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HOW I DO IT

# A Step-By-Step Surgical Protocol for the Treatment of Perianal Fistula with Adipose-Derived Mesenchymal Stem Cells

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## Abstract

Anal fistula is a challenging condition both for surgeons and patients. Recurrent fistula, Crohn's disease, or autoimmune disorders add further complexity to this situation. Numerous clinical trials have now demonstrated that cell-based therapy appears to be a good complement to fistulous surgery. As in any new treatment, especially that involving living cells, appropriate application is paramount to achieve optimal outcomes. As stem cell-based treatments are gaining a strong foothold in fistula management worldwide, we herein aim to share our mesenchymal stem cell surgical protocol. With the goal of optimizing results of this emerging therapy, we have improved and refined our protocol over the past 17 years of working with stem cells in clinical trials. The protocol consists of nine reproducible steps for mesenchymal stem cell application inside the fistulous tract, and has proven to be safe and effective in several studies, including international phase III clinical trials.

**Keywords** Mesenchymal stem cells · Perianal fistula · Surgical protocol

## Introduction

Once bleeding, infection, and pain control have been achieved in a perianal fistula tract, surgical options come into play in order to maximize treatment efficacy. Options for surgical techniques to treat perianal fistula, seen as a sequence of actions performed by the surgeon with or without specific instruments, have been maximized, and the complication rates, including incontinence, for a number of procedures remains surprisingly high.[1,2] Thus, the field welcomed a novel therapeutic approach of using cell-based therapy to enhance tissue regenerative capacity, thus improving the surgical results.

During the past decade, cell therapy-based treatment approaches have emerged for the management of several digestive tract diseases, especially perianal fistula.[3] Most of these use allogeneic or autologous mesenchymal stem cells (MSCs) harvested from bone marrow or adipose tissue (adipose-derived stem cells, ASCs). Although the results from preclinical investigation and early clinical trials are promising, only one treatment has successfully completed all the three phases of a clinical trial: the treatment of complex perianal fistula using ASCs.[4,5]

Unfortunately, there are no clear surgical guidelines for stem-cell therapy application. There are two main stem cell administration methods: (1) systemic (mainly intravenous injection) and (2) local (injection or scaffold mediated). The systemic route could be a reasonable option for systemic diseases, except pulmonary trapping limits their use.[6] However, for most localized digestive tract diseases, local application and delivery seems more logical because side effects can be minimized and cells are kept in direct contact with the at-risk tissue. Furthermore, there is no consensus on how to inject the

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cells (needle type and caliber, speed, concentration, suspension solution) in order to maximize the beneficial effects of this delicate biological treatment.

In this context, we believe sharing our current step-wise protocol, matured continuously since 2001, the year we treated our first patient adipose-derived mesenchymal stem cells,[7,8] may be a valuable contribution for other groups involved in stem cell application for perianal fistula (Fig. 1).

We herein describe in detail the preparation, surgical procedure, and intraoperative techniques of cell handling. For the first time in surgical history, we are dealing with a "living drug" for surgical purposes, thus the "user manual" goes far beyond simple technical considerations and implications: staff education and coordination, delicateness of cell handling, comprehensive knowledge of basic biology, and numerous possible physical, physiological, and pharmacological factors that could alter the cell viability and activity; these could all impact surgical outcomes in the setting of cell-based therapy. Given the recent approval of expanded human allogeneic mesenchymal adult stem cells extracted from adipose tissue (Alofisel, previously Cx601) by the European Commission, for the treatment of complex perianal fistulae in adult patients with non-active/mildly active luminal Crohn's disease, and its expected European commercial release in 2018, all these issues will need critical review for the upcoming extension of these cells from trial into clinical practice worldwide. Once commercially available, the use of cell therapy will likely increase significantly, especially if outcomes and safety profiles continue to look promising.

In addition, we believe the MSC isolation process and culture details will be of interest for those who aim to implement cell-based therapy protocols in their clinical practice for fistula or other gastrointestinal pathology.

## Indications and Safety of Stem Cell-Based Fistula Treatments

We described the first effective MSC-based treatment, both with autologous and allogeneic cells, in phase III multicenter international trials.[4,5] At the present time, the only definitively accepted indication for MSC-based treatment of perianal fistula is complex perianal fistulas in patients with Crohn's disease. Other indications could gain further importance in the future, as complex perianal cryptoglandular fistulas, enterocutaneous fistulas, pouch-vaginal fistulas, rectovaginal fistulas, and complex fistulas in a variety of locations, in which we have experience outside phase III clinical trials.

The safety profile of the MSCs has been widely studied in vitro and in vivo, and no major adverse events have been detected when treating study patients, even with allogeneic MSC applications.[4,9] However, new treatments should be implemented with caution in order to minimize any possibility for undesired events. It is important to emphasize that stem cells should not be used in patients with active malignancies; patients with a history of cancer should be free of disease for at least 5 years before considering stem cell-based therapy.

## Infrastructure and Legislation

Since 2008, stem cells intended for human use are considered pharmaceuticals by the European Medicinal Agency. Therefore, cell selection and expansion requires special conditions based on the GMP (good manufacturing practice) guidelines and only few laboratories that cover the required standards exist in each country. The time for cell expansion depends on the quantity and quality of fat tissue lipoaspirate.

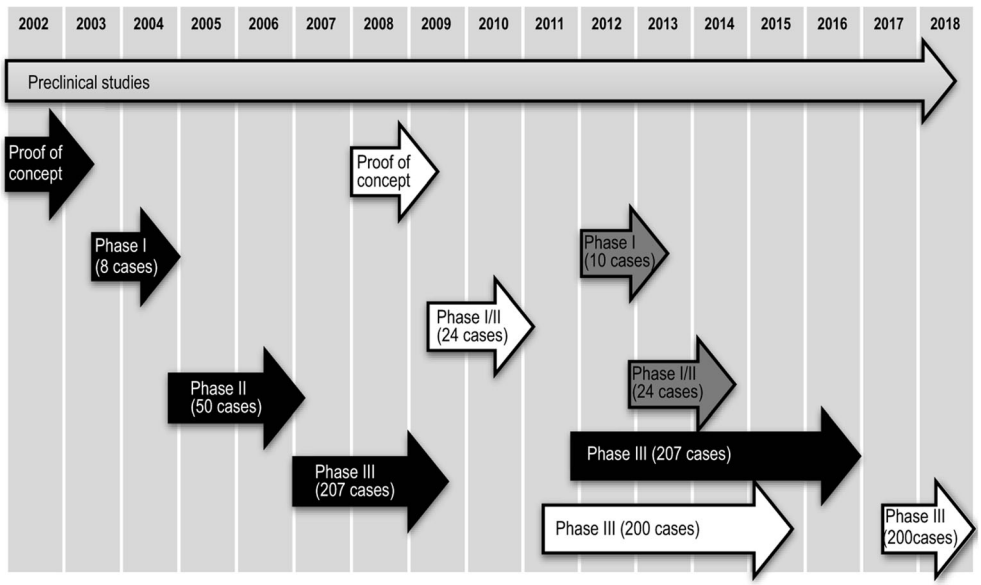



**Fig. 1** Clinical studies developed so far by our team. Black arrows: autologous use of ASCs; white arrows: allogeneic use of ASCs; gray arrows: rectovaginal fistula

Generally, it takes 2–3 weeks to obtain 100 million cells. In allogeneic setting with previously expanded and maintained stock of frozen cells with clinical GMP quality, it takes 96 h to thaw the stem cells and prepare them for application. Furthermore, for autologous use or any other form of stem cell application different from the allogeneic expanded stem cells approved for use in Europe (Alofisel), the therapy is considered experimental. Therefore, special permissions from government drug agencies are mandatory before use in clinical application. Until recently, stem cell treatment for complex fistulas (Crohn's and non-Crohn's) was offered only within officially registered clinical trials and, on rare occasion, with a protocol of "Compassionate Use" after institutional approval for single complex cases. However, when the drug (Alofisel) becomes officially available, its use would be guided by the national drug legislation as any other pharmaceutical.

## Cell Selection

### Adipose-Derived Stem Cells

While there are many sources of MSCs (e.g., placenta, bone marrow, adipose), we favor adipose-derived stem cells for cell-based therapies, as they are easy to harvest, either by liposuction or subcutaneous biopsy of fat tissue. Furthermore, they can be used in several ways:

- In raw: autologous use in which the cells are extracted and injected without expansion (stromal vascular fraction, SVF). The dose of cells injected will be much less and variable among the patients (~ 3% of SVF is MSCs), but it allows cells to be deployed during the same surgical procedure as the fat extraction. European legislation allows SVF use only when extraction and application are performed within the same procedure. This could be achieved only when the process of SVF obtaining is performed manually[10] in laboratory conditions, and this is not feasible in the majority of hospitals. While closed systems for SVF obtaining are commercially available (price 2200–5400€), they need up to 90–200 h to complete the process which obviously precludes their use during the same procedure as extraction. From previous studies, it seems that increasing the number of implanted cells can improve efficacy, making the actual indications and application of SVF very limited.[4,10]
- After expansion, autologous: large number of cells may be grown through cell passage and expansion. At present, this option could be applied only within a clinical trial or with a "compassionate use" in rare cases.
- Allogeneic: no liposuction is needed for cellular harvest. Their safety has been confirmed in numerous studies and they possess great homogeneity. Due to very low level expression of HLA class I and lack of HLA class II or co-stimulatory molecules, allogeneic cells may not generate clinically relevant antibodies, hindering efficacy with subsequent use. We consider their use especially valuable in Crohn's disease cases in order to minimize autoimmunity. Moreover, they can be delivered in a point-of-care fashion, as they are prepared ahead of the planned surgical procedure.

## Preoperative Assessments

Clinical history and physical exam will provide the complex fistula diagnosis and the indication for treatment. We recommend preparing at this moment a detailed description of the fistulous tracts (single or multiple) and a preoperative plan to calculate the necessary volume for each case, by a diagram representing the tracts and internal and external openings and mapping the injections (Fig. 2). As the intention is to simplify the stem cell application, we recommend using the same volume and quantity of cells in all cases. We describe in detail below that the half the total dose is to be applied to the internal orifice, and the rest is to be distributed among the fistula tracts, proportional to the length of the tract. In the successful phase III clinical trial of allogeneic stem cell application in Crohn's fistula, a total of 120 million cells suspended in 24 ml (usually Ringer lactate's solution with 2% albumin) were used in all the patients.

In our opinion, magnetic resonance imaging (MRI) is useful preoperatively for the description of cavities and complex branching tracts, but during follow up, it may not add any important information for the treatment efficacy as fibrosis may be mistaken as active fistula tract and vice versa.

Endoanal ultrasound provides information regarding fistula type and location but is not a reproducible test so we favor MRI over this diagnostic procedure in the context of clinical trials.

## Surgical Protocol

Below is a comprehensive description of our nine-step reproducible protocol for stem cell therapy application in treating a perianal fistula.

### Step 1—Perioperative Antibiotic Use

Crohn's disease patients frequently present with perianal sepsis. Prior to delivery of stem cell therapy, active infection should be controlled by means of surgical incision and drainage of any liquid collection/abscess > 2 cm, usually in combination with antibiotics.

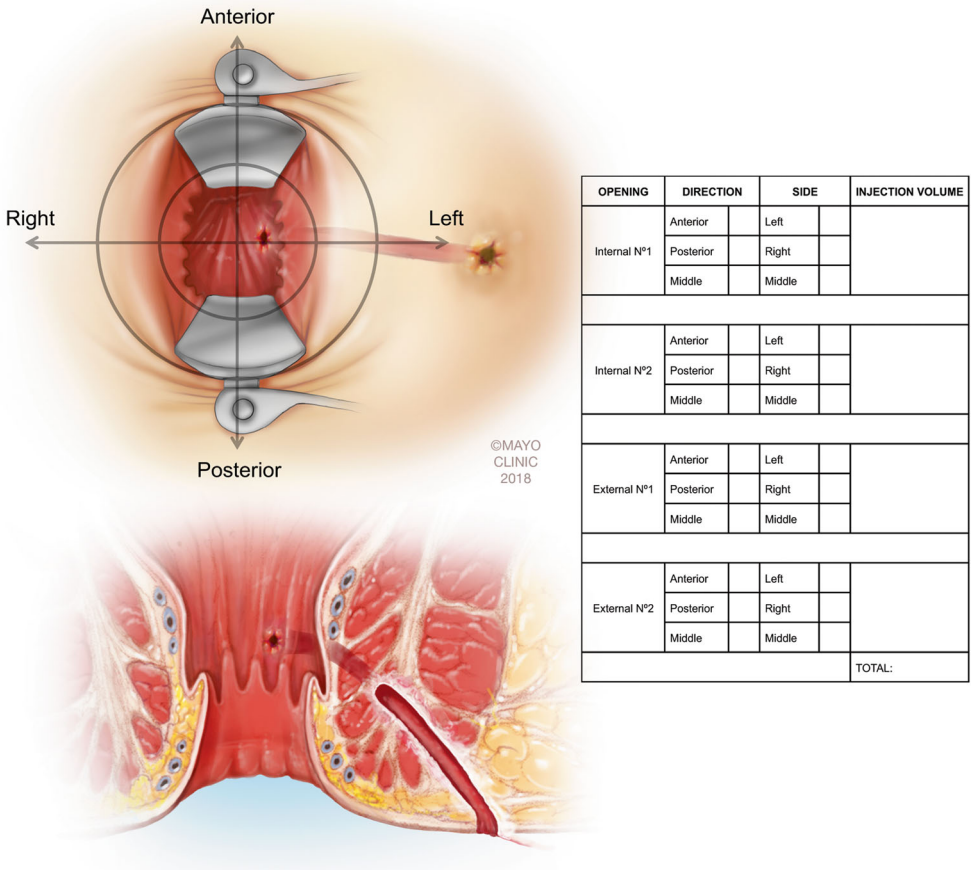



**Fig. 2** Preoperative diagram for stem cell injection planning. Gathers the fundamental information of the fistula tracts and openings and the cell product volumes to be applied to each of them

Currently, the knowledge of the potential effects of antibiotics on the MSC viability and function is scarce. However, some in vitro and animal studies suggest the most frequently used antibiotics (benzyl-penicillin, flucloxacillin, cefuroxime, and metronidazole) have not shown any detrimental effects on the stem cells, while gentamicin and vancomycin could downregulate MSCs proliferation and differentiation activity.[11,12] On the other hand, bone marrow MSCs are found to be able to uptake ciprofloxacin and release it to the tissues, which could further increase the antibacterial effect of the stem cell therapy.[13,14]

The authors do not use antibiotic prophylaxis prior anal fistula surgery. However, this issue is a subject of institutional preventive politics and is not a subject of this paper. In case antibacterial treatment is necessary once cells are implanted, we recommend avoiding gentamicin and vancomycin if other alternatives are available.

## Step 2—Anesthesia

Any anesthesia protocol may be chosen, taking into consideration that the surgical insult is minimized with this technique. However, local anesthesia should be used with caution, due to the possible direct cytotoxic effect of the most frequently used anesthetics (amide-type: ropivacaine, lidocaine, bupivacaine, and mepivacaine) to the MSCs, described after in vitro exposure of the cells to each of the drugs.[15] Furthermore, it was found that local anesthesia could directly and indirectly affect the anti-inflammatory capacity of MSCs, by altering the microenvironment, and modulating the macrophage inflammation and MSC secretion.[16] As local anesthesia in anal surgery is applied in most cases in the form of pudendal block, the contact of the injected cells with the local anesthetics is not expected to occur and thus the surgical protocol may not be changed substantially. Even though, if not strictly necessary, we recommend local anesthesia should be avoided. The surgical approach for stem cell application in perianal fistula is designed to be less aggressive, especially in Crohn's disease, and thus less painful than other established surgical approaches. Pain control could be managed perioperatively in most of the cases with paracetamol alone.

This issue should also be taken into consideration when applying stromal vascular fraction (precursor of the ASCs, derived from liposuction) where local anesthetics are frequently used in the abdominal wall during liposuction, as this practice could hamper the surgical results by worsening the cell immunomodulation capacity and viability, even after removing the anesthetic agent with SVF isolation.[15,16]

## STEP 3—Antisepsis

Alcoholic, hydrogen peroxide and povidone-iodine solutions should be avoided in surgical preparation due to their toxicity to the cells. Polyhexamethylene biguanide (PHMB), octenidine dihydrochloride, and chlorhexidine (non-alcoholic) solutions seem to have the optimal profile for this purpose.[17,18] We tend to simply use normal saline so that the preparation will not interfere with cell viability.

## STEP 4—Internal Fistula Orifice Location

Internal orifice location and management are the keys to successful treatment of perianal fistulas. Surgeons often inject hydrogen peroxide solution through the external opening to identify the internal opening. However, when stem cells are to be applied, in order to avoid cytotoxic effects of hydrogen peroxide, other methods should be employed. Probes or pure saline solution are appropriate for this purpose. This task is obviously easier when a seton has been previously placed. There may be pros and cons of using setons in patients who will receive cell therapy. On one hand, we could recommend avoiding seton as it sometimes causes an extensive fibrosis around the fistula that could decrease the blood supply to the implanted cells. On the other hand, a seton can maintain a clean fistula track and avoid abscess formation. Although a preoperative fistula protocol MRI was routinely performed in most fistula clinical trials, we have found a very low rate of correct identification of the internal opening on MRI. Thus, we consider an exam under anesthesia (EUA) the *gold standard* for defining fistula anatomy. Endoanal ultrasonography (EAU) can also aid in localizing the internal opening but the treatment will ultimately be based on the intraoperative features at the time of cellular delivery.

## STEP 5—De-Epithelization of the Fistula Tract

Another important aspect of the wound management is the debridement and de-epithelization of the fistula tract. This is especially important if a seton was previously placed. Extensive debridement of the epithelialization creates an appropriate wound bed for the cells by exposing healthy tissue. We perform a deep mechanical debridement (curettage) especially of the internal orifice (Fig. 3). Curettage is the single most effective and recognized part of the fistula treatment. Bleeding from the external and internal opening should be observed to assure adequate debridement.

## STEP 6—Cleaning of the Cavities and Fistula Tracts

The tracts are cleaned with saline solution in order to remove devitalized tissue debris following curettage (Fig. 4).

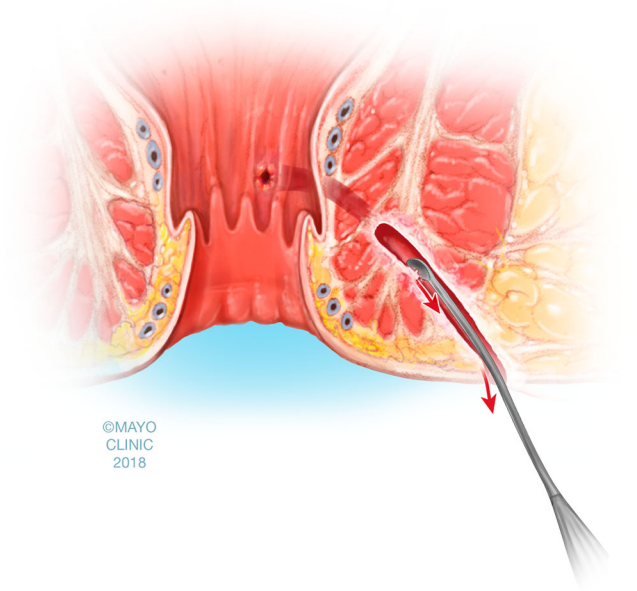



**Fig. 3** Mechanical debridement (curettage) of the fistula tract

## STEP 7—Closure of the Internal Opening

We believe this surgical act should not be very aggressive. The closure should be achieved by simple 2/0 absorbable suture (Fig. 5). The stitch must include full thickness bites, performing a figure of eight suture and snug pressure. Smaller and tighter bites may tear the fibrotic tissue. There is no restriction on the material of the suture to be used; we prefer 2/0 poliglactine 910. The success of the closure is confirmed by the injection of saline solution through the external opening (Fig. 5).

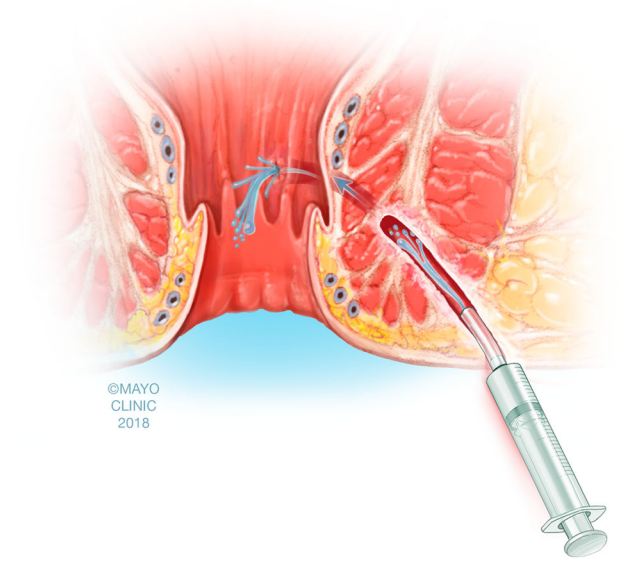
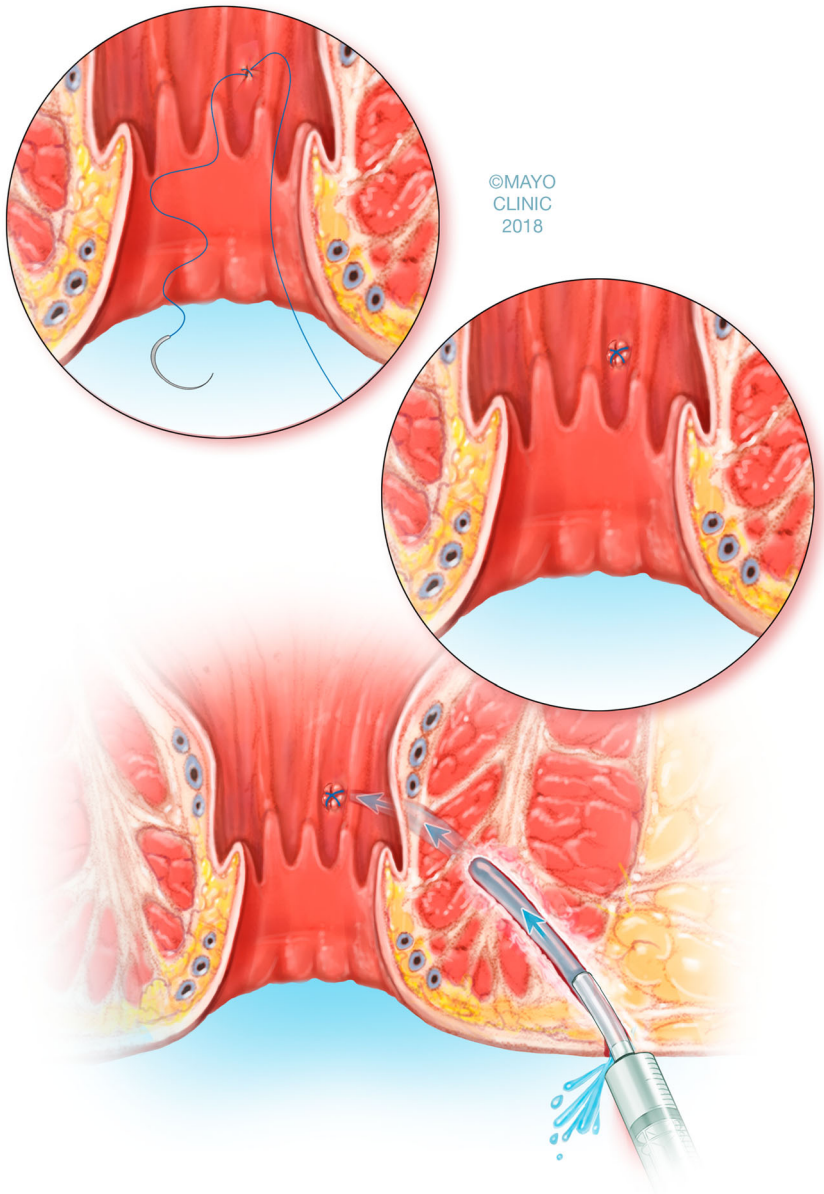



**Fig. 4** Fistula tract cleaning

**Fig. 5** Internal orifice closure




## STEP 8—Stem Cell Handling and Resuspension

Stem cell handling is a critical point. This is a biological living drug that comes to the operating theater in the form of several transparent (usually glass) vials and can be stored for a very limited time (24 h since reception). Usually, the concentration used is 5 million cells/ml. As this is a personalized treatment (either in the form of autologous or allogeneic application), a confirmatory checklist (dose, patient ID, allogeneic, or autologous) should be implemented to assure the correct dose, patient, and cell origin have been selected. The vials are transported from the laboratory to the operating room within a box that maintains stable temperature (between 15 and 25 °C) and prevents mechanical damage prior to the procedure. Therefore, it is of great importance that the surgical procedure is well scheduled (usually first patient of the day) and patients are well prepared in order to decrease the possibility of cancelation or excessive delay that could hamper the cell quality. We recommend not taking the vials out of the box until the surgical debridement and closure of the internal orifice have been completed. Cells should be gently resuspended by soft swinging movements, with care to avoid vigorous shaking. MSCs are characterized by their capacity to adhere to plastic surfaces. Therefore, they should be extracted from the vial in one step and slowly just before the application and should not be moved to a different receptacle before the procedure.

### STEP 9—Cell Injection

We recommend a slow injection process (to avoid high cell friction and cell mortality) through a fine and long needle (e.g., Abocatt 22G; Terumo). Studies have shown that up to 26G bore size needles are suitable for injecting MSCs without viability and functional capacity alterations of the cells even after three passes through the needle.[19]

We recommend injecting at least half of the total dose in the tissues around the internal orifice or orifices (Fig. 6). The other half should be injected through the external orifice into the fistula walls throughout all its length, creating several microblebs that are no deeper than 2 mm from the fistula tract (Fig. 6). Care should be taken not to inject the cells neither within the fistula lumen (as the cells will most certainly be lost with the postoperative secretions) nor too far (> 2–3 mm) from the fistula walls (as their effect is local and thus is less likely to contribute to fistula healing).

In some therapeutic strategies, stem cells could be applied in combination with fibrin glue. However, as this is rarely performed, there is no clearly defined protocol for its use (mixture with fibrin glue or application of fibrin glue within the tract together with stem cell injection in the fistula walls) and there is no sufficient scientific evidence to recommend it. Therefore, this issue has not been further discussed in the current protocol paper.

Care should be taken to avoid solution leaks during the application. The presence of a fibrotic tissue in chronic perianal fistula is frequent and could increase the difficulty for a correct distribution of cells during the treatment. For this reason, occasionally, the volume of the treatment should be adjusted. In small fistulas without cavities, treatment should be delivered in smaller volumes to avoid leakages. In order to maximize the number of injected cells, a small amount of saline is used to wash down the syringe so that the cells located in the end of the syringe and inside the needle after the injection can be utilized.

## Special Conditions

### Crohn's Disease

Crohn's disease is a well-known immune condition, with altered mechanisms of the immune response. This explains, in Crohn's-related fistulae, the high rates of failure with the conventional treatments (standard surgical techniques, fibrin glue, other implanted substances, etc.…) and the improved results with adipose-derived stem cells, as it was demonstrated in a phase III clinical trial.[4]

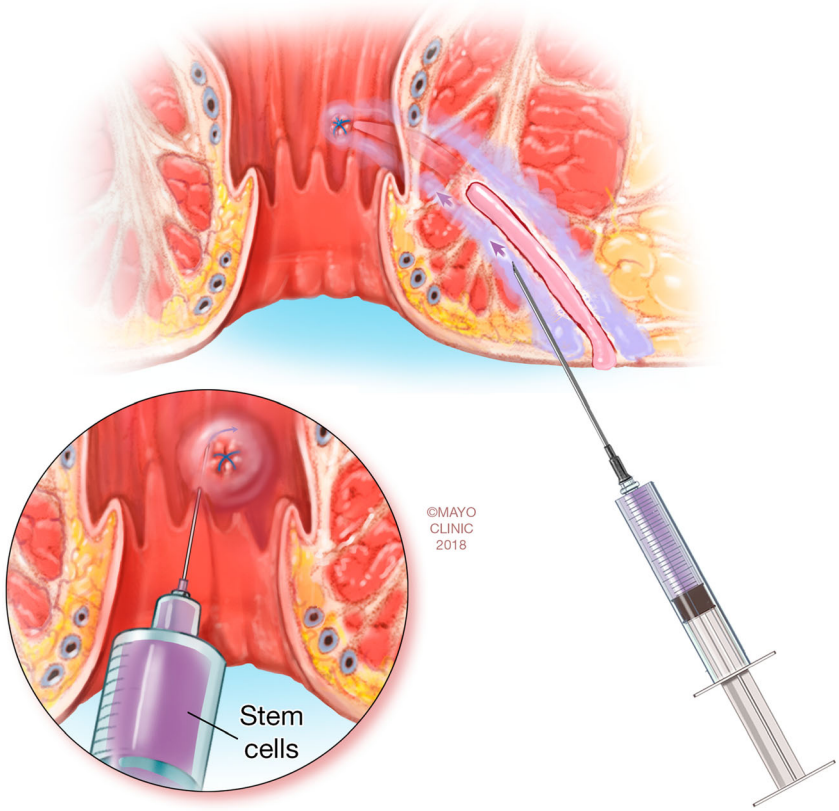



**Fig. 6** Stem cell application to the internal orifice and fistula tract walls

If Crohn's disease has a long course and complex perianal disease is present, a previous local exploration under anesthesia is recommended in order to:

- Prepare the surgical field, draining all the liquid collections, and leaving loose setons where needed
- Taking biopsies systematically due to the low but well-known risk of malignant transformation of the perianal Crohn's lesions and tracts.[20] Injection of cells in a tumor environment is contraindicated as some studies have found tumor-promoting effects of the MSCs.[21,22] So, any local neoplasia must be ruled out before MSC application
- Crohn's medical treatments generally should not be discontinued, as their effects in combination with stem cells may even be potentiated.[23] However, prolonged use of corticoids could decrease the stem cell motility and therefore in our studies, patients were excluded if they received corticosteroids within the 4 weeks prior to the treatment[24]

### Internal Orifice Not Found

If an internal orifice is not found, further dissection should not be performed. Even though, half of the cells should be injected in the suspected internal orifice area and the other half in the fistula tract as indicated.

## Expected Outcomes

Four to 6 months later, the patient is re-evaluated and the outcome of the initial treatment checked. According to a recent meta-analysis of the published clinical trials in perianal Crohn's disease, cell therapy achieves a 57% (95% CI 44–69%, $n = 251$) success rate.[25] Compared to the controls, mesenchymal stem cells improved fistula healing rates (6 to 24 weeks, OR = 3.06 (95% CI, 1.05–8.90); $p = 0.04$) and the success rate remained stable even after longer follow up (24 to 52 weeks, OR = 2.37 (95% CI, 0.90–6.25); $p = 0.08$).[26] In a non-Crohn complex perianal fistula, there are two published clinical trials (a phase 2 and a phase 3 studies, $n = 235$), both of them from our team with a success rate of 71 and 57%, respectively.[5,27] However, these recent non-Crohn complex fistula studies were performed with autologous stem cells, with much lower quantity (20–60 million cells) and without placebo-controlled group, failing to prove healing improvement compared to fibrin glue alone (healing rates at 24–26 weeks with ASCs alone, ASCs with fibrin glue, and fibrin glue alone were 39.1, 43.3, and 37.3%, respectively).[5] Even though, stem cell-based treatments provide longer lasting results with less relapses (healing rates at 1 year with ASCs alone, ASCs with fibrin glue, and fibrin glue alone were 57.1, 52.4, and 37.3%, respectively).[5]

## Future Directions

The field of stem cell applications in perianal fistulous pathology is constantly evolving and future modifications of protocols can be expected based on new data that could influence outcomes. These changes could be introduced at any step of the described protocol as this new biological treatment can be influenced by numerous factors, some of them underestimated in early studies. However, this treatment is about to become commercially available and enter in the clinical armamentarium of the perianal fistula treatment protocols, and an increasing number of surgical teams will need to be well versed in basic considerations for cell delivery. Furthermore, we believe the description of the steps of our protocol will increase the interest in applying stem cells in complex cases of perianal fistula by providing a reproducible surgical technique for this application.

## Take Home Messages

- When treating perianal Crohn's disease, the possibility of malignant transformation should be considered and ruled out with biopsies before stem cells are delivered, especially in high-risk patients (10 years from initial Crohn's diagnosis).
- The area being treated with stems cells should be free of active sepsis. Abscesses larger than 2 cm may decrease cell effects. In such cases, a preparative surgical exploration may be indicated with seton placement, or administration of antibiotics prior to definitive cell therapy.
- Do not explore fistula tracts with hydrogen peroxide due to its cytotoxic effect.
- Fistula tracts must be aggressively cleaned of debris before injection of cells, especially the internal opening of the fistula.
- Avoid local injection of lidocaine or other local anesthetics.
- At least half of the overall cell dose should be injected in the internal orifice.
- Resuspension of cells should be done gently! Do not shake vials!
- Avoid leakages by adjusting the volume to the areas being treated.

**Author Contributions** T. Georgiev-Hristov: gathering and interpretation of the protocols and data from the different studies published by the team and drafting and final approval the manuscript.

H. Guadalajara: final surgical protocol design and drafting, revision, and final approval of the manuscript.

M.D. Herreros: design of the initial and final surgical protocol and drafting, revision, and final approval of the manuscript.

A. L. Lightner: protocol design revision and drafting, revision, and final approval of the manuscript.

E. J. Dozois: protocol design revision and drafting, revision, and final approval of the manuscript.

M. García-Arranz: cell manipulation, delivery, and application protocol and drafting and final approval the manuscript.

D. García-Olmo: design of the initial and final surgical protocol and revision and final approval of the manuscript.

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