University of Tamil Nadu for Master of Surgery- Branch I (General Surgery) examination to be held in April 2015.

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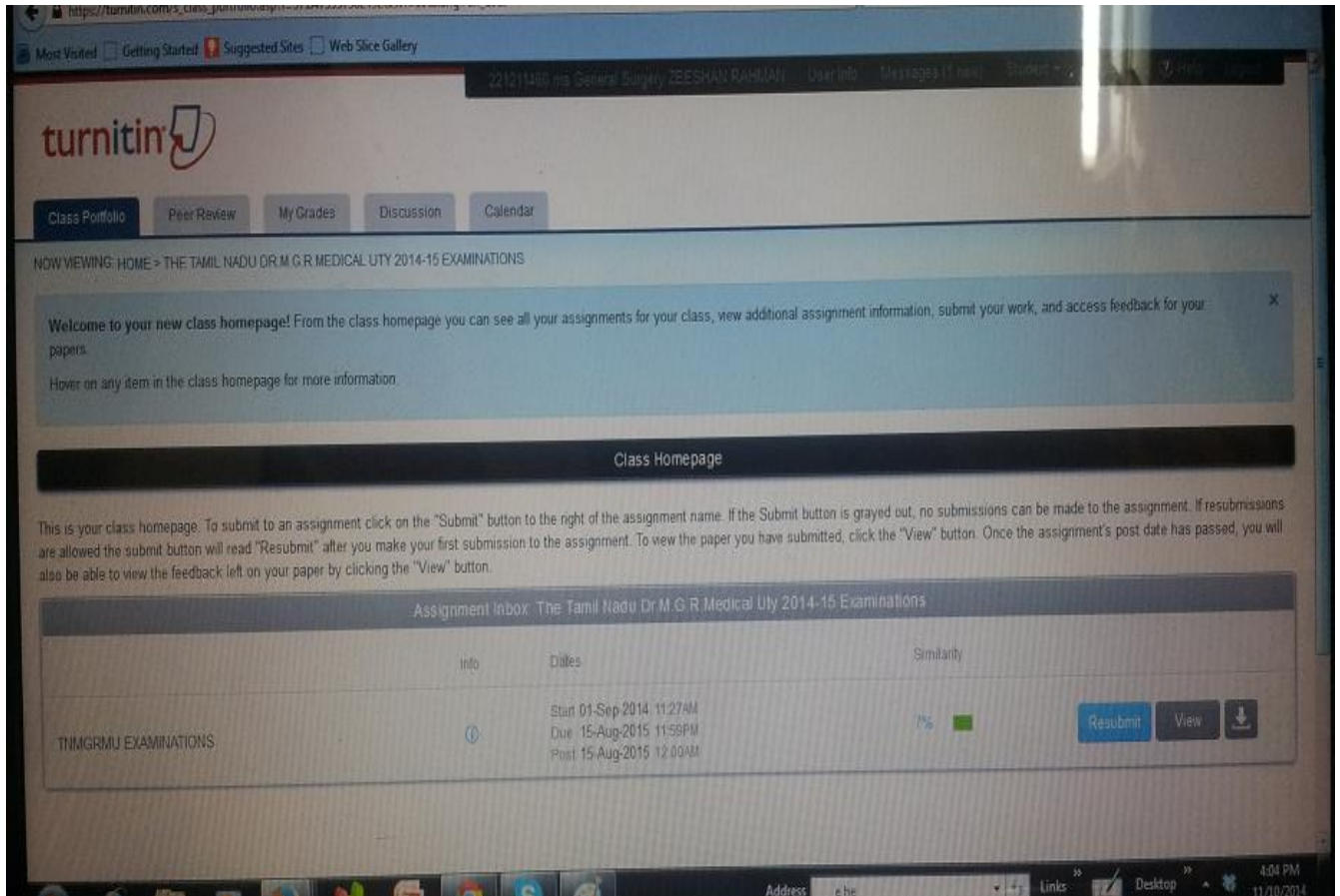
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April 4, 2013

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PG Registrar  
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Sub: **FLUID Research grant project NEW PROPOSAL: (RED IRB)**  
A prospective, interventional, Phase 2 trial to evaluate the use of placental derived mesenchymal stromal cells (MSC) in the treatment of recurrent, complex fistula-in-ano.  
Dr. Zeeshan Rahman, PG Registrar, General Surgery, Dr. Sukria Nayak, Surgery, Dr. Alok Srivastava, Dr. Vikram Mathews, Haematology, Dr. Anuradha Mittal, Radiology.

Ref: IRB Min. No. 8147 dated 19.12.2012

Dear Dr. Zeeshan Rahman,

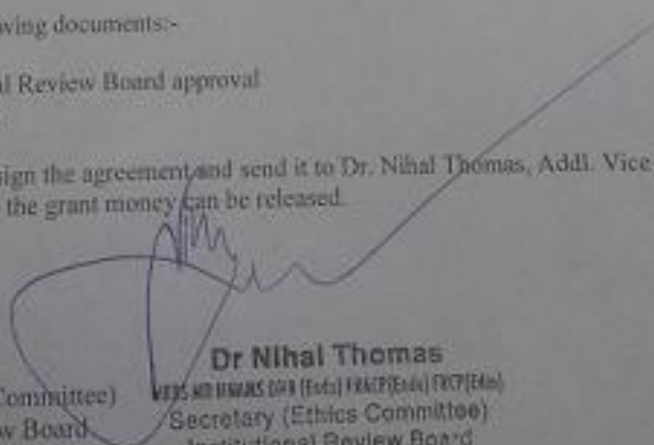
I enclose the following documents:-

1. Institutional Review Board approval
2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.


With best wishes,

Dr. Nihal Thomas  
Secretary (Ethics Committee)  
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**Dr Nihal Thomas**  
MD, MNAMS, DNB (Endo), FRACP (Endo), FRCP (Ethics)  
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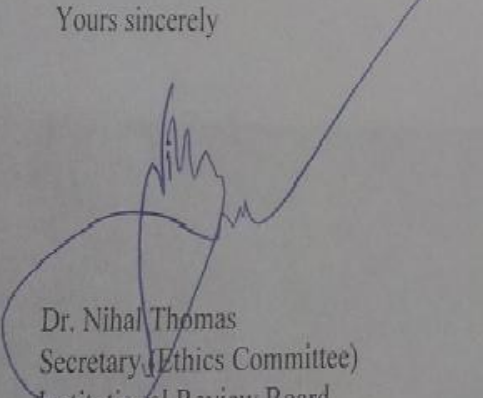
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We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent. And on completion of the study you are expected to submit a copy of the final report.

A sum of Rs. 80,000/- (Rupees Eighty Thousand only) for 2 years. A sum of Rs 40,000/- will be sanctioned for 12 months after receipt of the revised proposal, subsequent installment of 40,000/- each will be released at the end of the first year following the receipt of the progress report.

Yours sincerely

  
Dr. Nihal Thomas  
Secretary (Ethics Committee)  
Institutional Review Board

# Acknowledgements

- My patients who having understood the experimental basis of the study gave their complete trust and followed it all the way through the follow-up period.
- Dr. Sukria Nayak whose idea formed the basis of this research and whose belief that it may benefit many patients if successful drove me to pursue it.
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# ABSTRACT

**TITLE OF THE STUDY** To study the safety and efficacy of Mesenchymal Stromal Cells (MSC) in the treatment of recurrent high fistula-in-ano - A pilot study.

**DEPARTMENT:** Surgery unit IV

**NAME OF CANDIDATE:** Dr. Zeeshan Rahman

**DEGREE AND SUBJECT:** MS (General Surgery)

**OBJECTIVES:**

- To assess the feasibility and safety of Mesenchymal stromal cells (MSC) as a treatment option for recurrent high fistula-in-ano.
- To study the efficacy of Mesenchymal Stromal Cells (MSC) in the treatment of recurrent high fistula-in-ano.

**METHOD:**

Patients with recurrent high fistula-in-ano of crypto glandular origin (n = 7) underwent injection of heterologous Mesenchymal stromal cells (MSC), 40 million units into the fistula tract and were followed up for a total of 2 months. If healing was not detected clinically at the end of 1 month, the patients underwent a repeat injection of Mesenchymal stromal cells (MSC) into the fistula tract. All patients were followed up at the end of 2 months clinically and MRI was done for patients who had clinical healing

only. All patients were followed up for a duration of 4 months or till they were labeled as recurrence requiring another form of intervention.

#### **RESULTS:**

There were no adverse effects, reactions or worsening of primary disease in all 7 patients who underwent injections of Mesenchymal Stromal Cells (MSC) into the fistula tract. All patients were followed up for a period of 4 months and the procedure appeared to be safe, with no additional risk.

At the end of 1 month fistula healing was observed in 4 out of 7 patients (57%) assessed clinically. Two patients had persistent fistula tracts and underwent a repeat sitting of MSC injection. At the end of 2 months 3 out of 7 (43%) patients had clinically healed fistula tracts and at the end of 4 months 1 out of 7 patients had clinically healed.

Imaging done at 2 months following procedure revealed persistence of the fluid filled fistula tract in all the patients.

#### **CONCLUSION:**

The injection of heterologous Mesenchymal Stromal Cells (MSC) in the treatment of chronic non-healing crypto glandular fistula-in-ano appeared to be safe. However it did not heal the fistula tracts in this pilot study.

#### **KEYWORD:**

Mesenchymal Stromal Cells (MSC), Recurrent high fistula-in-ano, Cryptoglandular.

# **AIMS AND OBJECTIVES**

## AIM AND OBJECTIVES

**Aim:** To study the effect of Mesenchymal stromal cells (MSC) in the treatment of recurrent high fistula-in-ano.

### **Objectives:**

- To assess the feasibility and safety of Mesenchymal Stromal Cells (MSC) as a treatment option for recurrent high fistula-in-ano.
- To assess the effectiveness of Mesenchymal Stromal cells (MSC) in healing of recurrent high fistula-in-ano.

# **INTRODUCTION**

# Introduction

Fistula-in-ano is defined as an abnormal communication between two epithelium lined surfaces. It usually has an external opening on the skin of the perianal / perineal region and an internal opening at the ano-rectal junction.

References to fistula-in-ano dates back to more than 2000 years back. Hippocrates, in 420 BC had made references to the surgical therapy of fistula-in-ano and was the first person to advocate the use of setons (from the Latin *seta*, a bristle) in the treatment of chronic fistula-in-ano.

In 1376, English surgeon Dr. Ardene described fistulotomy and seton use in his publication *Treatises of Fistula in ano; Hemorrhoids and Clysters*.

In the 19<sup>th</sup> and 20<sup>th</sup> centuries numerous prominent surgeons such as Goodsall and Miles, Thompson, Lockhart-Mummery and Milligan and Morgan had made substantial contribution to the methodology of treatment of fistula-in-ano.

Since the progress of early times there has been little change in the understanding of pathophysiology of fistula-in-ano. There have been many techniques described to minimize recurrence rates and prevent incontinence. However, despite 2000 years of knowledge of the disease process, fistula-in-ano still remains a perplexing disease.

Most fistulas are thought to result from blocked anal glands present in the anal valves resulting in cryptoglandular infection, leading to formation of an abscess, adjacent to the ano-rectal tube. These fistulae are a consequence of rupture of perianal abscesses. As the abscess cavity ruptures it creates a tract between the anal canal and the epithelial lining of the perineal skin. The abscess is considered an acute inflammatory event, whereas the fistula is a result of a chronic inflammation.

Symptoms usually affect the quality of life significantly and can range from minor discomfort with hygiene problems to frank septicemia.

Surgery is usually the treatment of choice for these patients with the aim of removal of infection, excision of the fistula tract and preventing recurrent or persistent disease while preserving continence.

The treatment of the fistula-in-ano has been evolving over the past 30 years. Usually these fistulas were surgically drained with the belief that removal of focus of infection would result in healing of the tract.

Specific medical therapy like Infliximab is indicated for fistulas arising as a result of Crohn's disease.

The surgical options include lay-open of the tract or insertion of a seton. This results in loss of quality of life and difficulty in activities of daily living. A patient has to undergo repeated operations to change the seton till it is low enough to remove. This requires recurrent visits & hospitalization, and escalates the cost of treatment.

Currently newer techniques such as injection of glue or anal fistula plugs have been used to treat fistula-in-ano. However the success rates of these procedures are moderate with high recurrence rates and are costly.

Newer methods of surgical management of fistula-in-ano have been mentioned recently like mucosal advancement, Ligation of Fistula Tract (LIFT), Bio-LIFT. All have been found to have some success rate, with various recurrence rates. These can be practiced in specialized centers and when prosthetic materials are used, it makes the treatment costly.

It has been theorized that these Mesenchymal stromal cells (MSC) extracted from bone marrow/adipose tissue/placenta have the capability to differentiate into fibrogenic precursors leading to fibrosis of the non-healing fistula tract. These stromal cells also have the ability to suppress inflammation.

As a pilot project to a larger trial we proposed to study the safety and efficacy of heterologous Mesenchymal stromal cells (MSC) in the healing of recurrent high fistulas.



# **REVIEW OF LITERATURE**

## Review of Literature

### Epidemiology:

The true prevalence of fistula-in ano is unknown. The worldwide incidence of anal fistulas secondary to perianal abscess ranges from 26 – 38%<sup>(1, 2)</sup>.

The incidence of fistula-in-ano associated with underlying Crohn's disease is 6 in 1,00,000 and for Ulcerative colitis is 8 in 1,00,000<sup>(3)</sup>. This data is mostly from the western population. There have been no studies done in India to ascertain the true incidence of perianal fistulas.

The mean age at presentation is usually 40 years. The age group ranges from 20 years to 60 years<sup>(3)</sup>.

Males are twice more likely to develop fistulas as compared to females<sup>(4)</sup>.

# Etiology

The most common etiology in development of perianal fistulas is the formation of ano-rectal abscess<sup>(2)</sup>.

Other causes of fistula-in-ano are:

- **Inflammatory bowel disease** – Crohn’s disease: Fistulas associated with Crohn’s disease are deep with persistent non-healing tracts. These fistulas are associated with bowel symptoms related to inflammatory bowel disease.

The incidence of fistulas in Crohn’s disease is as high as 17-50%<sup>(5)</sup>.

- **Malignancy**

Carcinoma rectum is associated with perianal fistula formation in locally advanced disease process. However, the incidence is low.

- **Radiation proctitis**

Usually associated with radiation given to the pelvis for genitourinary or rectal malignancy<sup>(6)</sup>.

- **Rectal foreign body**

On insertion of rectal foreign body there is trauma to the mucosa and submucosa. With the presence of a foreign body there is superadded infection leading to a perianal abscess formation and fistula formation<sup>(7)</sup>.

- **Actinomycosis**

Although very rare, there has been evidence of perianal actinomycosis infection in immunocompromised patients resulting in non-healing fistulas<sup>(8,9)</sup>.

# Anatomy of Anal canal

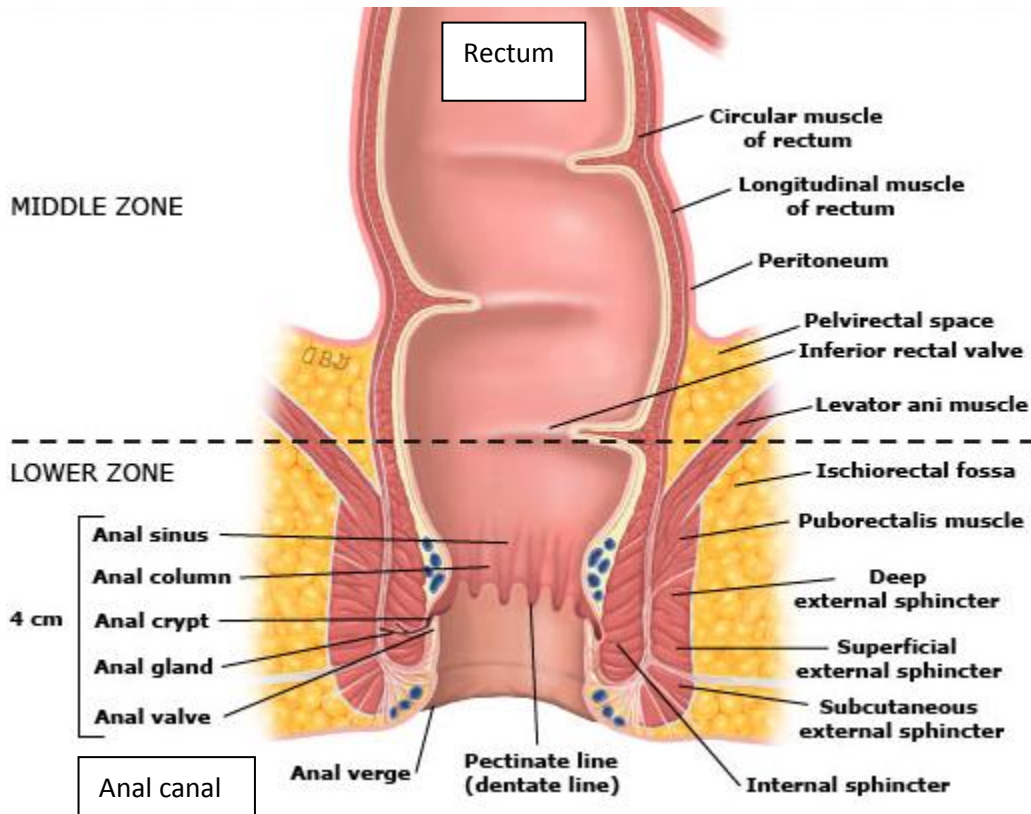


Figure 1: Anatomy of anal canal<sup>(10)</sup>

This is a diagrammatic representation of the anal canal and rectum showing the anatomical details of the external and internal anal sphincter and the location of the anal glands.

## Relevant anatomy

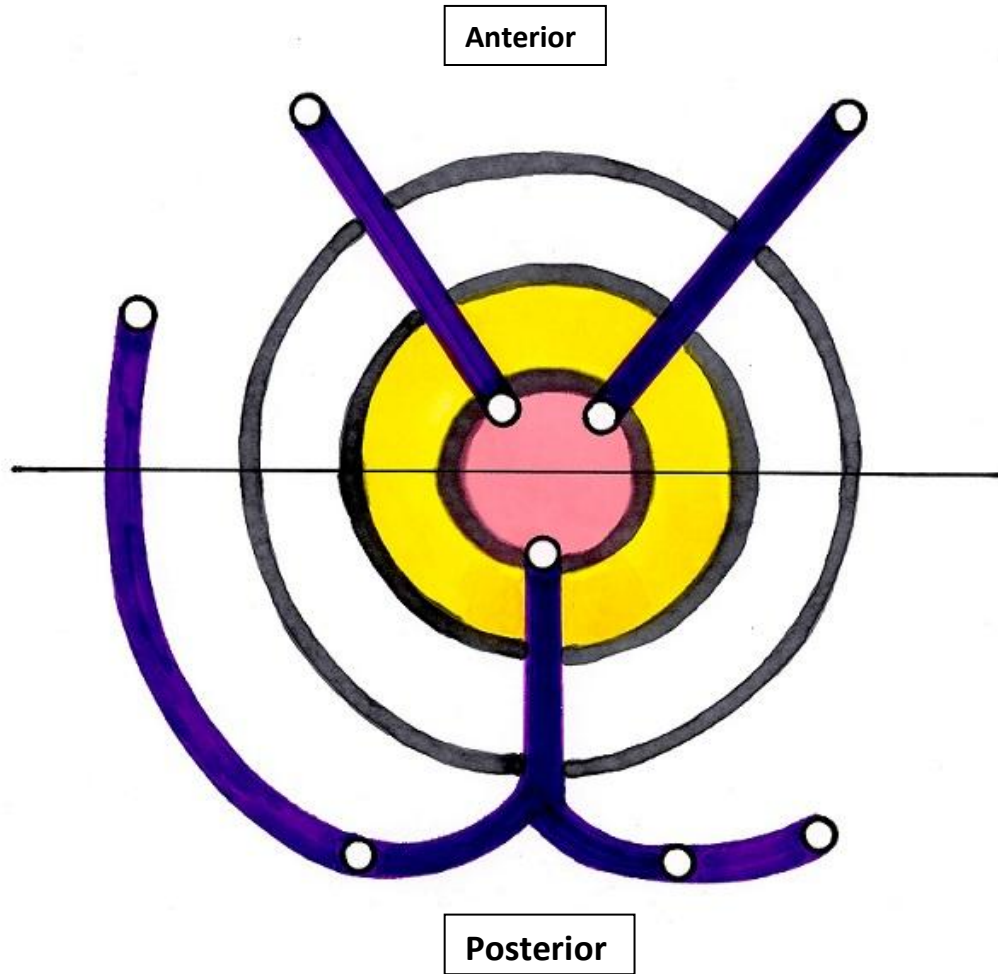
The external anal sphincter is a voluntary muscle belonging to the striated muscle type which has 3 components: submucosal, superficial and deep muscles. The deep component is continuous with the puborectalis muscle and forms the anorectal sling which is very well palpable on digital rectal examination (DRE). The internal anal sphincter is a smooth muscle which lies in the muscularis layer and is an extension of the circular muscles of the rectum.

At around 2.5 cm from the anal verge there are anal valves forming the dentate line and this signifies the union of the proctodaeum and the stomodaeum at this point. Most of the anal glands open in to the crypts of the anal valves. The blockage of these openings and the infection of the glands result in formation of perirectal abscess. After drainage of the abscess it leads to formation of a fistula-in-ano.

### Goodsall's Rule

This rule states that fistulas with external openings lying anterior to a plane passing transversely from 3 o' clock to 9 o' clock position through the centre of the anus follows a straight radial course to the dentate line. The fistulas with external openings posterior to this line usually follows a curved tract to the posterior midline.

The long anterior fistula is an exception to the rule.<sup>(11)</sup>



**Figure 2: Goodsall's rule<sup>(12)</sup>**

### **Pathogenesis**

The formation of fistula-in-ano has been explained by the widely accepted Cryptoglandular theory which is responsible for 90 % of perianal sepsis. There are about 8-10 anal crypt glands arranged circumferentially within the anal canal at the level of the dentate line in the inter-sphincteric plane. The ducts of these glands penetrate the internal sphincter and open at the crypts of the anal valves. The anal crypt glands get obstructed due to inspissated debris leading to infection within these glands and

formation of abscess. This suppuration follows the path of least resistance and drains into the perineal spaces presenting acutely as a perianal abscess. Anorectal abscess is an acute manifestation of this cryptoglandular infection and rupture of the abscess onto the perineal skin lead to formation of a fistula which leads to chronicity.

In 10% of patients, the cause of sepsis varies from inflammatory bowel disease, fungal infection, trauma, neoplasm or tubercular infections. These fistulas are classified as secondary fistulas and are usually complex.

### Park's classification

The Parks, Hardcastle and Gordon classification (known as the Parks Classification) is the most clinically relevant classification for fistula-in-ano. The classification defines four types of presentations of fistula-in-ano resulting from cryptoglandular infections:

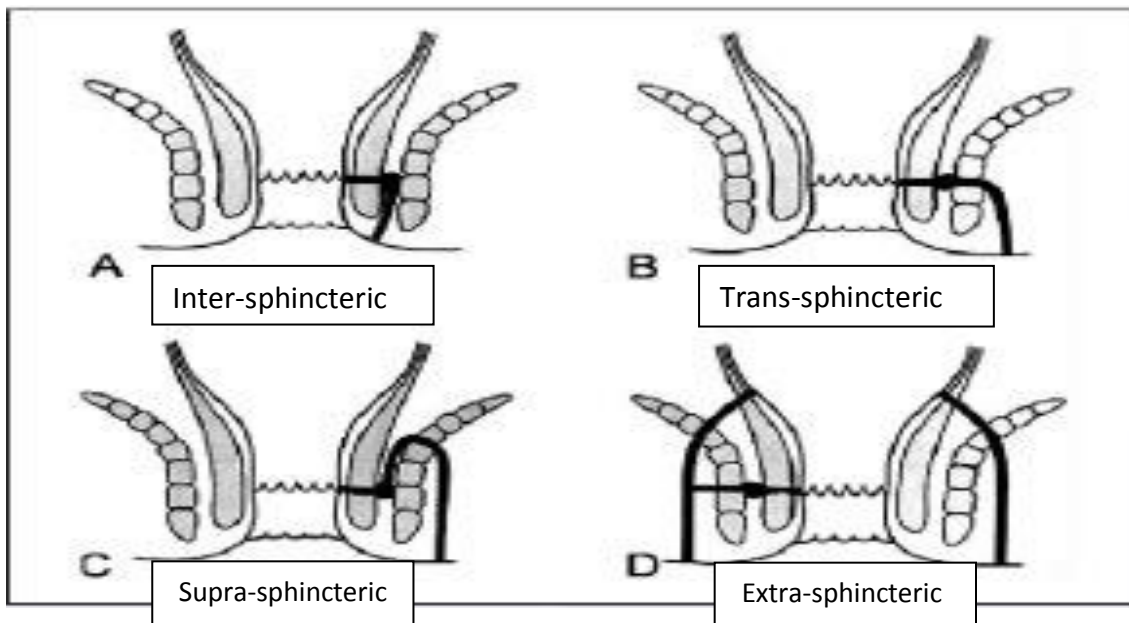


Figure 3: Classification of fistula-in-ano<sup>(13)</sup>



**A – Intersphincteric fistulas:** These fistulas begin at the anal verge and traverse the intersphincteric plane to end in the perianal skin.

- It usually results from a perianal abscess.
- Course: It begins at the dentate line due to blocked anal glands and tracks  
Common course - It begins at the dentate line and traverses the internal anal sphincter to reach the inter-sphincteric plane. It points to the skin by tracking along this and terminates in the perianal skin or perineum.
- Usually is associated with severe perianal pain and swelling.
- Incidence - 70% of anal fistulas.
- Other possible tracts – There may be no perineal opening or it may have a high tract traversing the puborectalis muscles.

**B – Trans-sphincteric fistulas:** These fistulas begin at the anal verge and traverses across both the internal and external anal sphincters.

- It usually results from the tracking of ischiorectal fossa abscesses.
- Course: It originates at the dentate line and traverses across both the internal and external anal sphincters to lead to the ischiorectal fossa and finally on the perianal skin.
- Incidence - 25% of anal fistulas.
- Other possible tracts – High tracts with blind opening may coexist with the main tract.

**C – Suprasphincteric fistulas:** These fistulas begin at the anal verge and traverse above the external and internal anal sphincters to reach the ischioanal fossa

- It usually results from a rupture of supralelevator abscess.
- Course - It originates at the dentate line and traverses the internal anal sphincter to reach the intersphincteric plane. In this plane the tract curves upwards to track superior to the puborectalis muscle and traverses it lateral to the external anal sphincter complex to reach the ischioanal fossa and further till the perianal skin.
- Incidence - 5% percent of anal fistulas
- Other possible tracts – High blind tracts are a possibility.

**D – Extrasphincteric fistulas:** These fistulas are very high fistulas traversing the sphincter apparatus including the levator ani and terminated in the overlying skin.

- It arises from varied etiology ranging from Foreign body penetration of the rectum, Crohn's disease, penetrating injuries of the perineum, pelvic inflammatory disease or carcinoma of rectum.
- Course – It courses from the skin through the ischioanal fossa and tracks further up through the levator muscles till the rectal wall all the while lying outside the sphincter mechanism.
- Incidence - 1% of anal fistulas.

## **Diagnosis**

The diagnosis of fistula-in-ano is mainly clinical. A history of perianal abscess drainage followed by persistent discharge from the perineum is highly suggestive of a fistula. A physical examination is the cornerstone of diagnosis. The entire perineum should be examined by the examiner to look for external opening as an opening or an elevated area of granulation tissue. There may be spontaneous discharge of blood or pus from the external opening which may be apparent on a digital rectal examination.

A proctoscopic examination may show the internal opening. However, many a times it may not be identifiable, if the fistula is not active. It also indicates if the rectum is diseased with an underlying pathology such as Inflammatory bowel disease / Malignancy.

## **Radiological Imaging**

Usually radiological imaging is not required for the diagnosis of fistula-in-ano. However if the diagnosis is not clear or it is a complex fistula radiologic imaging is helpful. Many a times, especially in recurrent fistula-in-ano it is used as a roadmap for planning of surgical procedure. There are several imaging modalities available and the efficacy of each is reviewed.

## **1. Fistulography**

This involves injection of iodinated contrast via the external opening followed by taking radiographic images in antero-posterior, lateral and oblique views to delineate the course of the tract and its opening into the anorectum.

It was used as a diagnostic test in the past but currently is not in use.

Its accuracy rate has been questioned and it ranges from 16-48%(14)

Due to various limitations, fistulography is usually done in cases in which there is a suspicion of fistulous connection between the rectum and urinary bladder or vagina.

## **2. Endoanal / endorectal ultrasonography**

This procedure involves passing a 7 – 10 Mhz ultrasound transducer into the anal canal to delineate the anatomy and differentiating intersphincteric from transsphincteric lesions. A balloon transducer is used to evaluate for extension of the fistula tract above the levator ani.

Addition of hydrogen peroxide has been shown to be beneficial in outlining the fistula tract course and helps in identifying missed internal openings.

This modality has been reported to be 50% more efficacious in comparison to physical examination alone in evaluating the tract and planning for treatment<sup>(15)</sup>.

### **3. CT scan**

Computed tomography scans have not been shown to be very helpful in fistula-in-ano. It is not accurate in delineating soft tissue anatomy. It appears to be more helpful in the setting of inflammatory disease of the rectum as it is good in delineating fluid pockets. It also not popular because of its invasive nature and use of intravenous contrast which is associated with side-effects such as contrast induced nephropathy.

### **4. MRI**

MRI is one of the most sensitive and specific investigation modality to evaluate for fistula-in-ano. It has shown 80-90 % concordance with operative findings.

Dynamic contrast MRI has shown a sensitivity of 97% and a specificity of 100%. Hence it has become the investigation of choice when evaluating for complex, recurrent fistula-in-ano. It also has shown to provide additional information regarding other secondary tracts and procedures which deal with it have shown to reduce recurrence rates<sup>(16)</sup>.

# TREATMENT OF FISTULA-IN-ANO

## SIMPLE FISTULA

Usually simple fistulas are low lying i.e. below the puborectalis sling. The treatment options for low fistula-in-ano include the following:

### (1) Fistulotomy

It involves laying open of fistula tract. A probe is passed from the external to the internal opening and the tract is laid open. In these patients partial division of internal sphincter does not affect the continence mechanism.

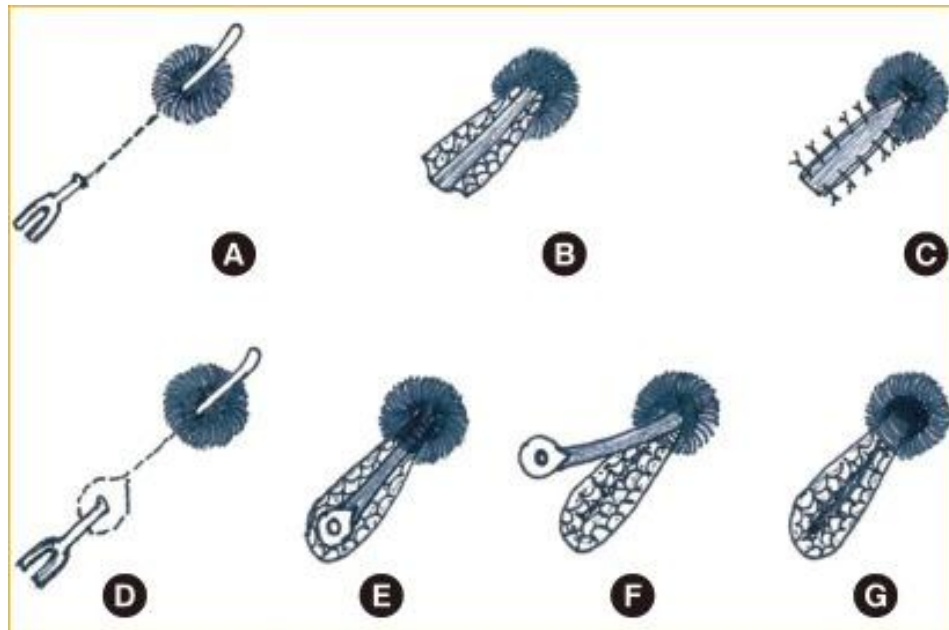


Figure 4: Technique of Fistulotomy ( A – C) and Fistulectomy (D – G)<sup>(14)</sup>

## **(2) Fistulectomy**

In this procedure a probe is passed from the external opening into the internal opening demarcating the entire tract. A key hole skin incision is made over the external opening which is deepened through the subcutaneous tissue and the entire tract is excised from the surrounding tissue. Towards the internal opening the anal musculature is divided. In comparison to fistulotomy, a fistulectomy results in longer wound healing time. The incidence of anal incontinence was almost the same in both procedure as shown in numerous trials<sup>(17)</sup>.

## **(3) Fibrin glue**

Fibrin glue is a combination of fibrinogen and thrombin along with other clotting factors (Ca, Factor XIII and Apoprotein), which on combining lead to formation of a gel like clot which obliterates the tract. It is a non-operative intervention which is associated with low incontinence rates. It is a simple day care procedure which does not require hospitalization and the loss of job hours. Its failure does not complicate further management of the fistula-in-ano. It has been used with good efficacy in primary, single tract fistulas and low lying fistula-in-ano<sup>(18)</sup>.

## **HIGH FISTULA-IN-ANO**

In patients with high fistula the tracts extend above the puborectal sling and hence encase a large mass of anal sphincter complex. The risk of incontinence in these patients following lay open of the fistula tract is very high. Surgical options for these patients include:

### **(1) Seton placement**

Setons are an option for treatment of high trans-sphincteric fistula in patients with very high probability of incontinence following surgery. The materials used as setons are non absorbable material like prolene, penrose drains, rubber bands, vessel loops and silastic catheters(19):

There are two types of setons used for management of fistula-in-ano.

The cutting type of setons incise through tissue while the non-cutting type of setons facilitate drainage of the fistula tract.

Non-cutting setons were mainly used for patients in chronic sepsis and chronic diseases like HIV positive patients. Currently setons are used in combination with fistulotomy as a staged procedure.

In Christian Medical College Hospital, Vellore Infant feeding tubes (# 6 or # 8) is used as setons.



The indications for use of setons are:

- Complex fistula in ano
- Recurrent fistula in ano
- Anterior fistula in female patients
- Patients with Crohn's disease

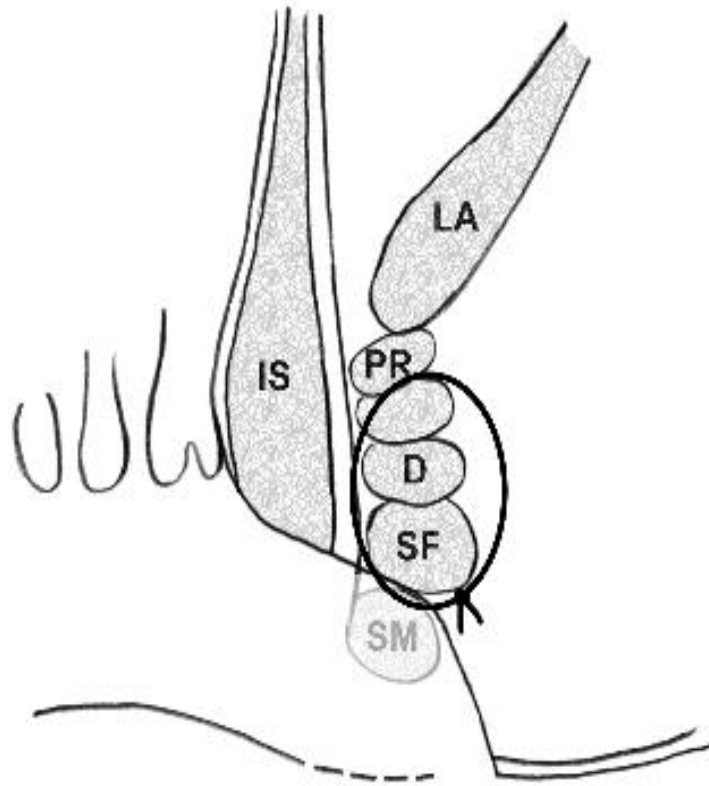
Setons can be used in 2 ways:

(i) Single stage seton:

The seton is tightened across the external and internal opening and is secured with a silk tie. The seton is pulled over 6-8 weeks and results in gradual descent of the seton with fibrosis and healing of the fistula tract.

(ii) Two staged seton:

The seton is passed around the deep portion of the external anal sphincter after opening the skin, subcutaneous tissue, internal anal sphincter and subcutaneous external anal sphincter. The seton is left loose to drain the inter-sphincteric space and promotes fibrosis unlike the cutting seton. Once the superficial wound heals the muscle component encompassed by the seton is divided making sure there is adequate sphincter muscle remaining to not adversely affect continence.



- IS – Internal anal sphincter
- PR – Puborectalis sling
- D – Deep external anal sphincter
- SF – Superficial external anal sphincter
- SM – Submucosal external anal

**High Transsphincteric Fistulotomy with Seton  
(divided muscle is dotted)**

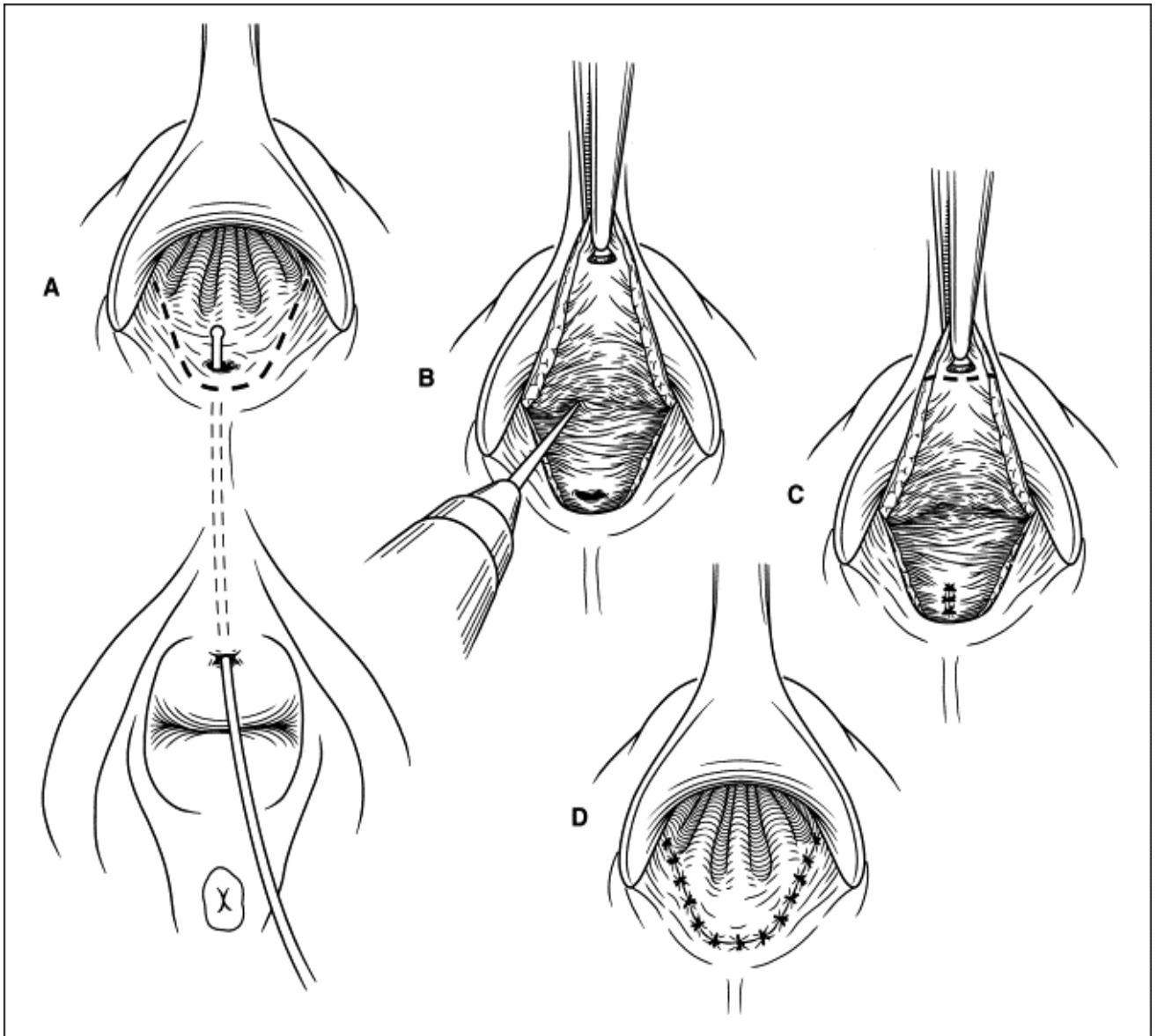
**Figure 5 : Relation of sphincter muscles to seton<sup>(12)</sup>**

## **(2)Mucosal advancement flap**

This technique is used for patients with high fistula in ano. The entire fistula is excised as a fistulectomy and the internal opening is sutured and covered using a mucosal flap from the adjacent mucosa with a base usually twice the apex of the flap. It is also known as the House advancement flap. The mucosal advancement flap is indicated in patients with high fistula-in-ano. The advantages of the procedure include a one stage procedure not requiring repeated hospitalization (as needed when seton is inserted).

The internal opening is sutured using absorbable suture material and the mucosal flap is covered onto the internal opening after raising it as a tongue shaped flap which is sutured over the internal opening using absorbable sutures.

It does not give good results with Crohn's disease or in the setting of acute infection.



**Figure 6 : Technique of Mucosal advancement flap(20)**

A- Passage of seton through the rectovaginal fistula

B- Mucosal flap raised in the rectum

C, D – Sutured mucosal flap after advancement – covering the internal opening.

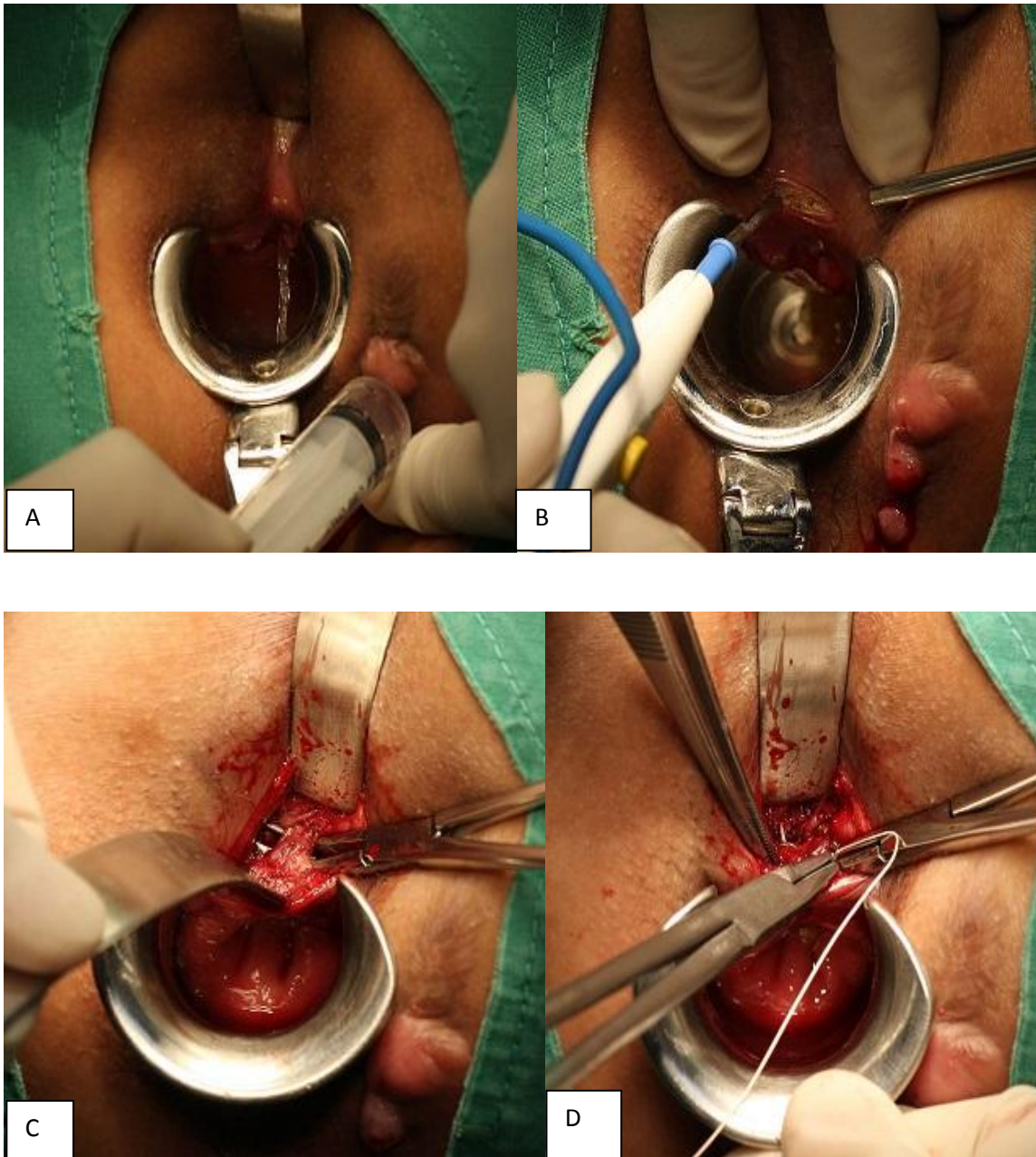
### **(3) LIFT procedure**

Ligation of Intersphincteric Fistula Tract (LIFT) is a technique used to treat high transsphincteric fistula in ano. The intersphincteric tract is identified and isolated. The entire tract from the external opening till the internal opening is excised after ligating it at the internal opening. The external anal sphincter is sutured using absorbable sutures and the fistula tract is excised. This technique has the added advantage of not affecting continence as the external sphincter mechanism is not disturbed in this procedure. The technique was founded by Dr. Arjun Rojanasakul in 2007 and he reported a preliminary healing rate of 97%<sup>(21)</sup>.

A small incision is made at the anal verge after delineation of the tract using fistula probe. The inter-sphincteric plane is dissected and the fistula tract is identified by careful dissection. The tract is then hooked using a right angled clamp and the tract is ligated at the internal anal sphincter. Following this hydrogen peroxide is injected through the external opening to make sure the right tract was identified and divided.

The external opening is then curetted with the remnant of the fistula tract till the inter-sphincteric plane. The inter-sphincteric incision is sutured loosely.

In a systematic review of studies the overall success rate of LIFT procedure ranged from 40 – 95% with a pooled success rate of 71%. It appeared to be an effective sphincter preserving approach for treatment of trans-sphincteric fistula-in-ano<sup>(22)</sup>.



**Figure 7 : Technique of LIFT**

A – Identifying the internal opening

B – Incision around the anal canal

C – Identification of the fistula tract

D – Suture ligation of the fistula tract

Various studies have compared the results of anorectal advancement flap technique to the technique of ligation of inter-sphincteric fistula tract (LIFT) which reveal almost similar efficacy and recurrence rates. The LIFT procedure is associated with more postoperative pain compared to mucosal advancement flap(23):

### **(3) Adhesives**

These are relatively new tissue adhesive materials used as plugs. They are less invasive and carry less postoperative morbidity, e.g. Fibrin. These materials are used to plug the fistula tract. They are associated with very high recurrence rates as the tract is not dealt with and these measures only close the external opening temporarily. They are effective in management of simple fistula-in-ano with low lying tracts but are not as effective in high fistula-in-ano<sup>(18)</sup>.

### **(4) Anal fistula plugs**

These are plugs made from lyophilized porcine small intestine submucosa shaped in a conical fashion. These plugs are inserted into the fistula tract and obliterate the cavity. It has shown mixed results in various trials conducted worldwide and further research is warranted into this field<sup>(24-26)</sup>.

# Newer therapies

## Mesenchymal stromal cells (MSC)

Adult stem cells have been shown to be useful in a number of areas of medical field.

These stem cells can be derived from bone-marrow or adipose tissue – either autologous or heterologous and are considered to be multipotent. These cells replicate as undifferentiated cells and have the ability to differentiate into lineages of mesenchymal tissue including cartilage, fat, tendon, muscles and cartilage stroma. They are termed as Mesenchymal stromal cells (MSC).

Due to their ability to alter both innate and adaptive immune response in individuals as well as their lack of immunogenicity they are considered to be a potent tool in regenerative cell therapy in autogenic as well as allogenic setting.

They have been used in various phase I/II clinical trials with striking results. The most successful example of their use is in Steroid-refractory graft versus host disease. There have been no adverse effects reported after injections of these Mesenchymal stromal cells (MSCs) proving their safety and efficacy in clinical use.

In the setting of inflammatory bowel disease these cells have been administered intraperitoneally<sup>(27)</sup>, intravenously<sup>(28)</sup> or directly into the colon surrounding the mucosal defects<sup>(29)</sup> which has proven to be of benefit in rat experimental models. In



humans intra-fistular injections of adipose derived mesenchymal stromal cells have been safely employed in the setting of Crohn's disease with success. These serial intra-fistular injections have proven efficacy in treating complex perianal fistulas of cryptoglandular origin as well as those associated with Crohn's disease.<sup>(30-33)</sup>

In chronic non-healing fistulas these Mesenchymal stromal cells affect wound healing by 2 mechanisms:

- (1) It can differentiate into fibrogenic precursors and result in fibrosis and healing of the active fistula tract
- (2) It has an anti-inflammatory action as it dampens the systemic inflammatory response

These mesenchymal stromal cells are isolated after extraction from the bone marrow aspirate of the donor after removal of the other cell lines and expanded in vitro. These cells maintain the potential to differentiate into fibrogenic precursors and have the capability to regenerate tissue damage and reduce inflammation and cause fibrosis. These cells are non-immunogenic as has been proved by various trials and can be safely transplanted in recipients as allogenic or autogenic ways.

Here we present a phase II clinical trial that investigates the safety and efficacy of heterologous Mesenchymal Stromal Cells (MSC) as a stem cell based therapy in the treatment of recurrent trans-sphincteric/ supra-sphincteric fistulas of cryptoglandular type. We have preferred the use of bone marrow derived Mesenchymal stromal cells as

their genetic stability has been assessed using conventional methods as well as molecular karyotyping.

# **MATERIALS AND METHODS**

## **MATERIAL AND METHODS**

This was a prospective, non-randomized, interventional study conducted in the Department of General Surgery, Unit IV, at Christian Medical College and Hospital, Vellore from the 1<sup>st</sup> of August 2012 to 1<sup>st</sup> September 2014 after obtaining appropriate approval from the Institutional Review Board.

### **Patient selection**

Patients were chosen for the study as per the following:

### **Inclusion criteria**

Patients included in the study had to meet the following criteria.

- Age above the age of 18 years
- Patients with cryptoglandular fistula-in-ano
- Recurrent fistula-in-ano (Failure of at least 2 surgeries is considered as recurrence)
- Trans-sphincteric / Supra-sphincteric fistulas

## **Exclusion criteria**

Patients with the following presentations were excluded from the study:

- Simple low fistula-in-ano
  
- Fistulae with underlying malignancy / post radiation / IBD.
  
- Complex fistula-in-ano with involvement of bladder, vagina and other viscera.

## **Sample size**

A total of 7 patients were enrolled in this study after obtaining prior informed consent as well as consent for photography from the duration of December 2012 till August 2014.

**ISOLATION OF  
MESENCHYMAL STROMAL  
CELLS (MSC's)**

## Isolation of Mesenchymal stromal cells

Bone marrow was harvested from each patient after informed consent and screening of patients. MSCs were prepared as described below:

The bone marrow (BM) aspirate was harvested under aseptic conditions. After checking the donor information (attached to the bone marrow aspirate container) with the donor information on the request and sampling of the BM aspirate at the Stem Cell Laboratory, sequential activities are performed in a clean room of the Good manufacturing practice facility in CMC leading to a qualified good manufacturing product that is administered to a patient. The product leaves the Good manufacturing practice facility in CMC as a closed system (Cryopreservation Bag) cell suspension to which DMSO-containing cryopreservation mix is added at the Stem Cell Laboratory, where also the actual cryopreservation takes place as well as the preparation for administration after cryopreservation is done.

The following reagents are used for expansion of Mesenchymal stromal cells in the stem cell research centre in Christian Medical College.

<b>REAGENTS FOR EXPANSION OF MESENCHYMAL STEM CELLS</b>		
<b>Reagents / Solutions</b>	<b>Reference</b>	<b>Manufacturer</b>
0.05% Trypsin - EDTA	25300 - 062	GIBCO, Invitrogen Corporation
2 - Mercaptoethanol	M 7522	SIGMA
Human Albumin (20%)	28E-3	Reliance Life sciences Pvt.Ltd, India
Human Albumin (5%)	642001	Biotest Pharma GmbH, Germany
Absolute Alcohol	200-578-6	Hayman Limited
ACD (Anticoagulant Citrate Dextrose)	PB - 1AC500J	TERUMO - PENPOL
Bacillocid	DD-102	RAMAN AND WEIL PVT LTD, INDIA
Dettol	D4612	Reckitt Benckiser, Mysore, India
Dimethyl Sulfoxide (DMSO)	USP060412A	WAK-CHEMIE MEDICAL GMBH
Fetal Bovine serum	SH30070	HyClone, PERBIO SCIENCE
Ficoll-Paque™ PREMIUM	17-5442-02	GE Healthcare Biosciences AB
Hydrocortisone	7904	StemCell Technologies
L-Glutamine	25030 - 081	GIBCO, Invitrogen Corporation

**Table 1: Reagents used by stem cell laboratory**



The following materials were used in expansion of these Mesenchymal Stromal cells (MSC).

<b>MATERIALS AND EQUIPMENTS FOR MESENCHYMAL STEM CELLS EXPANSTION</b>		
<b>Material/Equipment</b>	<b>Reference</b>	<b>Manufacturer</b>
15 ml Graduated, Conical Bottomed, Screw Capped Tubes	91515	TPP @, Europe
50 Graduated, Conical bottomed, Capped, Centrifuge Tubes	91550	TPP @, Europe
75 Sq cm Tissue Culture Flasks (T - 75), vented - BD Falcon™	353135	Beckton Dickinson Labware, NJ, USA
Benjamix (Luer Spike interconnector)	RMC3476	Baxter S.A
Blue cap (cover caps for cell factories)	167652	Nunc A/S, Denmark
Cell strainer BD	352360	BD Biosciences, MA, USA
Cover Glass	2mm square glass	Blue star
Cryocyte Bag	R4R9955	Baxter
Double layered Cell factory (1264 sq.cm surface area)	167695	Nunc
Filter for Cell factories	140050	Nunc
Fogging Machine	MICROMIST™	Sanosil Biotech Pvt Ltd
Funnel for Cell factories	140050	Nalgene Nunc International, NY, USA
GMP suites	...	MSL Steriwear
Inverted phase contrast microscope		Leica
CO2 incubator	Hera-240	Thermo
Laminar Air Flow System	KlenzFlo™	KLENZAIDS BIOCLEAN DEVICES (P), INDIA

**Table 1: Materials used in stem cell centre for isolation of Mesenchymal Stromal Cells**

(MSC)

<b>MATERIALS AND EQUIPMENTS FOR MESENCHYMAL STEM CELLS EXPANSTION</b>		
<b>Material/Equipment</b>	<b>Reference</b>	<b>Manufacturer</b>
Media Filtration Unit - Nalgene	569-0020	Nalgene Nunc International, NY, USA
Micro tubes (1.5 ml)	MCT - 175 - C	AXYGEN SCIENTIFIC INC, CA, USA
Modified Neubauer's Chamber	...	Feinoptick, Germany
Needle – 18 Gauge	MD-10-5652	Nipro Corporation, Osaka, Japan
Needle – 21 Gauge	918	Nissho Corporation, Osaka, Japan
Pipette (100 - 1000 µL)	3111 000.858	Eppendorf, Hamburg, Germany
Pipette (500 - 5000 µL)	3111 000.866	Eppendorf, Hamburg, Germany
Pipette (10 - 100 µL)	3111 000.831	Eppendorf, Hamburg, Germany
Pipette (20 - 200 µL)	3111 000.840	Eppendorf, Hamburg, Germany
Pipette (0.5 - 10 µL)	3111 000.815	Eppendorf, Hamburg, Germany
Pipette Tips (1 mL Maximum)	T – 1000 B	AXYGEN SCIENTIFIC INC, CA, USA
Pipette Tips (10 µL maximum)	T - 400	AXYGEN SCIENTIFIC INC, CA, USA
Pipette Tips (1-200 µL)	T - 200 -Y	AXYGEN SCIENTIFIC INC, CA, USA
Pipette Tips (5 mL Maximum)	9402030	Finntips, Thermo, Finland
Pipette Tips (5 mL Maximum)	0030 000..978	Standard tips, Eppendorf, Hamburg, Germany
Pipette Tips (2.5 mL Maximum)	0030 000.951	Standard tips, Eppendorf, Hamburg, Germany
Serological Pipette ( 10 ml) – GREINER BIO-ONE ® with plug	607180	Greiner Bio-one, Germany
Serological Pipette ( 25 ml) - FALCON ® with plug	357525	Beckton Dickinson Labware, NJ, USA
Serological Pipette ( 5 ml) - GREINER BIO-ONE ® with plug	606180	Greiner Bio-one, Germany
Serological Pipette ( 1 ml) - GREINER BIO-ONE ® with plug	604 181	Greiner Bio-one, Germany
Sterile Gloves	Surgicare 0434	KANAM LATEX INDUSTRIES PRIVATE LTD, INDIA
Syringes (50ml)	300144	BD, Singapore
Syringes (10ml)	Dispovan®	Hindustan Syringes and Medical Devices Ltd, India
Syringes (20ml)	Dispovan®	Hindustan Syringes and Medical Devices Ltd, India
Syringes (5ml)	Dispovan®	Hindustan Syringes and Medical Devices Ltd, India
Syringes (2ml)	Dispovan®	Hindustan Syringes and Medical Devices Ltd, India
Temperature Controlled Bucket Centrifuge	MULTIFUGE 3 s-r	Heraeus
Texwipe (Technicloth II)	TX1112	ITW texwipe Philippines, Inc
Vacuum pump	TID-25-S	Science House Pvt Ltd, Chennai, India

**Table 3: Materials used in isolation of Mesenchymal Stromal cells (MSC).**

## **Sampling and Counting**

The sample and the necessary reagents were placed in the safety box after wiping them with 70% ethanol. The clothes are changed according to Good Manufacturing protocol before entering the clean room. The Laminar air flow safety cabinet was disinfected and the UV lamp was switched on for 15 minutes. All products for use were placed in the laminar air flow safety cabinet. We took 0.5ml of Bone marrow aspirate for cell counting and the net bone marrow volume was determined. The Total white cell count was determined using the Sysmex counter.

### **Preparation of density gradient**

The bone marrow sample was layered over a volume of sterile Ficoll-Paque (2:1 ratio respectively) in a sterile tube and the tube was spun at 400g for 30 minutes with medium acceleration and deceleration. The interface was collected using a sterile pipette into a new tube. This supernatant was washed using sterile phosphate buffered saline and centrifuged for 15 minutes at 700g with slow acceleration / deceleration twice. The pellet then is re-suspended into 10ml of Mesenchymal stromal cell culture medium and 0.1ml of sample was obtained for cell counting and viability assessment using Sysmex and trypan blue.

## **Preparation of culture medium**

The amount of MSC culture medium needed is calculated (12 ml /  $11.25 \times 10^6$  cells) and then prepared using the below mentioned reagents.

### **(1) Fetal Bovine Serum**

Commercially available Fetal Bovine serum (Refer SCRL – 008, section 4.1) is stored at -20°C as 50 ml aliquots in sterile centrifuge tubes and. The aliquots are used after thawing the required volumes.

### **(2) L-Glutamine**

Commercially available L-Glutamine is frozen at -20°C as 5 ml aliquots which are used as per the volume required. This

### **(3) Hydrocortisone**

Hydrocortisone powder (36.3 mg) is dissolved in 400µl absolute alcohol by vortexing at 37°C for 5 minutes. De-ionized water (9.6ml) is added to it. This solution is 10X in concentration and is stored at -20°C.

The working solution is prepared by mixing 1ml of the above stock solution with 9 ml distilled water, which is good for 30 days at 4°C.

#### **(4) 2-Mercaptoethanol**

0.7 ml of 99% 2-mercaptoethanol is made up to 100 ml with de-ionized water. This is filtered under sterile conditions and stored as 0.5 ml aliquots at -20°C

#### **(5) Penicillin/Streptomycin**

Commercially available Penicillin-Streptomycin solution (is stored at -20°C as 0.5 ml aliquots.

All the components required for the preparation of the MSC culture medium that are frozen are thawed by placing them either at 4°C (refrigerator) overnight or at 37°C in a water bath if they are to be used the same day. The above thawed components and Alpha MEM is taken into the CR by placing them in the pass box after cleaning their containers with 70% ethanol.

0.5 ml Penicillin/Streptomycin, 50 ml of Fetal Bovine Serum, 5 ml of L-glutamate (100X), 0.5 ml of 2-mercaptoethanol, 0.5ml of Hydrocortisone and 445 ml Minimal Essential Medium-Alpha is mixed together and filtered and plated to prepare the culture medium.

## **Trypsinisation**

The amount of 0.05% Trypsin/EDTA needed is calculated and is added to the culture medium and incubated in a CO<sub>2</sub> incubator for 5 minutes and is checked under the inverted microscope whether the cells have detached from the bottom of the flasks. Following this a cell count is done using a Neubaur chamber and viability checked using Trypan blue. These cells are then re-plated through 3 passes.

## **Cryopreservation**

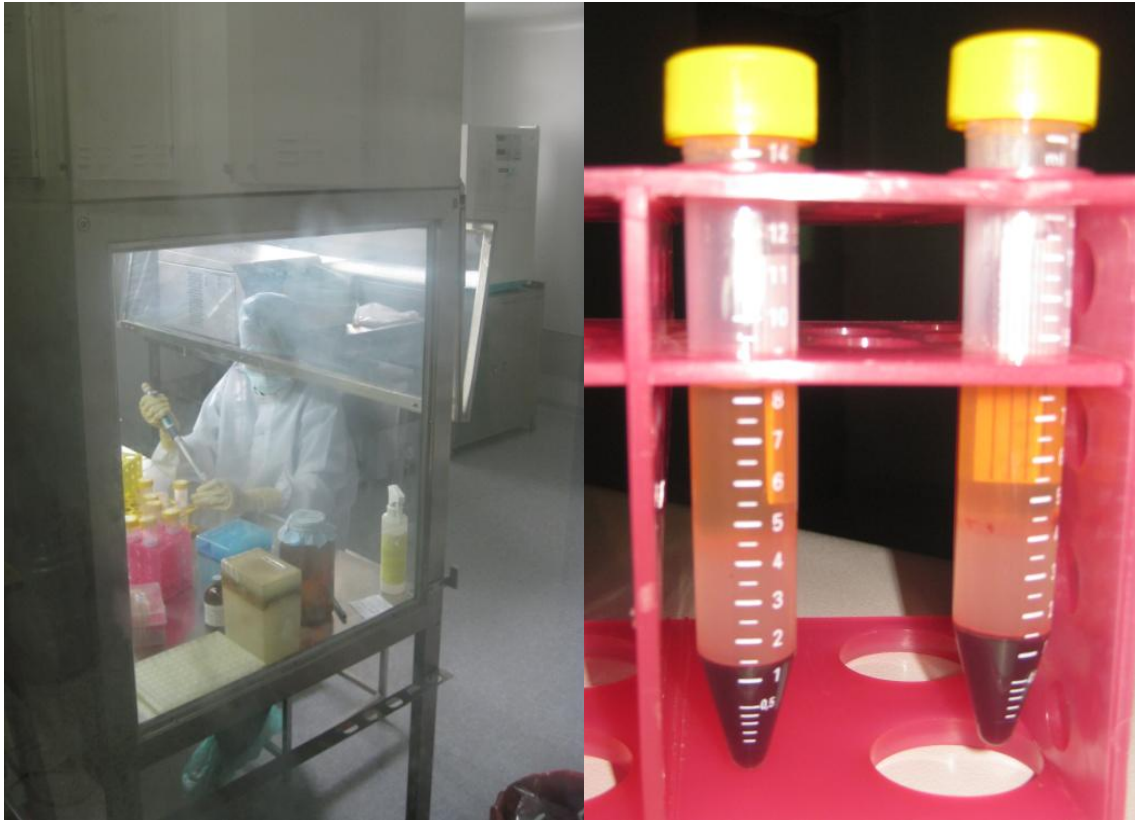
### **Preparation of Cryoprotectant**

The cryoprotectant (Mixture of Dimethyl Sulfoxide, Human Serum Albumin and Plasmalyte A in the ratio 1:1:3 respectively) is prepared by chilling and the amount of Mesenchymal stromal cells are added along with the cryoprotectant in the cryo-bags. These bags are frozen till -90 degrees and are then transferred to storage.

### **Preparation of Mesenchymal stromal cells for administration**

The cells are centrifuged at 700g for 15 minutes with slow acceleration and deceleration after thawing and resuspended in 5% human serum albumin in PlasmalyteA.

The cell suspension is transferred to the transfer bag(s) using syringe and needle after dilution as per required for the study

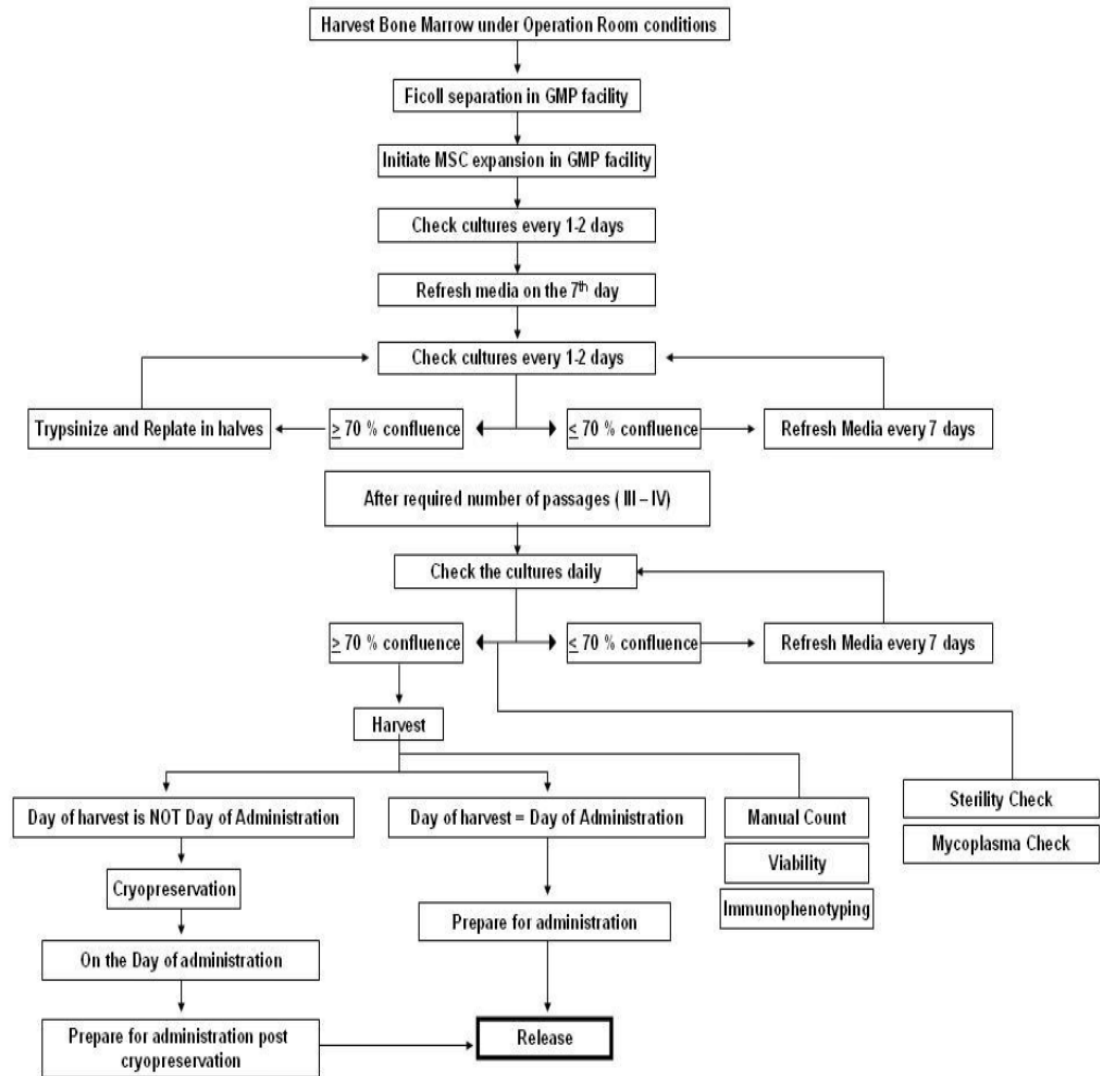


**Figure 8 : Preparation of Mesenchymal Stromal cells (MSC) extract.**

### **Concentration of cells used**

The Mesenchymal stromal cells (MSC) were used in a concentration of 40 million cells diluted in 10 cc of normal saline prepared after thawing the cryoprecipitate.

## The protocol of extraction of Mesenchymal stromal cells



**Table 2 : Schematic representation of protocol of extraction of Mesenchymal Stromal cells (MSC).**



## **Pre-Surgical procedure**

All patients were explained the nature of study and an informed consent was obtained for procedure as well as photography.

All patients underwent MRI scans of the pelvis to look for the extent of the tract and the presence of any collections.

Preoperative blood investigations were done which included

- Hb
- Creatinine
- Blood borne viral screen
- Chest X-ray
- ECG

All patients received preoperative IV antibiotics: Metronidazole and Ciprofloxacin given after induction of anaesthesia and before the commencement of procedure.

## **Injection of stromal cells**

All patients were given the injection of Mesenchymal stromal cells (MSC) under aseptic measures in the operation theatre. The following steps were followed for each case.

- (1) The Fistula tract was identified by locating the external opening and identifying the internal opening.
- (2) Fistula tract was instilled with hydrogen peroxide on a syringe through the external opening and the internal opening was confirmed.
- (3) The tract was gently curetted
- (4) The internal opening was sutured using absorbable sutures (3-0 Vicryl).
- (5) A lumbar puncture needle was used and the Mesenchymal stromal cell (MSC), 40 million diluted into 10ml of normal saline was injected in to the wall of the tract, at the internal opening site and the whole tract outwards.
- (6) The external opening was enlarged
- (7) Gauze and pad used for dressing.

The time between preparation of the inoculums and injection of the Mesenchymal stromal cells was less than 1 hour.

# **CLINICAL PHOTOGRAPHS**

## Clinical photographs depicting methodology

### Step 1

Curettage of the fistula tract.



Figure 9 : Fistula tract being curetted.

## Step 2

### Ligation of the internal opening

The internal opening was identified after injection of hydrogen peroxide from the external opening and visualizing the point of entry of hydrogen peroxide into the anal canal.

The internal opening was suture ligated using 3-0 vicryl.



**Figure 10: Fistula tract internal opening is identified and sutured.**

### Step 3

#### **Injection of Mesenchymal stromal cells into fistula tract.**

The mesenchymal stromal cells in a concentration of 40 Million units diluted in 10 cc of normal saline are injected around the fistula tract after ligation of the internal opening.

Injection of Mesenchymal stromal cells in and around the fistula tract is done using a Lumbar puncture needle loaded onto the 10 cc syringe. The injection is done in a clockwise manner making sure all the walls of the fistula tract are injected with the MSC.



**Figure 11: Injection of MSC into the fistula tract.**

**Procedure of injection of MSC**



**Figure 12 : Procedure of injection of Mesenchymal Stromal Cells (MSC) into the fistula tract**

## **Post-operative care**

The patients were started on normal diet after the procedure. Postoperatively sitz bath was avoided. All patients were discharged the following day if the patients were tolerating orally, pain free and had passed flatus and stools.

## **Histopathological analysis**

All patients had tissue from the fistula tract sent for histopathological analysis.

## **Follow-up schedule**

- Patients were reviewed at 1 week, 1 month, 2 months and 6 months to assess healing of the fistula tract.
- If healing had not occurred in the immediate postoperative period (after 4 weeks) the patient underwent a repeat injection of 40 Million units of Mesenchymal stromal cells (MSC).
- At 2 months review, all patients were assessed clinically and/or by MRI to define the status of the fistulous tract as closed or open.

Patient's who clinically had shown no response to the treatment after 2 months were labeled as failed cases and MRI was not done for these cases. These patients were given the option of surgical repair of the fistula.

A fistula is classified as open or closed according to the Fistula Drainage Assessment:



▶ OPEN FISTULA:

1. Purulent material is expelled with gentle pressure
2. External opening not completely epithelialized
3. Active fistula tract.

▶ CLOSED FISTULA

1. A fistula remains closed for 2 consecutive visits (at least 4 weeks apart), with no discharge or opening.
2. MRI shows fibrosis of the fistula tract with no collection after the end of treatment.
3. The patients are to be followed up till the duration of 1 year either by visit or phone to assess the healing of the fistulous tract.

# **CLINICAL CASES**

## Patient 1



**Figure 13: Clinical photograph before procedure**

Fistula tract – Before injection of Mesenchymal stromal cells

Fistula tract- External opening at 11 o' clock position 3cm from the anal verge and internal opening at 11 o' clock 2 cm from the anal verge.

The tract was radial with pus pointing at the external opening.

## Pre-Procedure MRI

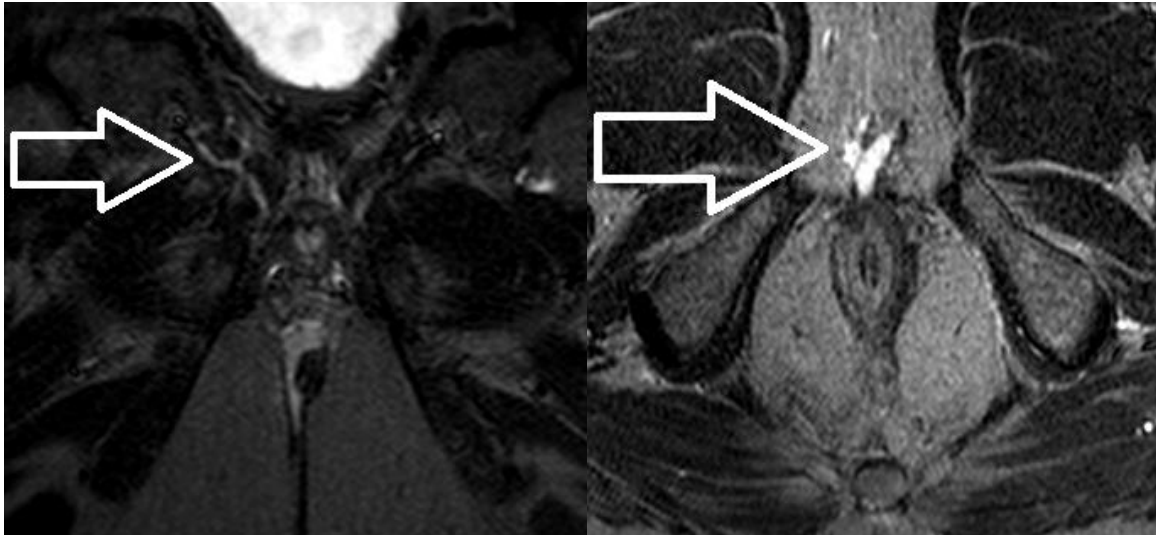


Figure 14; MRI of the fistula tract prior to procedure

Description of the fistula tract
Single tract
Inter-sphincteric
Mid anal canal
No inter-sphincteric, supralelevator, ischiorectal collection
Ext opening- Right anterior quadrant
Internal opening – 12 o’ clock mid anal canal

Table 3: Description of the fistula tract

## Patient 1

After 2 months of follow up



Figure 15; Clinical photo after 2 months.

Clinically- There was no healing of the fistula tract.

## Post-procedure MRI

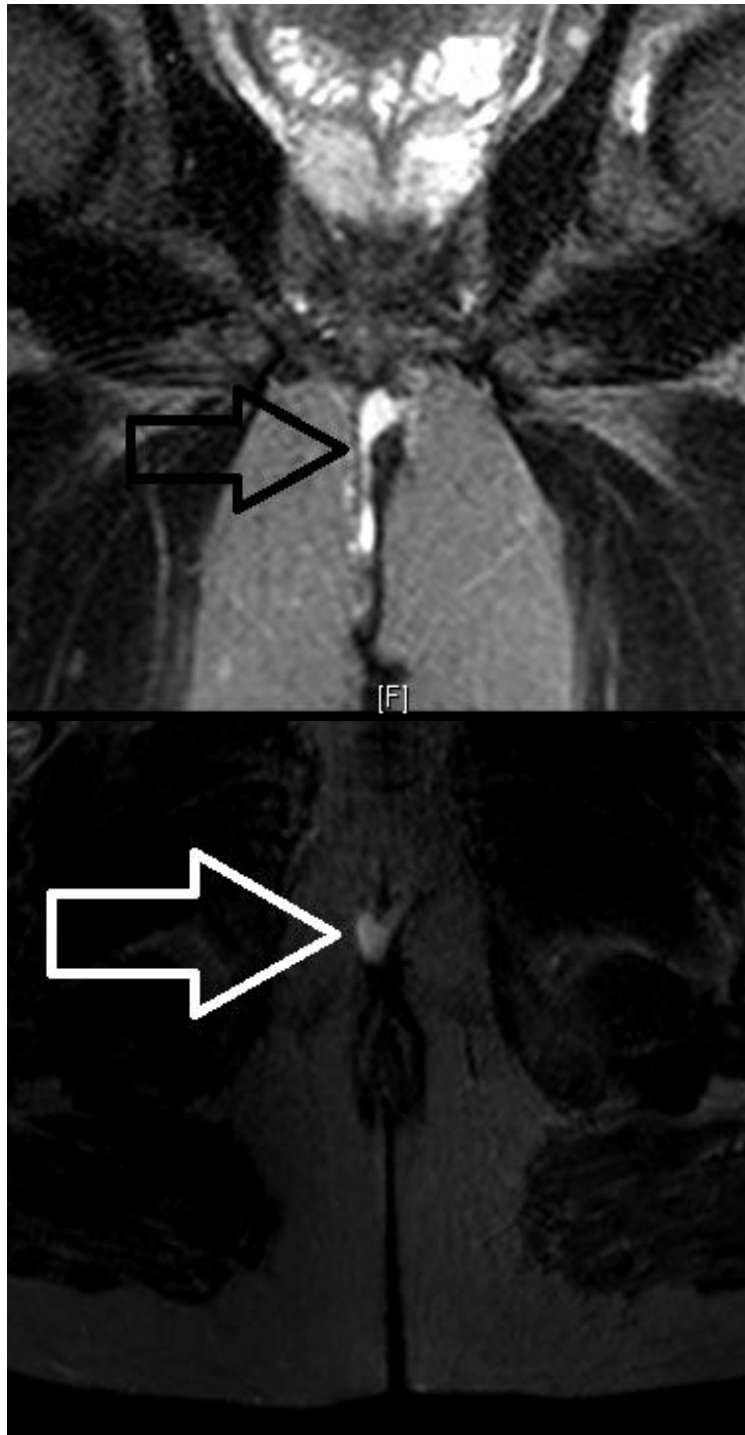


Figure 16 : Post- procedure MRI

**Table 4: Description of the fistula tract**

<b>Description of the fistula tract</b>
Persistence of the fistula
No fibrosis of the fistula tract
T2 hyper intensity – Same as prior to procedure
No new disease present

Patient underwent examination under anaesthesia and laying open of fistula under anaesthesia.

Intra-operative findings:

External opening was present at 11 o' clock 3 cm from the anal verge.

Internal opening was present at 12 o' clock 2 cm from the anal verge.

## Patient 2

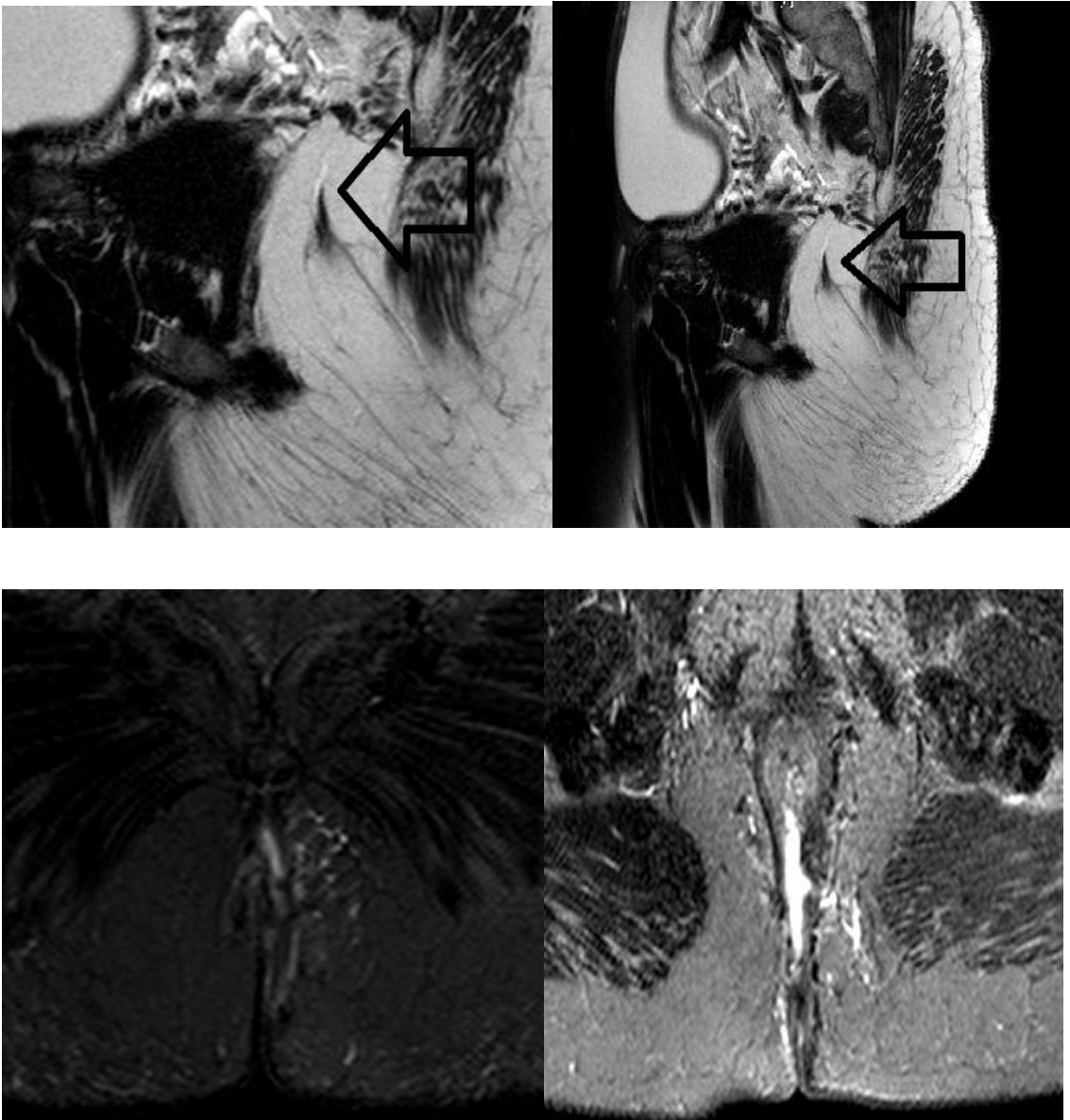


**Figure 17: Fistula tract before the injection of Mesenchymal stromal cells.**

Fistula tract - Scar on the left side of anal verge. Opening at 5 o'clock, 5 cm from the anal verge, on the scar. The tract was trans-sphincteric with no intersphincteric extension, and lined with granulation tissue. Rectal mucosa was normal.



## Pre-Procedure MRI



**Figure 18 : Pre-Procedure MRI**

**MRI coronal and sagittal cuts revealing the fluid filled fistula tract (T1 and T2W images)**

<b>Description of fistula tract on MRI</b>
<p>Single tract</p> <p>Trans-sphincteric fistula-in-ano</p> <p>Mid anal canal</p> <p>No inter-sphincteric collection</p> <p>No Ischiorectal collection</p> <p>No supralelevator collection</p> <p>External opening – Left posterior quadrant</p> <p>Internal opening – 6 o’ clock position mid anal canal.</p>

**Table 5: Description of the fistula tract prior to procedure**

## Patient 2

### After 2 months of follow-up



**Figure 19: Appearance of the fistula tract on 2 months follow-up.**

Fistula in ano with internal opening at 6 o' clock position at the anal verge

External opening was at 5 o' clock position 4 cm from the anal verge.

Discharge was present at internal opening and scar had healed by secondary intention but the fistula tract was patent. As the fistula tract was patent clinically after 2 months- MRI was NOT done. Patient underwent operative procedure.

### Patient 3



**Figure 20: Fistula before the injection of Mesenchymal Stromal Cells (MSC).**

There was a high trans-sphincteric fistulous tract with external opening at 8'o clock position, 5 cm from the anal verge & an internal opening at 6'o clock position at the PR sling with scars of previous surgery present.

## Pre-Procedure MRI

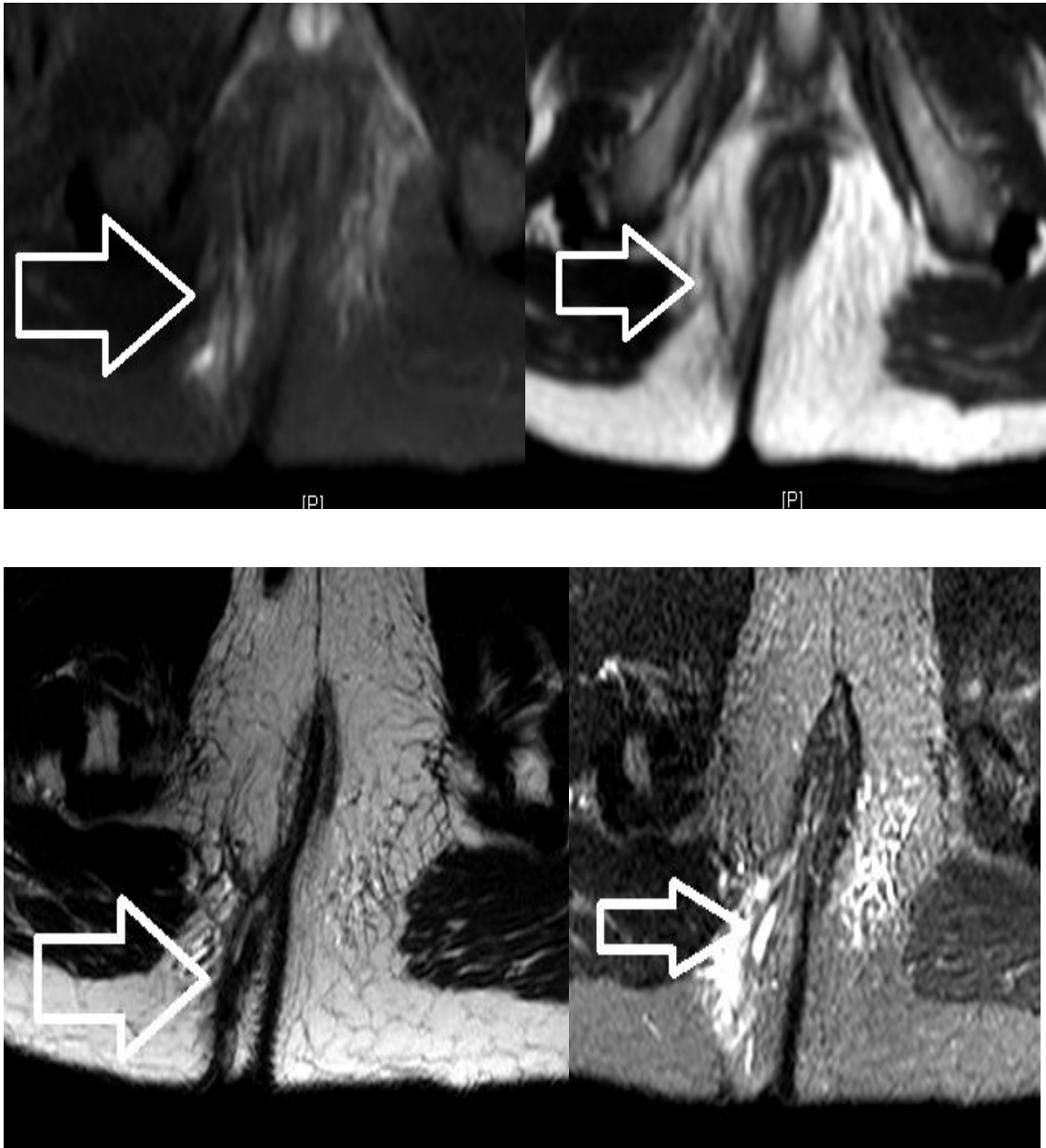


Figure 21: MRI images revealing the active fistula tract.

Description of fistula tract
<p>Single tract</p> <p>Suprasphincteric fistula</p> <p>High anal canal</p> <p>Inter-sphincteric collection present</p> <p>Supra-levator collection present</p> <p>Ischiorectal collection present</p> <p>Ext. opening- Right posterior quadrant</p> <p>Int. opening- 6 o' clock high anal canal</p>

**Table 6: Description of fistula tract prior to procedure.**

**At 1 month**

Patient was detected to have a patent external opening and underwent a repeat injection of Mesenchymal stromal cell injection (40 Million units/mm<sup>3</sup>).

**Intra-operative findings:**

External opening was on the scar close to the anal verge. The internal opening was closed and not evident on probing and hydrogen peroxide infiltration. The tract was filled with granulation and there was a small collection.

## Patient 3

After 2 months of follow-up



**Figure 22: Appearance of fistula tract at 2 months of follow-up.**

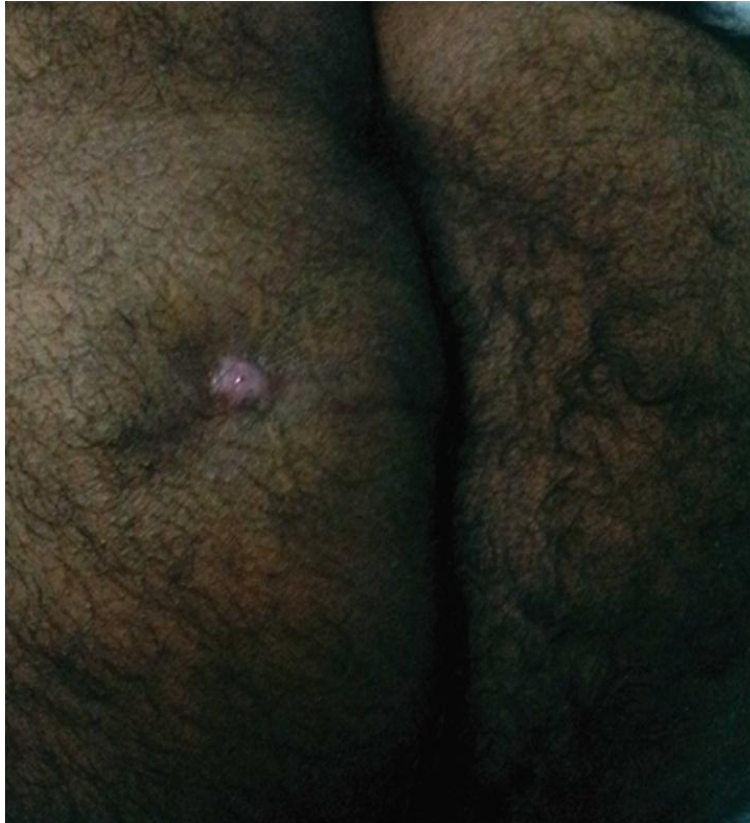
Re-epithelialized external opening with white scab formation over the external opening of the fistula tract.

However after 3 months the external opening was patent with breakdown of epithelialisation. Post-procedure MRI was not done as clinically the tract had recurred.

He underwent examination under anaesthesia and seton insertion.



## Patient 4



**Figure 23: Appearance of the fistula tract prior to procedure**

There was a 5 cm scar present at 9 o' clock position. There was induration and fluctuation in the lateral end of the scar. Internal opening was at 6 o' clock position just beyond the anal verge.

## Pre-Procedure MRI

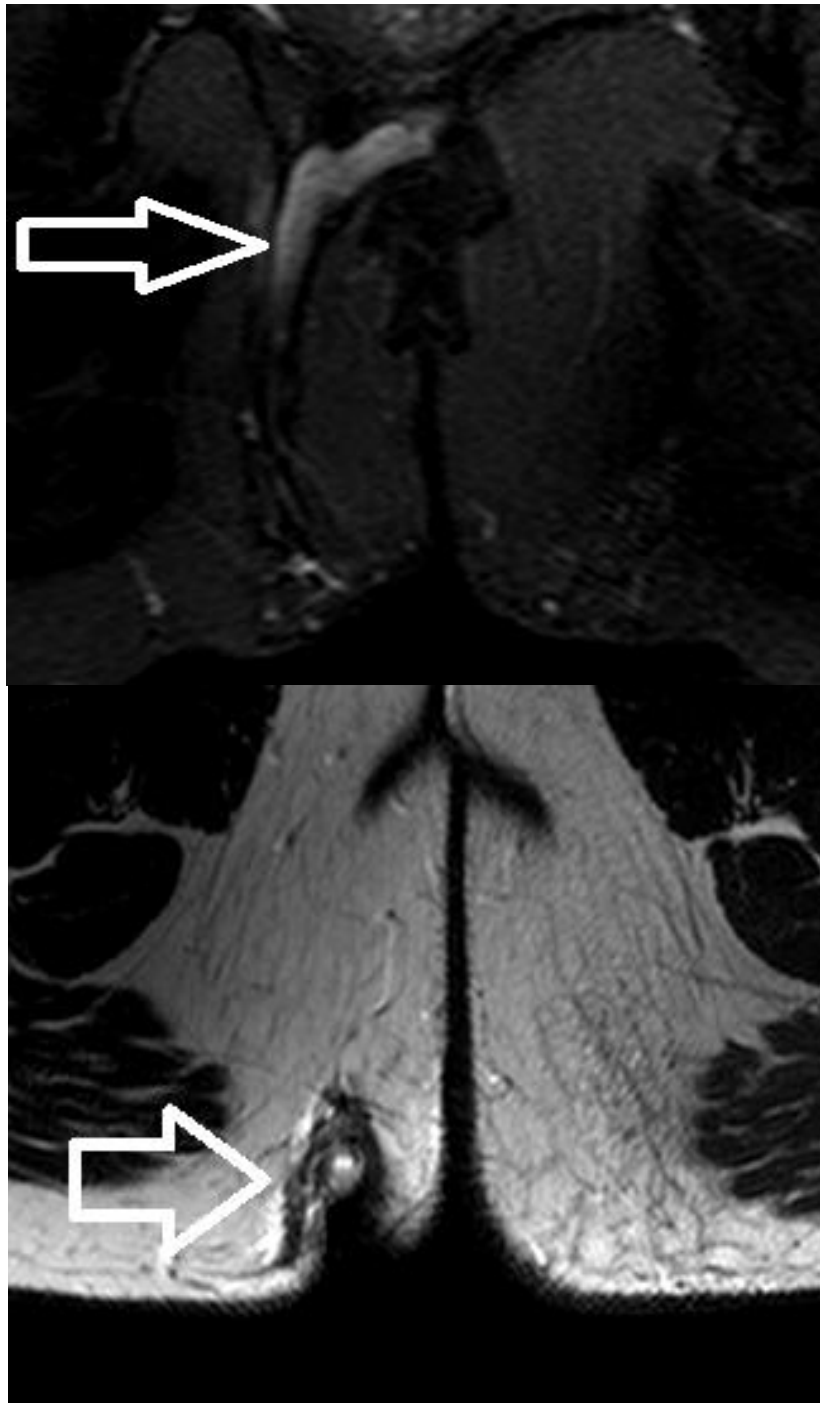


Figure 24: MRI images revealing the active fistula tract

<b>Description of the fistula tract</b>
<p>Single tract</p> <p>Trans-sphincteric fistula</p> <p>High anal fistula</p> <p>No inter-sphincteric collection</p> <p>No supra-levator collection</p> <p>No Ischiorectal collection</p> <p>Ext. opening – Right posterior quad. Internal opening – 6 o’ clock</p>

**Table 7: Description of the fistula tract**

## Patient 4

After 2 months follow-up



**Table 8: Clinical appearance of the fistula tract 2 months following injection of Mesenchymal Stromal Cells (MSC).**

Clinical examination revealed a healed external opening.

## Post-Procedure MRI

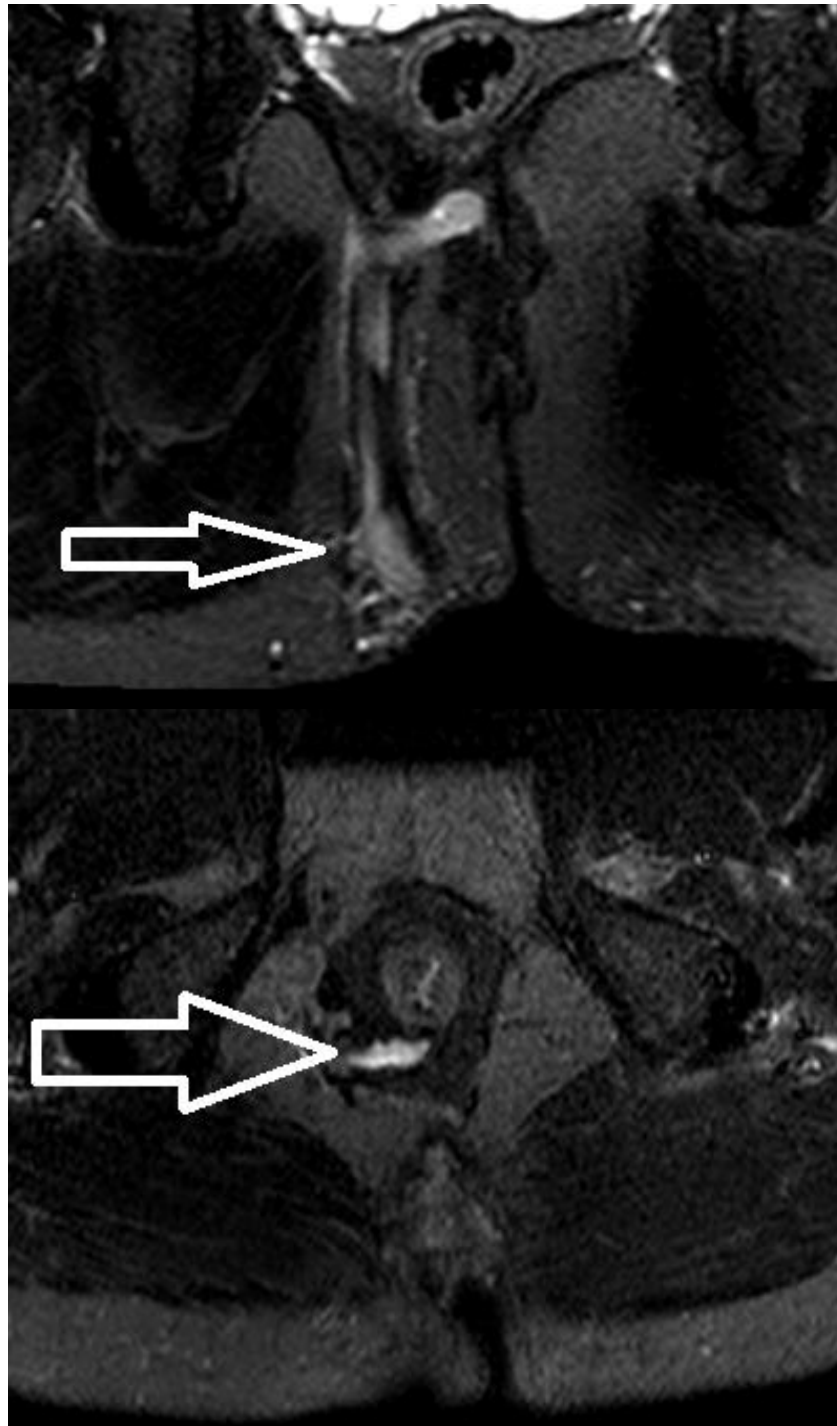


Figure 25: MRI appearance of the fistula tract following procedure.

<b>Description of fistula tract</b>
Persistent fistula after 2 months
No evidence of fibrosis
T-2 Hyperintensity same as prior to procedure
No evidence of new disease

Patient had recurrence of symptoms after 3 months.

He underwent examination under anaesthesia and seton insertion.

Intraoperative findings:

There was an external opening at 7 o' clock position about 6 cm from anal verge.

Internal opening at 6 o' clock position about 1cm from anal verge. There was no collection.

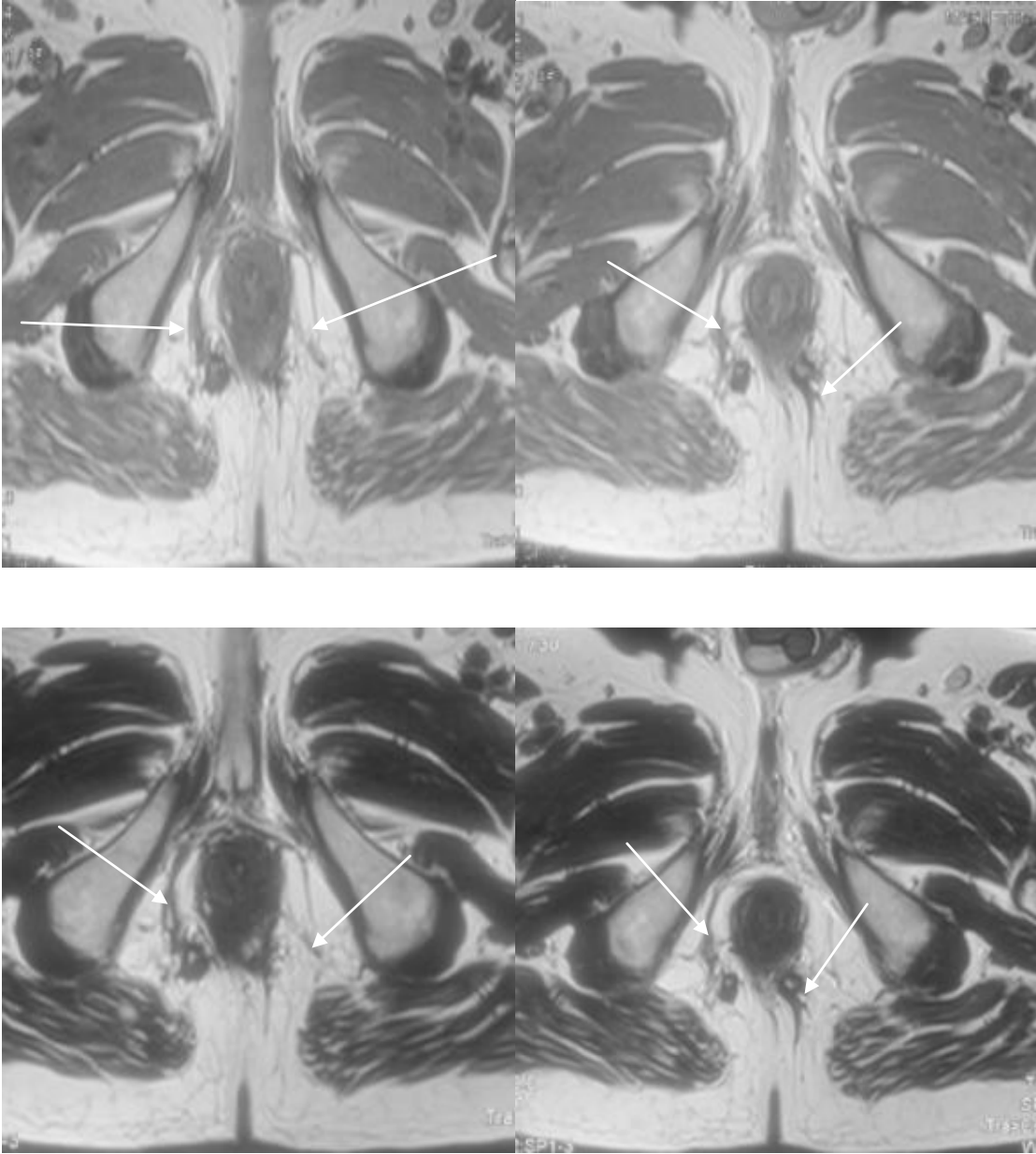
## Patient 5



**Figure 26: Clinical photograph prior to injection of Mesenchymal Stromal Cells (MSC).**

Fistula-in-ano: A fistula in ano was noted with two external openings, one at 4'O' clock, 4cms from the anal verge and another at 9'O'clock, 3 cm from the anal verge. The internal opening was at 6'O' clock position about 2cm from the anal verge. There was presence of serous discharge from the external openings.

## Pre-procedure MRI



**Figure 27: MRI visualizing active fistula tracts.**

This patient was detected to have two tracts as seen in the MRI



## Description of the tracts

First tract – (Right sided)	Second tract – (Left sided)
Trans-sphincteric tract	Trans-sphincteric tract
High anal canal	Mid anal canal
No inter-sphincteric collection	No inter-sphincteric collection
No supra-levator collection	No supra-levator collection
Ischiorectal collection present	No Ischiorectal collection
External opening – Right posterior quadrant	External opening – Left posterior quadrant
Internal opening- 7 o’ clock high anal canal	Internal opening- 4 o’ clock mid anal canal

**Table 9: Description of the fistula tracts prior to injection of Mesenchymal Stromal Cells (MSC).**

This patient underwent Mesenchymal stromal cell injection into both the fistula tracts and was followed up after 1 month and 2 months respectively.

## Patient 5

After 2 months of follow-up

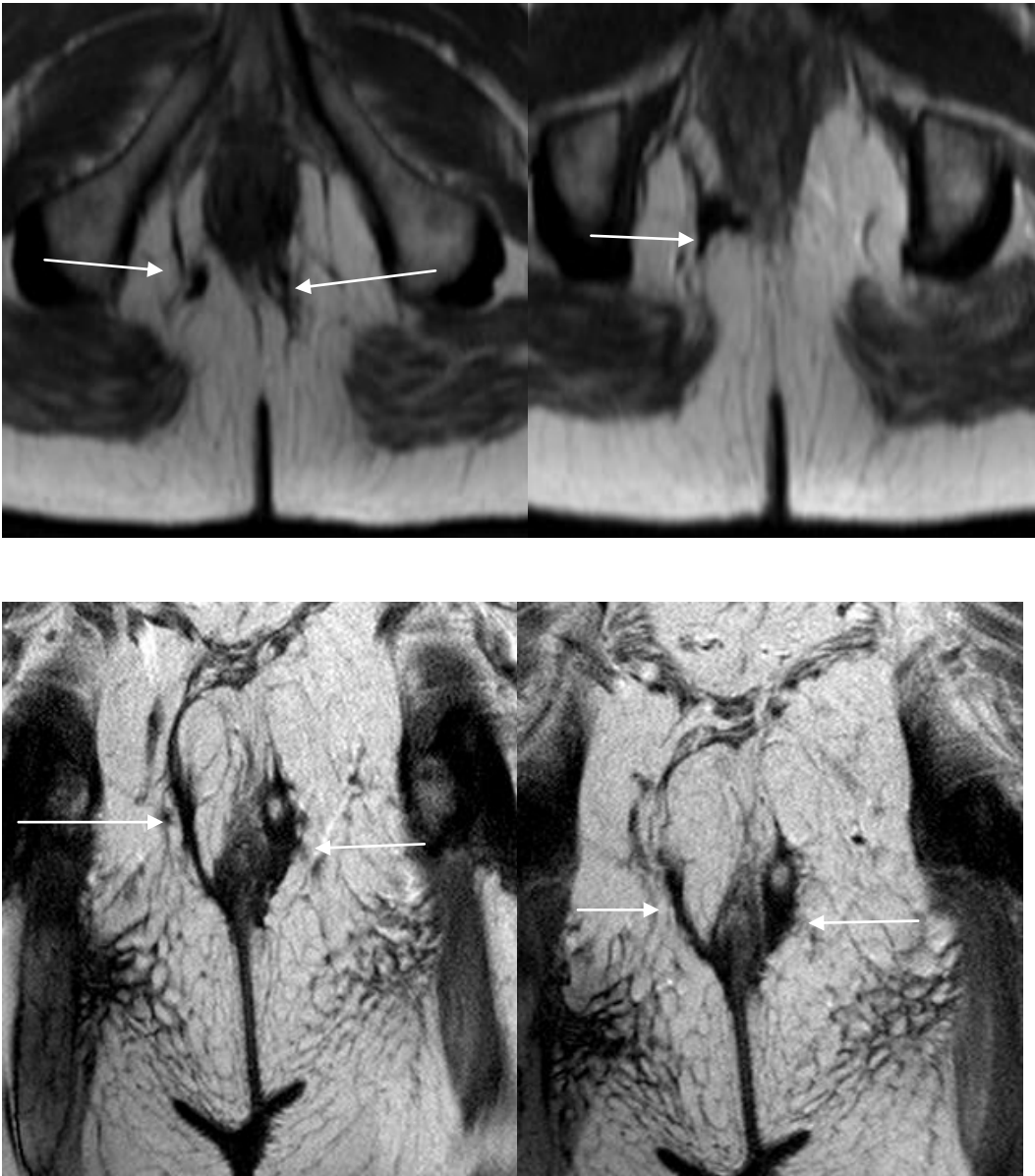


**Figure 28: Clinical photograph on 2 months of follow-up.**

Clinically – Healed external opening at 2 months but on follow-up there was break down of epithelialisation of the external opening at 3 months.

Recurrence of symptoms of perianal discharge at 3 months.

## Post-Procedure MRI



**Figure 29: MRI appearance of fistula tract on 2 months of follow-up.**

In the MRI films the right sided tract shows complete fibrosis with no remnant collection in the right ischioanal fossa- right sided tract healed completely. The left sided tract continued to be patent.

## Post-Procedure comparison of both tracts

Right sided tract	Left sided tract
No persistence of tract	Tract still persistent
Tract fibrosed	No fibrosis
T2 hyper intensity absent	Same as prior to procedure
No new disease	No new disease

**Table 10: Description of the fistula tracts at 2 months of follow-up**

The patient was asymptomatic for 3 months but had recurrence of symptoms and discharge following that.

He underwent Examination under anaesthesia and partial lying open of fistula tract with insertion of seton.

Operative findings were:

External opening at 4 o clock position

High internal opening at 6 o clock with a collection in fistula tract.

The fistula on the right side was completely fibrosed.

## Patient 6



**Figure 30: Clinical photograph of fistula tract prior to injection of Mesenchymal Stromal Cells (MSC).**

Fistula-in-ano: External opening was present at 8 o'clock position 5cm away from anal verge and an internal opening was present at 6 o'clock position 1cm from anal verge. There were no palpable mass felt. Sphincter tone was normal.

## Pre-procedure MRI

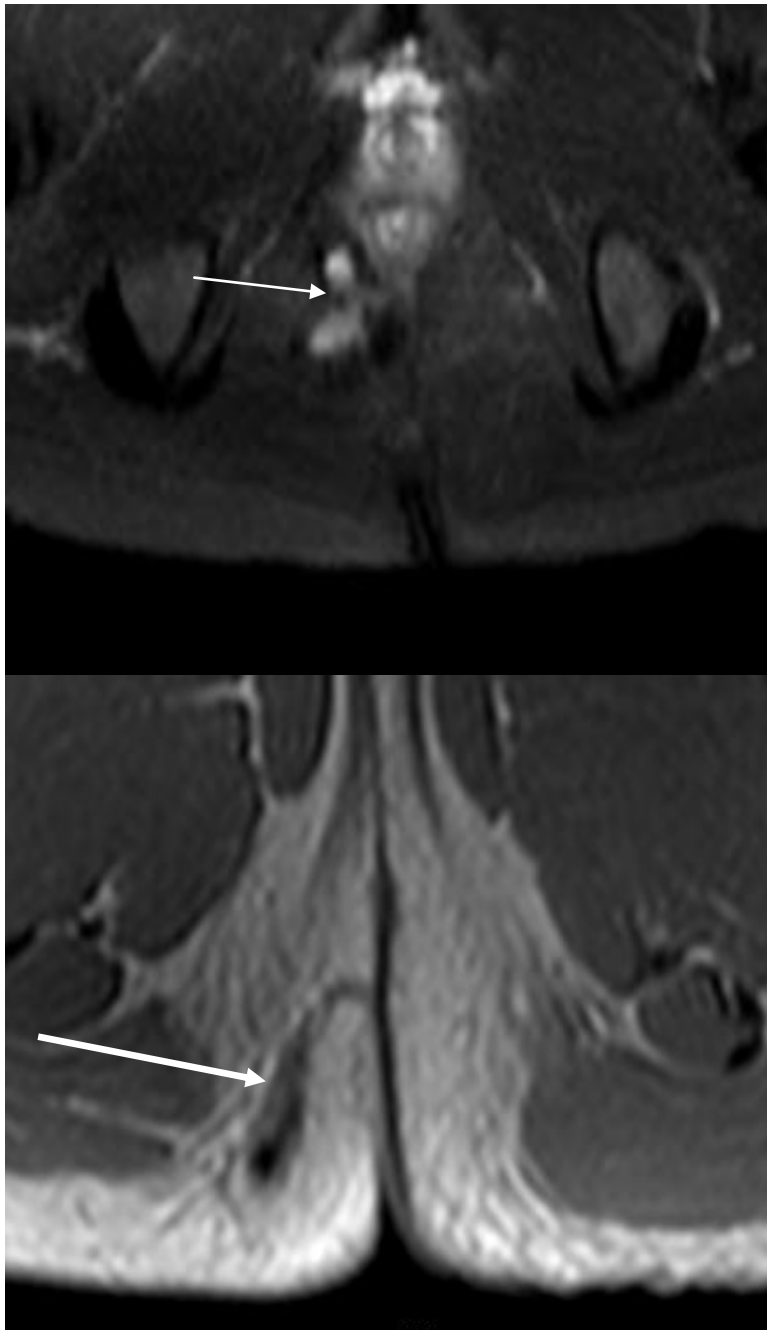


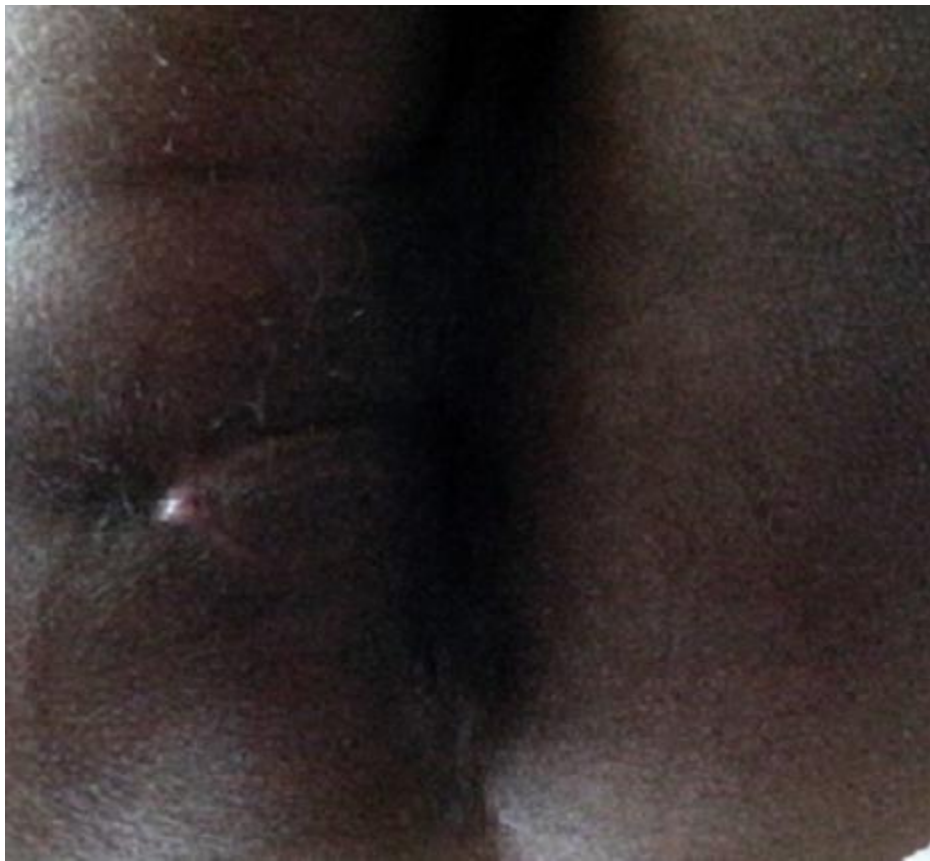
Figure 31: MRI of the fistula tract prior to procedure

<b>Description of the fistula tract</b>
<p>Single tract</p> <p>Supra-sphincteric</p> <p>High anal canal</p> <p>Inter-sphincteric collection present</p> <p>No Ischiorectal collection</p> <p>No Supra-levator collection</p> <p>Ext. opening – Right posterior quadrant;</p> <p>Internal opening 6 o’ clock mid anal canal</p>

**Table 11: Description of the fistula tract**

## Patient 6

After 2 months of follow-up



**Figure 32: Appearance of the fistula tract following procedure.**

After 2 months -- Clinically persistent tract- Internal opening felt as nodule.

External opening patent; Hence MRI was not done.



## Patient 7



**Figure 33: Appearance of the fistula tract prior to procedure**

### **Clinical photograph**

**Fistula-in-ano:** external opening at 4 o' clock position about 5cms from the anal verge and an internal opening at 6 o' clock position about 2 cm from the anal verge.

## Pre-Procedure MRI

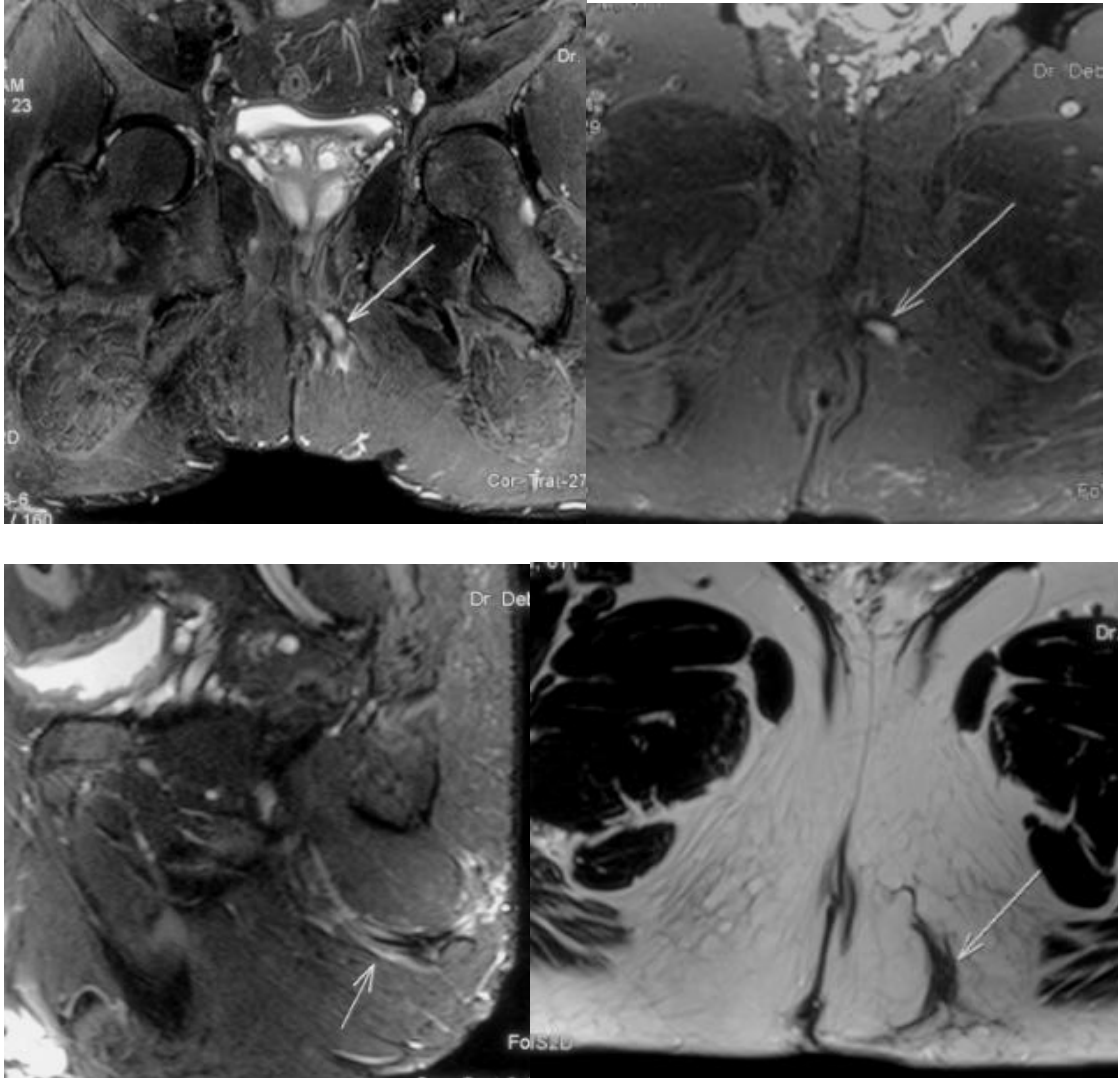


Figure 34: MRI of the fistula tract prior to procedure

## Description of the fistula tract

Trans-sphincteric tract

Mid anal canal

No inter-sphincteric collection/ No ischiorectal collection/ No supralelevator extension

External opening - Left posterior Quadrant;

Internal opening – 12 o' clock mid anal canal.

**Table 12: Description of the fistula tract.**

## Patient 7

After 2 months of follow-up

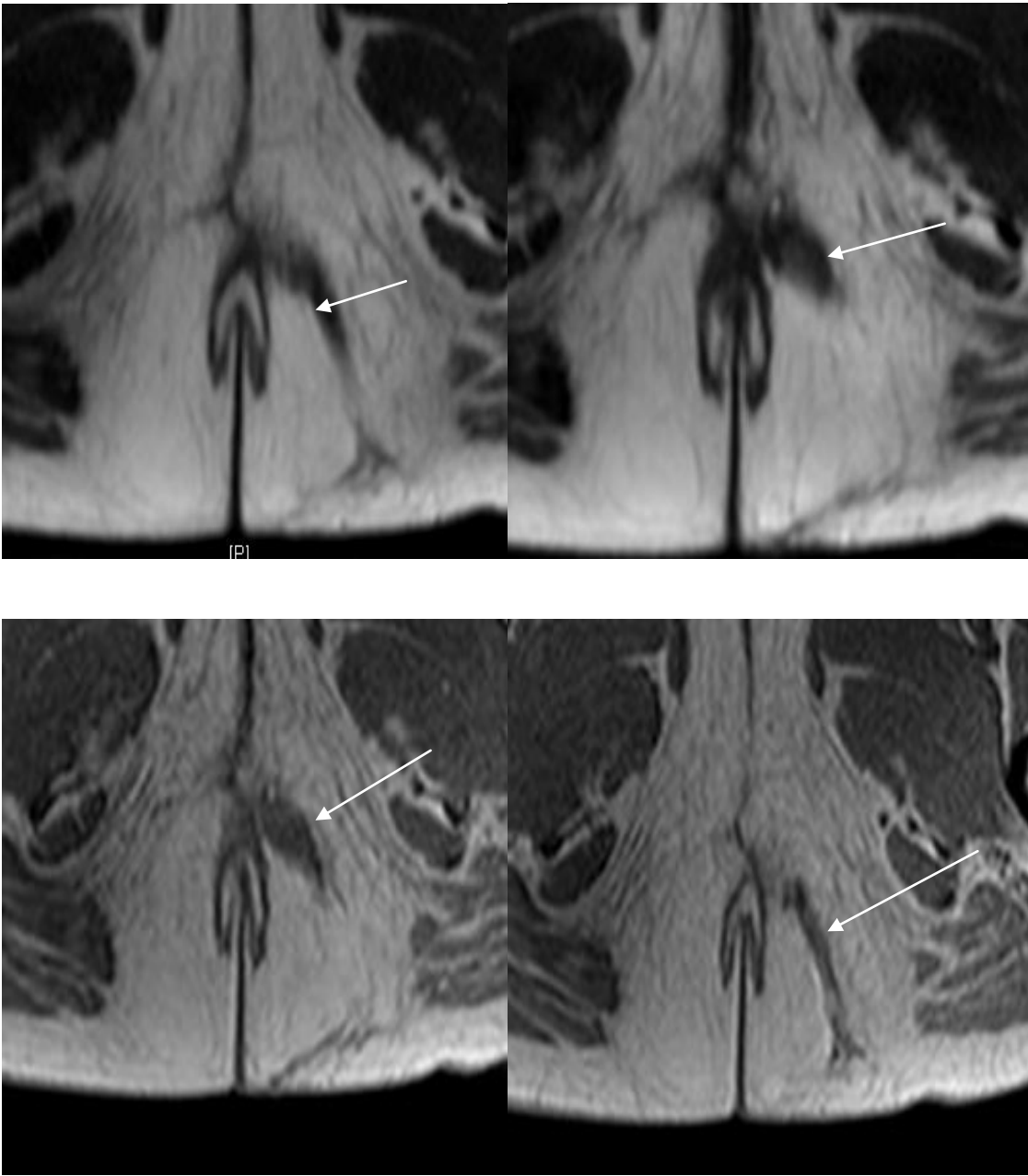


**Figure 35: Appearance of the fistula tract after the procedure**

### **Fistula-in-ano:**

External opening: There was fibrosis of the external opening with no further discharge from the fistula tract. Symptoms of perianal discomfort and discharge did not recur at 6 months of follow-up.

**Post-Procedure MRI**



**Figure 36: MRI of the tract following procedure**

<p style="text-align: center;"><b>Description of the fistula tract</b></p>
<p>Fistula tract – Persistent</p> <p>No evidence of fibrosis</p> <p>T2 hyper intensity – Increased in post-procedure MRI</p> <p>No evidence of new disease</p>

**Table 13: Description of the fistula tract following procedure**

# RESULTS

## Results

A total of seven patients were included in the study and underwent injection of Mesenchymal Stromal cell (Table 15). There were no patients who were excluded and no-one was lost to follow-up. All the seven patients had a diagnosed high cryptoglandular fistula-in-ano clinically as well as radiologically (using MRI). Each patient had undergone a minimum of 2 operations before being included in the study as a non-healing fistula-in-ano. The entire patient group consented for the procedure as well as intraoperative photography.

All patients had a pre-procedure MRI done to document the type of fistula tract and the extent of disease. This would also give us an idea of the extent of healing following the Mesenchymal Stromal Cells (MSC) injections when compared with post-procedure MRI.

Mesenchymal Stromal Cells (MSC) obtained from consenting donors were cryopreserved and required to be thawed prior to injection of the fistula tracts. The timing between the thawing of the Mesenchymal stromal cells and injection into the fistula tracts was less than 1 hour on an average.

Patients underwent a clinical examination under anaesthesia on table followed by injection of Mesenchymal Stromal Cells (MSC) into the fistula tract.

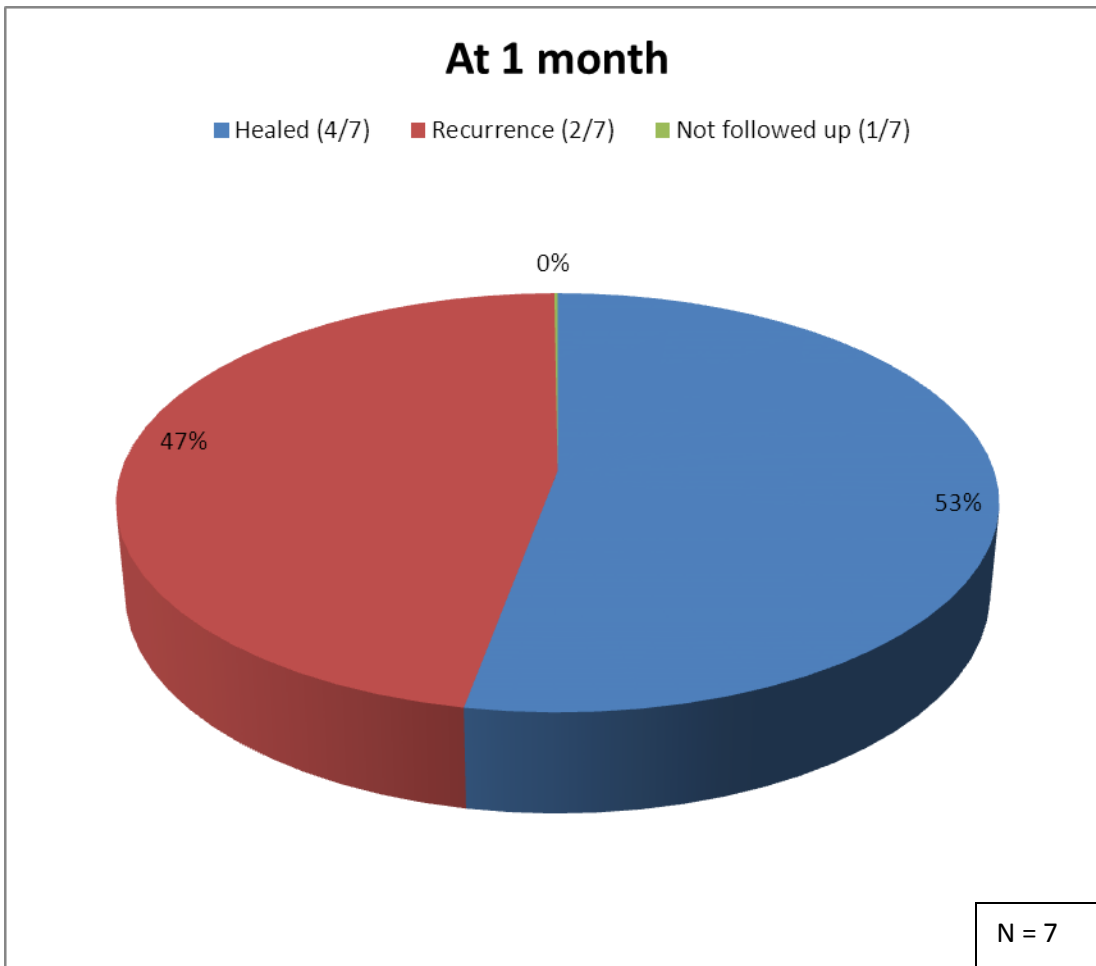


All seven patients were injected with the Mesenchymal stromal cells in a concentration of 40 million/mm<sup>3</sup> diluted in 10 ml of normal saline. All patients were followed up after 1 month to clinically assess for re-epithelialization of the external opening indicating closure of the tract.

In four patients there was healing of the tract clinically with epithelialization of the external opening with no further discharge at the end of one month. These patients were not subjected to repeat injection of Mesenchymal Stromal Cells (MSC) into the fistula tract and were reviewed after another month.

In two patients there was a patent external opening of the fistula with persistent discharge at the end of one month. These patients underwent a repeat injection of Mesenchymal Stromal Cells (MSC) (40 Million/mm<sup>3</sup> diluted in 10cc normal saline) and were asked to review after another month.

One patient did not review after 1 month and hence the need for a second sitting of Mesenchymal Stromal Cell (MSC) injection could not be assessed.



**Figure 37 : Follow-up at the end of 1 month**

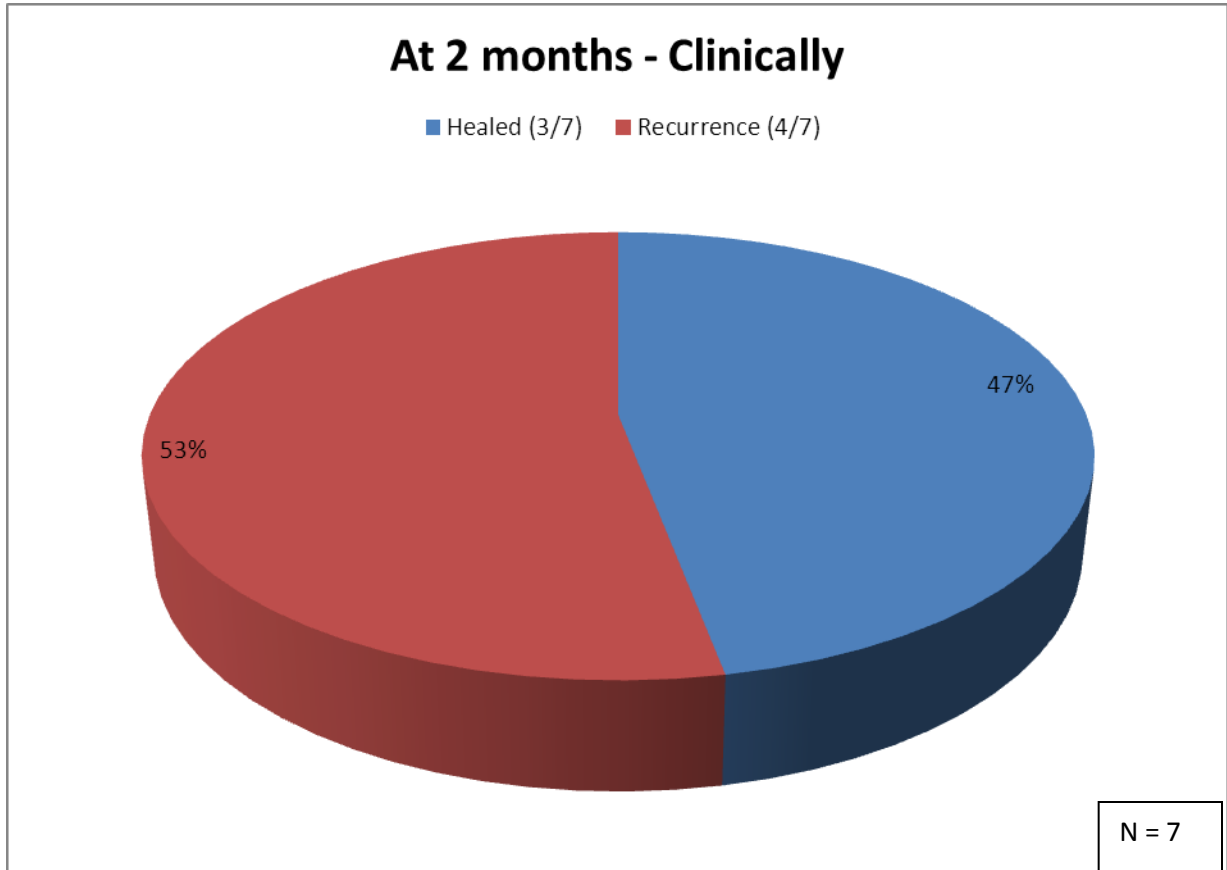
#### **After 2 months**

At the end of two months, three patients continued to have epithelialization of the external opening and no further discharge from the tract.

A total of four patients had persistent discharge from the fistula tract with no healing of the external opening. These patients were diagnosed to have recurrence on the basis of

clinical presentation and a repeat MRI was not done to assess healing of the fistula tract in three of the four patients.

## After 2 months



**Figure 38: Follow-up at the end of 2 months- Clinically**

There were 57% (4 out of 7) cases which appeared to have not healed whereas 43%(3 out of 7) cases still had epithelialisation of the fistula tract.

At 4 months of follow-up only one patient was found to have epithelialization of the external opening and the remaining six patients had a patent external opening with pus discharge.

The patients were diagnosed to have recurrence following the injection of Mesenchymal Stromal Cells (MSC) and underwent operative intervention in the form of seton placement.

A total of four patients underwent MRI scans to visualize the fistula tract following procedure. Of these patients one patient had 2 fistulas prior to procedure but on follow-up there was complete obliteration of one of the fistula tracts while the other tract continued to be patent and thus he was treated as a failure of procedure.

**Table 14:Final outcome**

No. of Patients	At 1 month		At 2 months		At 4 months	
	Healed	Not healed	Healed	Not Healed	Healed	Not Healed
7	4	3	3	4	1	6

**Table 15: Patient details**

Study patients								
Pt	Age	Gender	Type of fistula	Clinical assessment (1month)	Passage	MSC (x 10 <sup>6</sup> )	After 2 months Clinically	After 2 months Radiologically
1	60	M	Cryptoglandular	Healed	1	40	Not Recurred	Recurred
2	55	M	Cryptoglandular	No follow-up	1	40	Recurred	Single tract healed
3	36	M	Cryptoglandular	Not healed	2	80	Recurred	Not done
4	31	M	Cryptoglandular	Healed	1	40	Recurred	Recurred
5	37	M	Cryptoglandular	Not healed	2	80	Recurred	Not done
6	31	M	Cryptoglandular	Healed	1	40	Not Recurred	Not done
7	41	M	Cryptoglandular	Healed	1	40	Not Recurred	Not done

M= Male; Age= Years

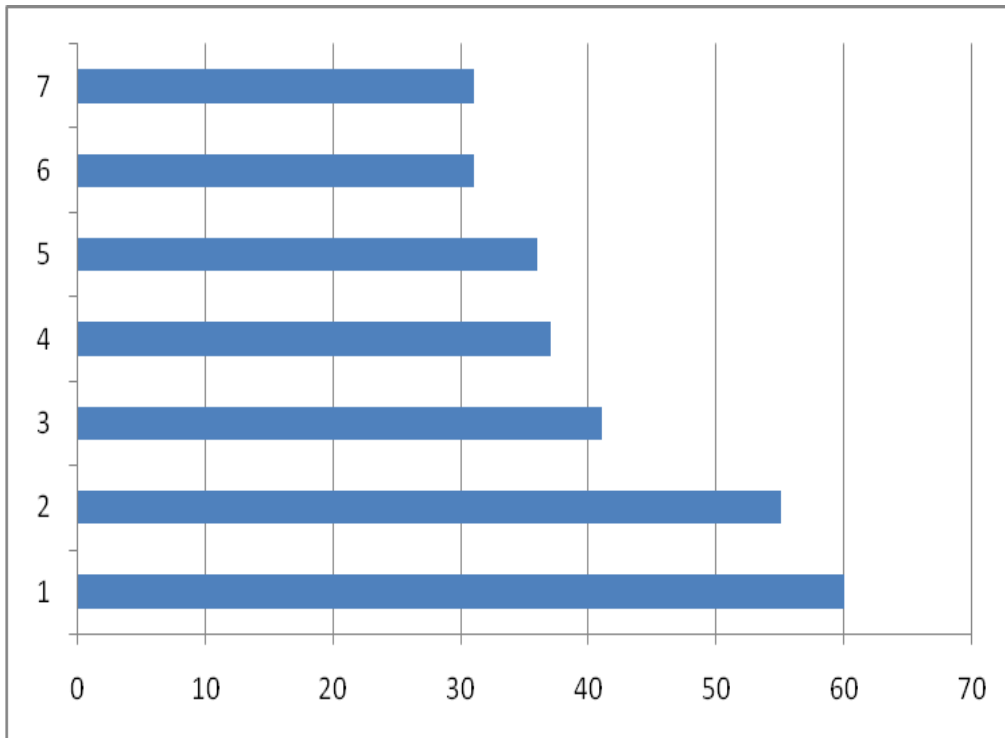
**Table 16: On long term follow-up**

Patient	Follow-up			Regular Surgery
	At 1 month	At 2 months	At 4 months	
1	Healed	Healed	Not recurred	Not Required
2	Healed	Recurred	xxxxxxx	Required
3	Not healed	Recurred	xxxxxxx	Required
4	Healed	Recurred	xxxxxxx	Required
5	Not healed	Recurred	xxxxxxx	Required
6	Healed	Healed	Recurred	Required
7	Healed	Healed	Recurred	Required

All the patients who were operated upon showed no evidence of adverse reactions to the injection of the Mesenchymal stromal cells.

## Data from results

### Age distribution:



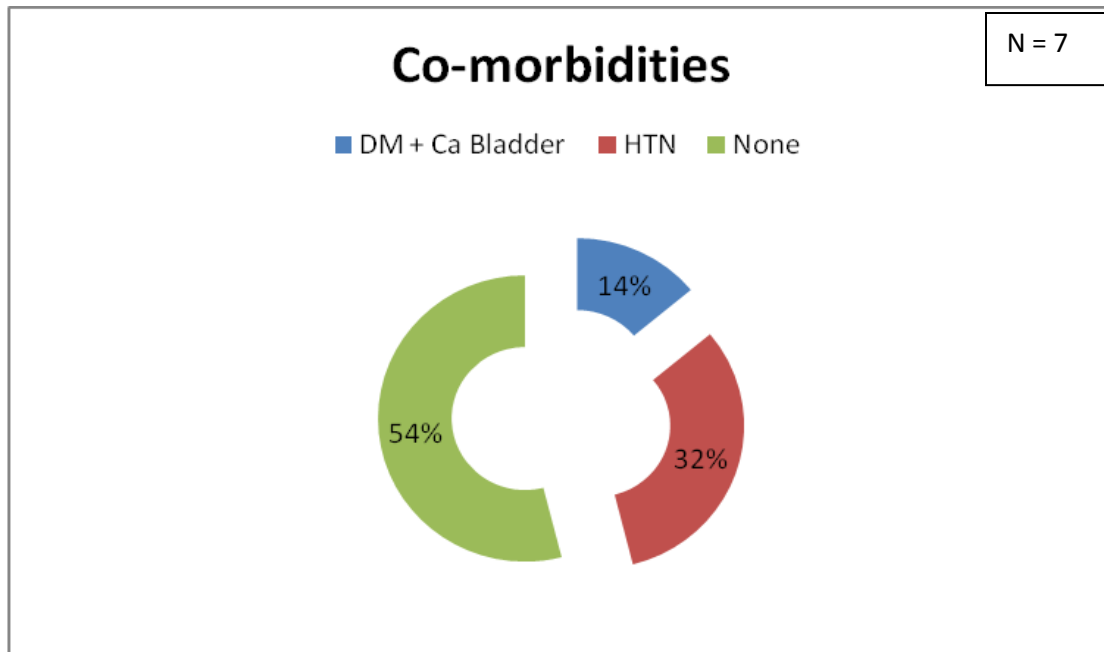
**Figure 39: Age distribution**

**X- axis: Patients**

**Y-axis: Age in years**

The oldest patient was 60 years old and the youngest patient was 31 years old. The mean age group of patients was 41 years.

## Comorbid Illnesses



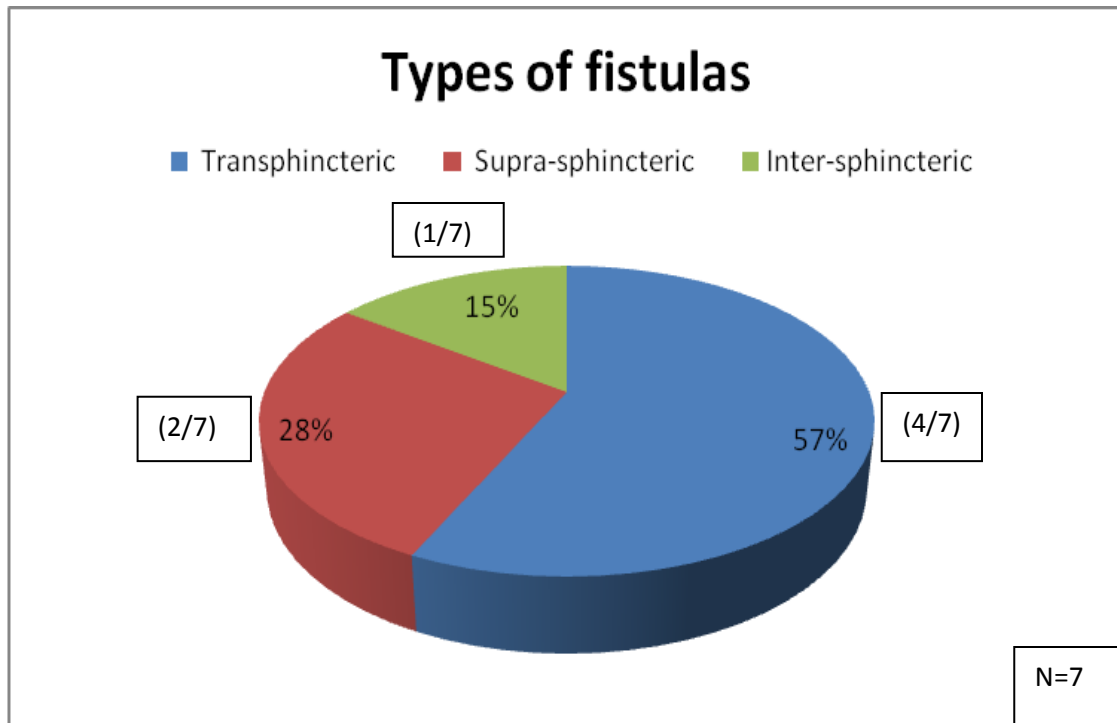
**Figure 40: Co-morbid illnesses of patients included in the study.**

There was one patient diagnosed to have Diabetes Mellitus and Carcinoma of urinary bladder who underwent transurethral resection of bladder tumor.

There were two patients diagnosed to have systemic hypertension and were on medication.



## Types of fistulas



**Figure 41: Types of fistulas**

### On Imaging :

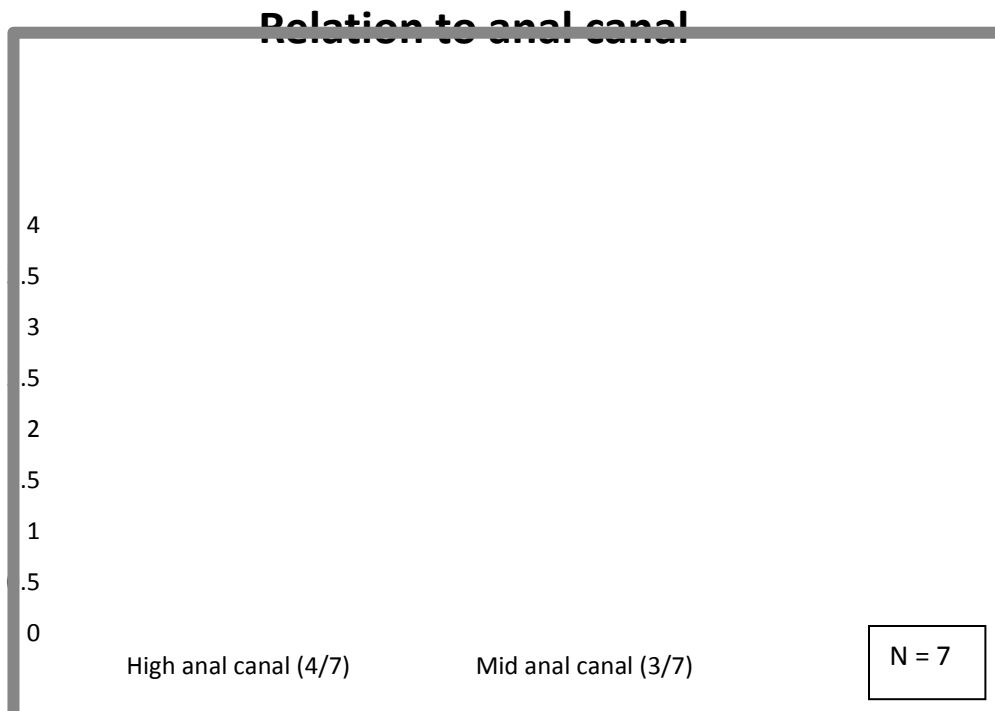
The types of fistulas encountered were:

Trans-sphincteric fistulas – 4

Supra-sphincteric – 2

Inter-sphincteric – 1

**Relation to anal canal (High/Mid)**



**Figure 42: Relation of the fistula to anal canal**

There were a total of seven patients who underwent the procedure of Mesenchymal Stromal Cell (MSC) injection and one patient had 2 separate fistula tracts.

Hence out of a total of 7 fistulas:

- 4 fistulas were opening into high anal canal
- 3 fistulas were opening into mid anal canal

### **Pathological diagnosis**

All patients underwent curettage of the fistula tract which was sent for biopsy.

Histopathology revealed all fistulas to have chronic nonspecific inflammatory granulation tissue.

# DISCUSSION

## Discussion

In various studies done as phase I and phase II trials worldwide the technique of injection of mesenchymal stromal cells have shown promise in the treatment of non-healing fistula-in-ano. Most of the studies have been done in patients with Crohn's disease who had fistula-in-ano which was not amenable to medical or surgical therapy.

Treatment of non-healing fistulas with surgery has been associated with high morbidity of repeated sittings of surgery as well as high recurrence rates. The patient often is in considerable distress due to insertion of a seton into the fistula tract. It affects the activities of daily living causing social handicap. Such patients lose time from activities of daily living due to repeated admissions and repeated procedures.

Mesenchymal Stromal Cells (MSC) aims at reducing repeated hospitalizations and the need for repeated sittings of surgery.

Mesenchymal Stromal Cells (MSC) has been shown to exhibit differentiation ability as well as immune modulatory function. In studies it has been shown that injection of mesenchymal stromal cells is safe and feasible with no side effect profile in the treatment of fistula-in-ano.

We recruited seven patients suffering from non-healing high cryptoglandular fistula-in-ano with failure to respond to conventional therapy (e.g. Surgery) after obtaining informed consent.

At the end of the study it was found that only one patient had complete resolution of symptoms although radiological resolution was not demonstrated. None of the patients had any adverse effects following injection of Mesenchymal Stromal Cells(MSC) and was found to be safe.

Six of these patients underwent examination under anaesthesia and laying open of the fistula tract due to recurrence of symptoms. One patient continues to have resolution of symptoms and is on close follow-up.

In conclusion, the injection of heterologous mesenchymal stromal cells in the treatment of chronic non-healing cryptoglandular fistula-in-ano although appeared to be safe as none of the patients revealed any side-effects of treatment, it did not reveal any benefit in the healing of these fistula tracts. Since the study recruited only seven patients and was designed as a pilot study more research needs to be pursued using Mesenchymal Stromal Cells in a larger patient population.

# LIMITATIONS

## Limitations

1. Most of the patients who were recruited in the study were not from the local area and needed to travel long distances for follow-up which led to financial and logistic restraints.
2. The procedure of procurement of Mesenchymal Stromal Cells (MSC) was done at a site which was a few kilometers away from the operation suite and thus we faced logistical challenges in transportation of the same.
3. There were monetary limitations in processing and procuring these Mesenchymal Stromal Cells (MSC) as well as preoperative and post-operative MRI scans.
4. This study was done as a pilot study on a small patient population and needs to be carried out on a larger scale.



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# INFORMED CONSENT

Christian Medical College, Vellore

Department of General Surgery Unit IV

*A pilot study to determine the feasibility safety and efficacy of injection of heterogenous Mesenchymal Stromal Cells (MSC) in the treatment of recurrent high cryptoglandular fistula-in-ano*

Information sheet

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## Patient Information Sheet.

Your doctor has informed you that you have a recurrent nonhealing fistula in ano which is unlikely to heal in the usual way by itself. He has also told you that there is a new method of treatment being evaluated which involves injecting Mesenchymal stromal cells around the fistulous tracts which is to help in closing of the fistulas. You are invited to take part in this study, but before you do so, it is important for you to understand why this research is being done and what would happen if you are involved. Please read the information below and do not hesitate to ask any questions.

### What is a recurrent fistula?

A recurrent fistula is an abnormal connection of the gastrointestinal tract to the external skin which has failed to respond to current method of management (Medical or Surgical) and is chronically discharging pus.

**What are Mesenchymal Stromal cells?**

These cells are found in various parts of the body such as the bone marrow or placenta. The special characteristics of this cell type is that it can change into virtually any type of cell the body needs. In this study we will be injecting these cells around the fistula tract and assessing the degree of healing.

**What is the purpose of this study?**

In order to see whether the injection of these cells help in the healing of recurrent fistulas which have not responded to medical or surgical therapy.

**Why are you chosen and do you have to take part?**

You have been chosen because you have a recurrent fistula in ano that has not responded to Medical/Surgical treatment. If you decide to take part, you will be asked to sign a consent form. If you do not take part, you will continue with your usual treatment and the care you will receive will not be affected in any way.

**What will happen if I take part?**

Once you sign the consent form you will be booked for examination under anaesthesia. You will be given a date to get admitted in the hospital. You will be examined under anaesthesia. The fistula tract may be photographed for documentation purposes. Haematology will provide the stem cells in vials which will be diluted to 10 cc and injected using a long large bore needle.

You will be required to follow up with us in OPD after 1 week, 1 month, 2 months and 1 year. At the end of 1 month your wound shall be reviewed and if there are no signs of healing you shall be subject to another injection of Mesenchymal Stromal cells around the fistula tract in a manner as mentioned above.

At the end of 2 months you will undergo a clinical as well as a radiological evaluation which includes an MRI scan of the pelvis to assess the fistula.

No further intervention shall be done henceforth.

You may be contacted over the phone or asked to follow up on OPD basis after the end of 1 year.

**What are the side effects of taking part?**

From the available literature it appears that infusion of volunteer bone marrow derived MSC is safe. There can however be occasional mild reactions when the cells are infused (infusional toxicity) which can very rarely be life threatening. It is possible to develop an allergic reaction to some of the components of the infusion.

**What will happen if you develop any study related injury?**

We do not expect any injury to happen to you but if you do develop any side effects or problems due to the study, these will be treated at no cost to you. We are unable to provide any monetary compensation, however.

**Will you have to pay for the treatment?**

The Mesenchymal stromal cells are provided by the Department of Haematology. The follow up MRI scan is being funded for by the fluid grant. Hence the only expenditure you shall incur would be the pre treatment MRI.

**Will my taking part be confidential?**

Yes. If you take part in the study, the doctors involved will look your charts and other documents. This study is coordinated by the Dept of General Surgery Unit IV, Christian Medical College, Vellore. The information about you is stored on a computer and in confidential charts. No one other than the personnel in the study will have access to your records.

**What will happen to the results of the study?**

The data will be analysed. If there is a significant result, then it will change the way in which chronic recurrent fistula in ano are treated.

**Who has approved this study?**

This study has been cleared by the Department of General Surgery, Department of Haematology and the Ethics committee, Christian Medical College, Vellore.

**If you have any further questions, please ask Dr.Zeeshan, Dr. Manbha or Dr. Sukriya Nayak(tel: 0416 2282441/ 09629229239) or email: [surgery4@cmcvellore.ac.in](mailto:surgery4@cmcvellore.ac.in)**

# CONSENT TO TAKE PART IN A CLINICAL TRIAL

**Study Title:** *A pilot study to determine whether direct injection of mesenchymal stromal cells into the fistula tract helps in the healing of the tract.*

**Study Number:**

**Participant's name:**

**Date of Birth / Age (in years):**

I \_\_\_\_\_  
\_\_\_\_\_, son/daughter of \_\_\_\_\_

(Please tick boxes)

Declare that I have read the information sheet provide to me regarding this study and have clarified any doubts that I had. [ ]

I also understand that my participation in this study is entirely voluntary and that I am free to withdraw permission to continue to participate at any time without affecting my usual treatment or my legal rights [ ]

I understand that mesenchymal cells derived from another human being after being processed will be injected in me and I give my consent for the same [ ]

I understand that the study staff and institutional ethics committee members will not need my permission to look at my health records even if I withdraw from the trial. I agree to this access [ ]

I understand that my identity will not be revealed in any information released to third parties or published [ ]

I voluntarily agree to take part in this study [ ]



Signature:

Name:

Date:

Place:

Witness Signature:

Name:

Date:

Place:

Investigator's Signature:

Name:

Date:

Place:

## **CONSENT FOR PHOTOGRAPHY**

I have been explained regarding my diagnosis of recurrent fistula in ano and have willingly enrolled in this study of use of heterologous Mesenchymal Stromal Cells(MSC) serial injection into the fistula tract.

I understand the need of pre procedure as well as post procedure photographs of the fistulous tract as well as the need for documentation of the procedure of injection of the stromal cells into the fistula tract.

I have been explained that these photographs may be used in future publications with my identity fully protected and I hereby give my consent for the same.

Patient signature

Doctor's signature