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Regenerative medicine for digestive fistulae therapy: benefits, challenges and promises of stem/stromal cells and emergent perspectives via their extracellular vesicles

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Abstract

Despite current management strategies, digestive fistulae remain extremely debilitating complications associated with significant morbidity and mortality, generating a need to develop innovative therapies in these indications. A number of clinical trials and experimental studies have thus investigated the potential of stem/stromal cells (SCs) or SC-derived extracellular vesicles (EVs) administration for post-surgical and Crohn's-associated fistulae. This review summarizes the physiopathology and current standards-of-care for digestive fistulae, along with relevant evidence from animal and clinical studies regarding SC or EV treatment for post-surgical digestive fistulae. Additionally, existing preclinical models of fistulizing Crohn's disease and results of SC therapy trials in this indication will be presented. The optimal formulation and administration protocol of SC therapy products for gastrointestinal fistula treatment and the challenges for a widespread use of darvadstrocel (Alofisel) in clinical practice will be discussed. Finally, the potential advantages of EV therapy and the obstacles towards their clinical translation will be introduced.

Keywords: Gastrointestinal fistulae, Crohn's disease, stem/stromal cells, post-surgical fistulae, extracellular vesicles

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1. Introduction

A fistula corresponds to an abnormal communication between two epithelialized surfaces [1]. Specifically, gastrointestinal fistulae arise as iatrogenic complications following surgery in approximately 75-85% of the cases [2,3]. Crohn's disease represents the main aetiology for primary gastrointestinal fistula occurrence in developed countries [4]. Other potential causes include neoplasia, trauma, infections, exposure to ionizing radiation, foreign bodies, etc [2-6]. Internal gastrointestinal fistulae can develop between any two parts of the gastrointestinal tract (entero-enteric fistulae, gastrojejunal fistulae, etc) or between any part of the gastrointestinal tract and another hollow structure (rectovaginal fistulae, colovesical fistulae, aorto-duodenal fistulae, etc) [1,4,7,8]. External gastrointestinal fistulae, or enterocutaneous fistulae, correspond to those fistulae that drain to the skin [1].

Gastrointestinal fistulae are challenging to manage and constitute an extremely debilitating complication for the patient, associated with prolonged hospital stays and dramatically increased morbidity and mortality rates [2,5]. Healing is not always achieved and a significant proportion of

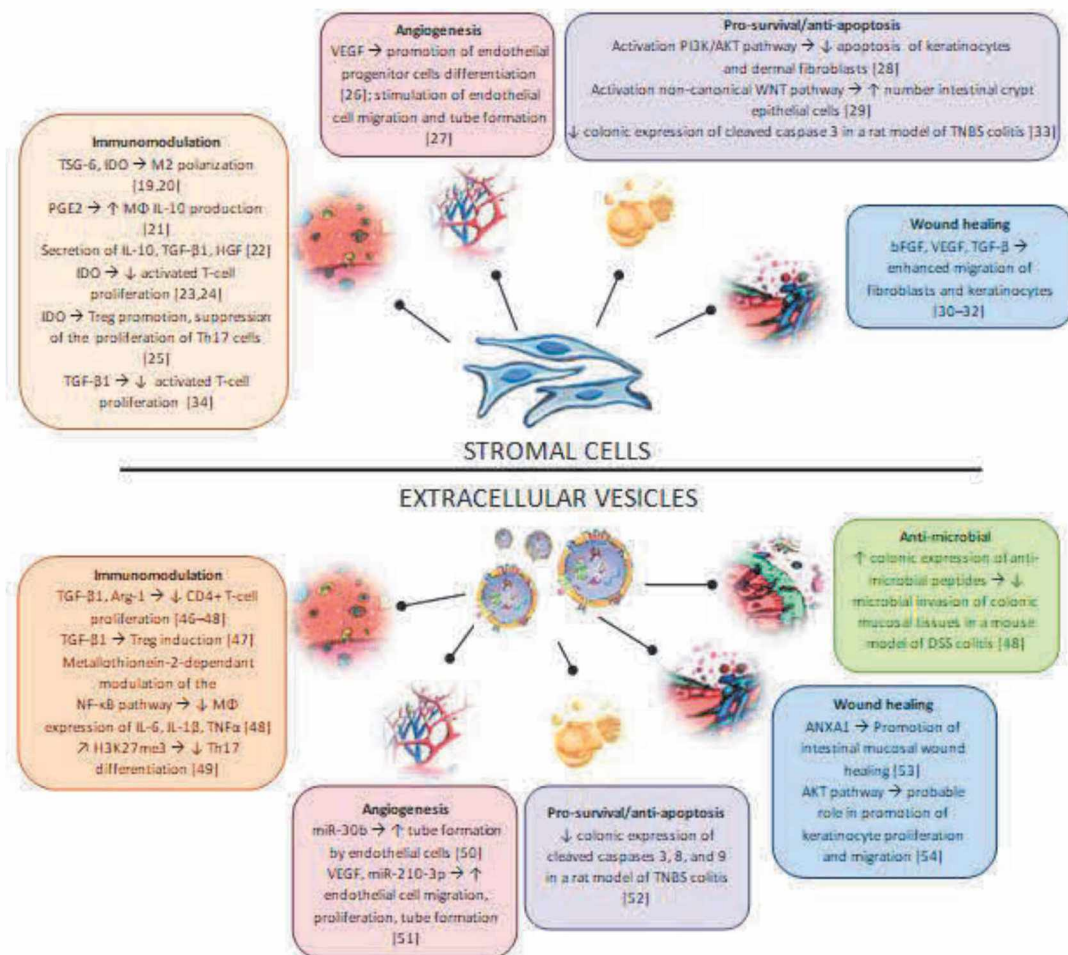


Figure 1: Some of the putative mechanisms of action of stromal cells and extracellular vesicles of interest for digestive fistula therapy. TSG-6: tumor necrosis factor-stimulated gene 6; IDO: indoleamine 2,3-dioxygenase; PGE2: prostaglandin E2; MΦ: macrophage; IL-10: interleukin 10; TGF-β: transforming growth factor β; HGF: hepatocyte growth factor; Treg: regulatory T-cells; Th17: T-helper 17; VEGF: vascular endothelial growth factor; PI3K: phosphoinositide 3-kinase; bFGF: basic fibroblast growth factor; Arg-1: arginase 1; NF-κB: nuclear factor-kappa B; IL-6: interleukin 6; IL-1β: interleukin 1β; TNFα: tumor necrosis factor α; H3K27me3: histone 3 lysine 27 trimethylation; miR-30b: microRNA 30b; miR-210-3p: microRNA 210-3p; TNBS: 2,4,6-trinitrobenzene sulfonic acid; ANXA1: annexin A1; DSS: dextran sulfate sodium

patients will have to live with a permanent diverting stoma [9], with a significant impact on their

1 quality of life. **Moreover, especially in Crohn's disease,** the recurrence of previously healed fistulae
2 constitutes a common occurrence [10,11]. These poor outcomes associated with gastrointestinal
3 fistulae have prompted continued experimental and clinical research for the development of innovative
4 treatment approaches, notably based on stem cell therapy, so as to add effective new alternatives to
5 current management algorithms.

6 Stem cell therapy has brought along tremendous hope in the medical community as a potential
7 solution for the treatment of a wide range of complex pathologies. Due to the ethical and safety issues
8 linked with the use of pluripotent stem cells [12], many clinical studies have focused on the use of
9 adult stem cells, such as stromal cells (SCs), a heterogeneous cell population also known as
10 mesenchymal stem cells. SCs are adult, non-hematopoietic, multipotent cells that can differentiate into
11 several cell types – such as osteoblasts, chondroblasts, adipocytes, tenocytes, etc. They are further
12 characterized by an adherent, spindle-shaped morphology, along with the expression of surface
13 markers such as CD73, CD90 and CD105 and the absence of expression of markers of the
14 hematopoietic lineage (CD45, CD19, CD14, CD11b, CD79 α , HLA-DR, etc) [13]. SCs were
15 historically isolated from the bone marrow. Other sources notably include umbilical cord blood,
16 **Wharton's jelly, tendons, the synovial membrane, the periosteum, etc.** Of particular interest for
17 regenerative medicine, a population of SC-like cells called adipose-derived stromal cells (AdSCs) can
18 easily be harvested at high yields from the adipose tissue through liposuction [14]. Initially, the
19 therapeutic effects of SCs were mostly attributed to their differentiation potential [14], thus providing
20 a replacement for compromised functional cells through homing to the injured tissues. However, the
21 prominent interest of SCs for regenerative medicine has rapidly gone beyond their ability to
22 differentiate into multiple cell types, with the preclinical and clinical demonstration of their propensity
23 to promote endogenous wound healing and angiogenesis, along with their immunomodulatory
24 properties and anti-fibrotic effects (**Figure 1**, [15–34]). Moreover, the results from several animal
25 studies and clinical trials led to challenge the assumption of a direct contribution of SCs to tissue
26 regeneration. On the one hand, it was shown that only very few SCs would durably engraft at the
27 injury site [29,35]. Indeed, after systemic administration, most of the injected cells become entrapped
28 in the pulmonary capillaries [29,35,36], and the presence of an inflammatory environment at the injury
29 site is highly deleterious to stem cell survival [29]. On the other hand, the therapeutic effect of SCs
30 often seemed to occur in an overly short time frame to be solely attributed to their direct differentiation
31 into functional cells [35]. These preclinical results, along with the observation of equivalent
32 therapeutic effects with SC-conditioned medium, prompted a change of paradigm in stem cell therapy
33 towards mostly paracrine mechanisms of action [37].

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44 Amongst all the potential effectors for these paracrine effects, SC-derived extracellular vesicles
45 (EVs) have gained increasing interest for regenerative medicine in recent years. EVs correspond to a
46 heterogeneous group of subcellular entities that are secreted by cells in a constitutive or induced
47 manner [38,39]. They are outlined by a membrane and are usually of a nanometric scale (40-5000nm).
48 Several subsets of EVs have been described depending on their biogenesis pathway, like exosomes,
49 which stem from the endosomal network and are released upon fusion of late endosomes with the
50 plasma membrane; microvesicles, which are formed by outward budding and rupture of the plasma
51 membrane; or apoptotic bodies, resulting from cell blebbing during programmed cell death [38,39].
52 However, it is now recommended to favor a classification of EVs based on parameters such as size or
53 biochemical composition, which are technically easier to assess [40]. EVs carry lipids, proteins,
54 mRNAs, miRNAs, and their contents depend both on their parent cells and on their method of
55 production [38,39]. Physiologically, EVs serve as mediators for intercellular communication [38,39].
56 SC-derived EVs have demonstrated anti-apoptotic properties, along with a propensity to increase cell
57 survival through the activation of the PI3K/Akt pathway, and to promote cell proliferation and wound
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healing [41–43]. They have also demonstrated anti-inflammatory, immunomodulatory and anti-fibrotic properties (Figure 1, [37,42,44–54]). Their potential for regenerative medicine has thus been shown in preclinical studies for a wide variety of pathologies, including myocardial ischemia/reperfusion injury, acute kidney injury, type 2 diabetes mellitus, liver fibrosis, intervertebral disk degeneration, etc [41,42,45,55,56], and emerging evidence tends to indicate their interest for the treatment of gastrointestinal fistulae [57,58].

This review aims at summarizing current knowledge and discussing the benefits, challenges and promises of SC-based and EV-based therapies for the treatment of fistulae of the digestive system. It will mostly focus on fistulae of post-surgical aetiology, including both gastrointestinal and pancreatic fistulae, and on fistulizing Crohn’s disease. The use of SC therapy for perianal fistulae of cryptoglandular origin, already reviewed in details elsewhere [59,60], will be shortly described in the supplementary materials (Supplementary table 1). First, we will present the physiopathology and treatment of post-surgical and Crohn’s-associated fistulae, then available evidence supporting their therapy with SCs and EVs will be exposed. Finally, current challenges and perspectives for their clinical translation will be discussed.

2. Digestive fistulae

Different types of digestive fistulae and the associated surgical treatment modalities are presented in Figure 2.

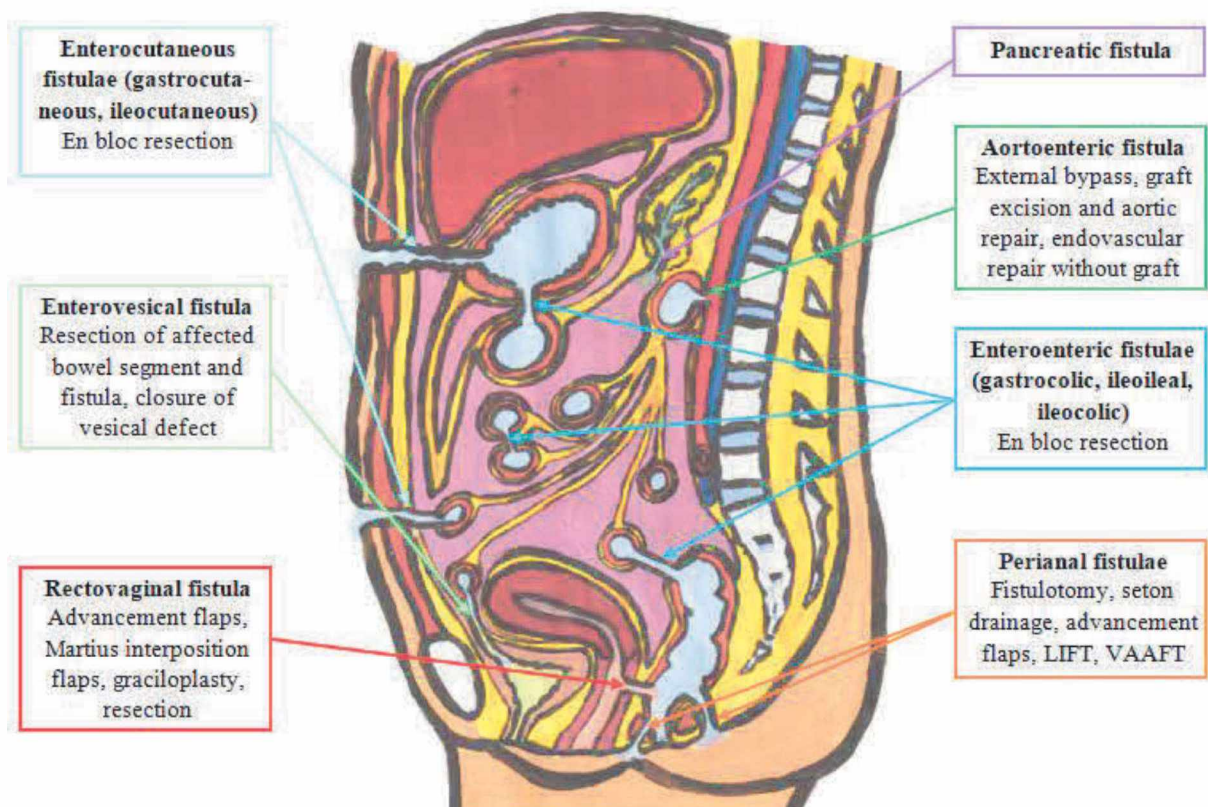


Figure 2 : Digestive fistulae – main strategies for definitive surgical closure. LIFT: Ligation of the intersphincteric fistula tract. VAAFT: Video-assisted anal fistula treatment.

2.1 Epidemiology and physiopathology

Gastrointestinal fistulae occur mostly as post-surgical complications (75-85% of the cases), especially following surgery for cancer, inflammatory bowel diseases and lysis of adhesions [2,3].

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Fistula incidence can vary drastically depending on the procedure (6.3% in non-colorectal abdominal surgery including bowel suture or anastomosis [61], 10-11% following surgery for gastric cancer [62,63], and about 15% of anastomotic failure following colorectal surgery [64]). Post-surgical fistulae are often linked with anastomotic failure or inadvertent enterotomies, leading to the leakage of luminal contents outside of the gastrointestinal tract [3–5]. Such leakages can cause severe, potentially lethal, infectious complications like abscesses or peritonitis. Progressive extension of an abscess by erosion of neighboring tissues results in the formation of a fistula tract [65]. Inadequate surgical technique (tension on the suture lines, devascularization resulting in local ischemia) and local factors (perianastomotic infection, involvement of diseased segments of the gastrointestinal tract) constitute predisposing factors to anastomotic failure [4]. Additionally, dense adhesions and/or distal obstruction [66,67], emergency surgery without adequate preoperative preparation, along with patient-related factors such as malnutrition or systemic hypotension [4], have been implicated in the physiopathology of post-surgical fistulae. The development of post-surgical fistulae significantly alters the patient's prognosis. Mortality rates ranging from 10 to over 30% have been reported for enterocutaneous fistulae in recent years [63,68–71], depending on their localization and output. Morbidity and mortality are often linked to malnutrition, fluid and electrolyte imbalances and infectious complications leading to sepsis [2,3]. Moreover, probably owing to these complications and the associated deterioration of the patient's overall condition, post-surgical fistulae incidence in cancer patients may result in their not receiving adjuvant chemotherapy [72], which will further decrease their prognosis. Gastrointestinal fistulae might cause malnutrition or fluid and electrolyte imbalances through several mechanisms [1,2]: internal fistulae bypassing large portions of the digestive tract may result in malabsorption and diarrhea; considerable loss of fluids, electrolytes and nutrients may be observed, especially in the case of high-output fistulae; poor oral intake is common; and finally, patients might be hypercatabolic due to septic complications. Severe haemorrhagic complications may also occur, notably in the case of aorto-enteric fistulae or arterial exposure to pancreatic secretions in the case of post-surgical pancreatic fistulae [4,73].

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In 15 to 25% of the cases, gastrointestinal fistulae will develop spontaneously, notably in the course of Crohn's disease [2,3]. Crohn's disease is a chronic inflammatory bowel disease affecting over 1,000,000 patients in Europe with over 75,000 new cases each year [74]. It is considered a multifactorial pathology, linked with an association of genetic and environmental determinants, alterations of the gut microbiota, an increased intestinal permeability, and a dysregulated immune response, resulting in lesions potentially all along the digestive tract [75–80]. Fistulization constitutes a relatively common complication, affecting up to 35-40% of Crohn's patients [1]. Penetrating Crohn's disease mainly includes fistulae of the ano-perineal sphere (Figure 3), but also rectovaginal fistulae, entero-enteric fistulae, enterocutaneous fistulae, etc. The pathogenesis of penetrating Crohn's disease is quite complex. Crohn's patients present impaired wound healing mechanisms, notably linked to a decreased migratory potential of colonic lamina propria fibroblasts [81,82]. These impaired wound repair mechanisms might trigger an epithelial-to-mesenchymal transition (EMT) in intestinal epithelial cells, initially in an attempt to repair an intestinal lesion [82,83]. EMT of intestinal epithelial cells is notably induced by TGF β and further increased by pro-inflammatory cytokines such as TNF α or IL-

13, resulting in the formation of myofibroblast-like cells called transitional cells [82,83]. Transitional cells then deeply penetrate into the gut mucosa and form a tube-like structure, which might extend to an adjacent organ or the abdominal wall [82,84]. This process is further facilitated by extracellular matrix remodeling through increased expression of matrix metalloproteinases such as MMP-3 and MMP-9 [82,83]. **The presence of underlying Crohn's disease constitutes a low prognosis factor for spontaneous fistula healing, especially in the case of uncontrolled inflammation [85].**

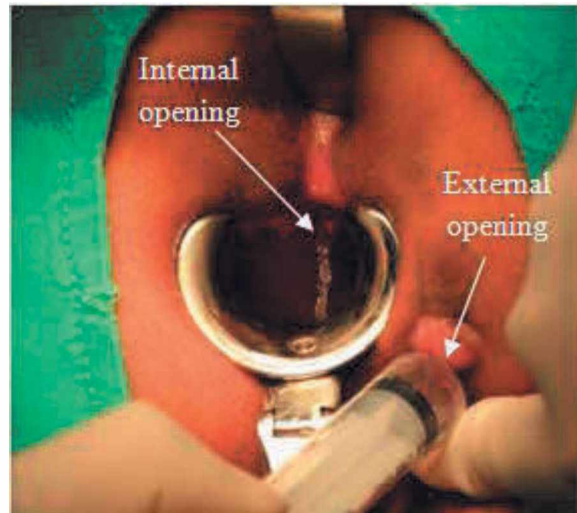


Figure 3: Perianal fistula. The injection of water, saline or dyes through the external opening facilitates the identification of the internal opening. Adapted with permission from Springer, *Techniques in Coloproctology* “LIFT procedure: a simplified technique for fistula-in-ano” A. Rojanasakul ©2009

2.2 Current management principles

Gastrointestinal fistulae management is a complex procedure necessitating combined medical, radiological, endoscopic and surgical treatment modalities. The main principles are first to limit the associated complications, and then to reestablish continuity of the gastrointestinal tract. In the acute phase, infection should first be controlled, notably through percutaneous, endoscopic or surgical drainage of the abscesses [2,3]. In the presence of signs of sepsis, broad-spectrum antibiotherapy should be rapidly initiated then adapted to microbiological tests results [3,5]. The patient should then be provided with nutritional support adapted to the anatomical location of the fistula, to the volume and contents of fistula output, and to their overall state [2,3,5].

To promote spontaneous fistula healing and reduce morbidity, fistula output should be decreased, and in the case of external fistulae, exposure of the skin to fistula secretions should be avoided to limit local complications, such as pain or infection [3,5]. Despite proper conservative care, spontaneous healing, which usually occurs in 4 to 6 weeks, remains rare (about 20-30% of the cases for enterocutaneous fistulae [5], depending on fistula localization [71]), with a frequent chronicization of the fistula.

Definitive surgical closure is therefore often necessitated [5]. Only patients with an adequate nutritional status and an absence of sepsis are eligible for definitive surgical closure [3]. These invasive procedures notably include en-bloc resection of the fistula tract and the involved bowel segments with restoration of bowel continuity through anastomosis, and may be associated with severe complications such as fistula recurrence or short bowel syndrome [3,5,65,66]. Temporary proximal intestinal diversion may sometimes be required to control fistula output before surgical treatment, and the risk of permanent stoma remains very high [9].

The treatment of primary fistulae arising in the context of Crohn's disease differs due to the presence of inflammation. Recent guidelines promote a combined medical and surgical therapy [86,87], to manage infectious complications, control luminal inflammation and promote fistula closure. Anti-TNF α biotherapies such as infliximab are currently recommended as first-line therapies for the induction and maintenance of remission in complex perianal fistulae in Crohn's disease [86]. However, results from the randomized controlled trial ACCENT II revealed significant rates of failure (30%) or loss of response (42%) [88] and data on their use in non-perianal fistulae remains limited [1].

1 Other biological agents, such as ustekinumab (anti-IL12 and IL23) or vedolizumab (anti-integrin $\alpha_4\beta_7$)
2 are currently under exploration for fistulizing Crohn's disease, however there is still insufficient
3 evidence to recommend their use in this indication [86].

4 Emerging therapies for persisting fistulae tend to advocate minimally invasive treatments through
5 endoscopic placement of stents, through-the-scope or over-the-scope clips [89], or the use of various
6 sealing agents such as fibrin glue [90], cyanoacrylic glue [91] or the thermoresponsive hydrogel
7 poloxamer 407 [92]. Amongst them, the possibility of stem/stromal cell therapy has received growing
8 attention over the years.
9

11 3. Post-surgical fistulae: a new hope provided by stem/stromal cells

13 3.1 Stem/stromal cells for the treatment of post-surgical fistulae – Evidence from animal studies

14 Due to the high morbidity and mortality rates still observed with post-surgical fistulae, preventing
15 their development constitutes a very attractive treatment strategy [5,73].
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17 In this regard, the potential of stem/stromal cells to improve colonic anastomoses healing has been
18 studied in rat models, however a preventive effect of stem cells on the development of anastomotic
19 fistulae could not be found, though the low basal rates of post-surgical fistulae incidence in those
20 models might have biased the results [93].
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22 Some better results were obtained in experimental studies of per-operative local cell sheet
23 placement to prevent post-surgical pancreatic fistulae development [94–96]. Rat models of post-
24 surgical pancreatic fistulae were established by splenic pancreatic duct transection [94,95] or gastric
25 and splenic pancreatic ducts division with distal pancreatectomy and splenectomy [96]. One study
26 investigated sheets formed with 1 million rat myoblasts on thermoresponsive culture plates [94].
27 Another compared murine AdSC multilayered sheets in the presence or absence of mannose, formed
28 with 0.5 million magnetically-labeled AdSCs in a collagen/Matrigel solution [95]. A third study
29 compared rat AdSC and bone marrow-derived stromal cell (BM-SC) sheets, formed with 1.1 million
30 cells on thermoresponsive culture plates [96]. With cell sheet treatment, less pancreatic leakage was
31 observed in the early post-operative course, as evidenced by intra-abdominal fluid volume [96] and
32 ascitic pancreatic amylase and lipase concentrations [94,95]. It also led to less associated intra-
33 abdominal damage [94,95], less inflammatory cell infiltration [94–96], an increased tissue
34 regeneration [96] and better survival at 1 month [94]. A different cell source [96] or the adjunction of
35 mannose [95] did not significantly alter these results. Moreover, a control experiment using collagen
36 sheets without cells enabled to exclude a mere physical effect as the mechanism of action of the cell
37 sheets [95].
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39 Overall, these 3 studies tend to show some beneficial effects of cell sheets for the prevention of
40 post-operative pancreatic fistula development. However, as underlined by Kim *et al.* [96], the
41 experimental models used in those studies were not completely representative of the clinical situation.
42 Therefore, further studies with a longer follow-up in more clinically relevant, large animal models are
43 needed before an eventual clinical translation.
44

45 The potential of stem/stromal cell therapy has also been studied in experimental models of
46 enterocutaneous fistulae, this time as a curative treatment modality [97–100]. These experimental
47 models, due to the absence of underlying pathology, were more representative of fistulae of post-
48 surgical origin. In spite of extensive differences in terms of protocol, all 4 studies evidenced some
49 promising results.
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51 A first study, in a rat model of enterocutaneous fistula established by performing a 5 mm
52 enterotomy on the caecum and suturing it to the abdominal wall, investigated the local administration
53 of 1 million human AdSCs either through local injection or through their adhesion to the suture
54 material used [97]. At 21 days post-administration, the area of the fistula had decreased considerably
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1 in both groups treated with cells, especially in the suture group, compared to the control; however this
2 was only evidenced by image analysis of successive photographs of the external opening, therefore
3 these results, though promising, still remain very preliminary. In another study, gastrocutaneous
4 fistulae were created in rabbits by gastrotomy, and, after two hours for leakage formation, the insertion
5 of a tube in the lesion as a drain. 2 weeks later, the drain was removed and autologous stromal
6 vascular fraction (SVF, containing AdSCs) was injected into the fistula tract [98]. At 3 weeks post
7 treatment, examination of the external openings and histological analysis of the fistula tract evidenced
8 patent tracts with draining, inflamed external openings in the untreated control group and near-
9 complete healing in the SVF group. However, this study presents a limited precision preventing a fully
10 objective comparison between both groups (for instance, the mean percentage of tract closure is given
11 for each group without any indication on intra-group variability). A more thorough study evaluated the
12 potential of human BM-SC sheets in a murine model of enterocutaneous fistulae created by
13 caecostomy [99]. On the third post-operative day, bi-layered sheets, containing 2 million cells either
14 labeled with both magnetic nanoparticles and a fluorescent dye or unlabeled, were positioned into the
15 fistula orifice. 2 weeks after transplantation, clinical evaluation, MRI, histological analysis and probe-
16 based confocal laser endomicroscopy results indicated better healing in both the labeled and unlabeled
17 sheet groups compared to the control with less inflammation and fibrosis, enhanced
18 microvascularisation, a lower fistula surface and a decreased maximal size of the fistulous orifice. The
19 mechanism of action of the sheets was investigated by following the fate of labeled cells post
20 transplantation and analyzing the expression of diverse signaling molecules, suggesting an indirect up-
21 regulation of host trophic factors rather than a direct contribution of cells to fistula repair. Such
22 experiments unraveling the fate and mechanism of action of the administered stem cells will be crucial
23 in the long run for clinical translation. A fourth study evaluated the benefits of local injection of
24 allogeneic rat AdSCs (4 million cells) in the perifistular tissues in a similar model of caeco-cutaneous
25 fistula in the rat 4 weeks after fistula creation [100]. The control group was injected with a similar
26 amount of saline instead. One month after treatment, 6 fistulae out of 16 had closed in the AdSC group
27 against 1 out of 16 in the control group (p=0.033); and no deaths or treatment-related adverse effects
28 were reported in either group. However, these clinical observations were not completed by either
29 radiological or histological analyses of the fistulous tract.

30 Overall, current evidence from small animal models highlights the potential of the local
31 administration of stromal cells as a curative treatment for enterocutaneous fistulae. However, more
32 experimental studies are warranted in large animal models, with longer follow-up periods, thorough
33 investigation of fistula healing through different modalities, and a more in-depth analysis of the
34 mechanism of action through which cells contribute to fistula healing.

35 3.2 Stem/stromal cells for the treatment of post-surgical fistulae – Evidence from clinical trials

36 As of today, few clinical trials assessing the potential of stem/stromal cell therapy for the
37 treatment of post-surgical fistulae have been conducted, however, current evidence, though scarce,
38 remains promising.

39 A first open-label phase I clinical trial investigated the safety and efficacy of autologous adipose-
40 derived cells (AdRCs) for post-surgical enterocutaneous fistulae [101]. Autologous AdRCs (similar to
41 SVF, non-expanded mononuclear cells from the adipose tissue containing a heterogeneous cell
42 population with relatively high concentrations of AdSCs) were isolated from gluteal subcutaneous
43 adipose tissue samples using the Celution® system. 6 patients with persisting refractory
44 enterocutaneous fistulae secondary to gastrointestinal surgery were included. All patients had
45 underlying inflammatory bowel disease (4 cases of ulcerative colitis, including one with a concomitant
46 rectal carcinoid, and 2 cases of Crohn's disease), and had developed enterocutaneous fistulae
47 following total proctocolectomy with ileal pouch-anal anastomosis, or low anterior resection, or
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1 ileocecal resection (with or without anterior resection). Fistula duration was 5-44 months. Treatment
2 was administered locally, with half of the cells injected in the neighboring tissues (endoscopically and
3 percutaneously), and the other half mixed with fibrin glue and injected into the fistula tract to seal it,
4 before closure of the external opening. The dose ranged from 9.6 to 142 million AdRCs. Fistula
5 closure was defined as the cessation of drainage with no recurrence of an abscess or fistula observed at
6 endoscopy or computed tomography. 5 patients out of 6 achieved fistula closure at 4 weeks, which
7 persisted to the end of the follow-up period (24 weeks) associated with ileostomy closure in 4 patients
8 and the possibility to resume oral food intake in 1. The 6th patient achieved complete re-
9 epithelialization only at week 24 and presented a recurrence at 10 months due to pouchitis relapse. No
10 serious adverse effect related to treatment was reported. Adverse effects related to treatment consisted
11 in pain and mild subcutaneous haemorrhage linked to liposuction. Overall, this pilot clinical trial
12 reveals encouraging preliminary results in terms of safety and efficacy of local AdRC therapy in this
13 indication, warranting further investigation to confirm them at a larger scale in randomized, placebo-
14 controlled clinical trials.

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18 Another phase I clinical trial, led by W.A. Faubion at the Mayo Clinic, investigating local AdSC
19 administration via a bioabsorbable fistula plug for post-surgical fistula treatment has recently been
20 completed (n°NCT02807389). These results are yet to be published. Their protocol was based on
21 previous clinical trials investigating AdSC delivery through a fistula plug for the treatment of
22 **fistulizing Crohn's disease (perianal and rectovaginal fistulae [102,103])** and cryptoglandular perianal
23 fistulae [104]. 15 patients were to be enrolled. The main inclusion criteria consisted in the presence of
24 a single-tract persisting symptomatic fistula secondary to gastro-esophageal resection, enteric or
25 colonic resection, or bariatric surgeries and refractory to standard therapy (including at least one
26 endoscopic attempt at fistula closure). Patients with inflammatory bowel diseases, with a malignancy
27 or previous irradiation at the location of the fistula, with sepsis, immunosuppressive treatments or
28 chemotherapy were excluded. Other exclusion criteria included non-enterocutaneous, multiple or end
29 fistulae, fistula tract below 2 cm in length and/or over 1 cm in diameter, fistula opening into an
30 abdominal wall defect or fistula output over 2L per day. The fistula plug, loaded with 20 million
31 AdSCs, was to be placed endoscopically. The follow-up period was scheduled to extend for 18
32 months. The primary endpoint was to be safety and tolerability, and efficiency was to be evaluated as a
33 secondary endpoint through the number of patients with fistula closure (defined as the cessation of
34 drainage).

4. Extracellular vesicles for the treatment of post-surgical fistulae

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1 gel+EVs group. Of note, a decrease in inflammatory cell infiltration and in fibrosis along with
2 increased wound healing and angiogenesis were observed in the treatment groups compared to the
3 control, but this was significant only for the gel+EVs group. Overall, this study provided the first *in*
4 *vivo* evidence of the interest of local EV administration for the treatment of post-surgical digestive
5 fistulae. A second study in a rat model of post-surgical enterocutaneous fistulae created by caecostomy
6 tended to confirm these results with a decrease in fistula diameter and drainage after administration of
7 6×10^{11} murine C3H/10T1/2- derived EVs in poloxamer 407 compared to poloxamer 407 alone or
8 saline [58]. A decrease in fibrosis and inflammation along with an increased angiogenesis could also
9 be evidenced histologically. Moreover, a biodistribution study over 7 days after radiolabeling with
10 [^{89}Zr] zirconium evidenced that local EV administration within poloxamer 407 enabled a better
11 retention of EVs at the fistula site than either local injection in saline or intravenous administration
12 [58]. However, further studies are warranted to replicate these promising results with higher numbers
13 of animals and longer follow-up periods. Notably, a more thorough investigation of the mechanism of
14 action of EVs should be performed before any attempts at a potential clinical translation. Additionally,
15 it would be interesting to vary the amount of EVs administered so as to study a potential dose/response
16 relationship, to compare the therapeutic potential of EVs with that of parent cells in this indication, or
17 to further investigate potential links between mode of delivery, EV retention within the fistula tract
18 and therapeutic effect.

24 5. Stem/stromal cells and EVs for the treatment of fistulizing Crohn's disease

26 5.1 Preclinical models of fistulizing Crohn's disease for stem/stromal cell therapy applications

27 A number of experimental models of digestive fistulae have been established over the years to
28 gain a more thorough understanding of the pathophysiology of fistulizing diseases, explore potential
29 new therapies and unravel the mechanisms of action of the therapies used in clinical settings
30 (Supplementary Table 2). However, most of them are not relevant for the study of Crohn's-related
31 fistulae due to the lack of underlying inflammatory bowel diseases.

32 A first model of fistulizing Crohn's disease was reported by Rivera-Nieves *et al.* in 2003 in
33 rodents [105]. Mice of the Samp1/YitFc strain spontaneously develop severe chronic ileitis whose
34 histological features strongly resemble those of Crohn's disease. Less than 5% of these mice
35 additionally presented perianal involvement, which included the occurrence of ano-cutaneous fistulae
36 in 40% of the cases. Therefore, though this mouse strain certainly constitutes an interesting model of
37 Crohn's disease, its relevance for the development of new therapies for perianal fistulizing disease is
38 hampered by this limited overall fistula incidence.

39 Canine anal furunculosis has also been suggested as a relevant model for perianal fistulizing
40 Crohn's disease [106,107], although the cutaneous sinus tracts do not usually communicate with the
41 rectum in this condition, unlike in Crohn's fistula-in-ano [107]. However, owing to the preclusion of
42 systematic autopsy, the financial costs and the logistics involved in trials with companion animals
43 [108], its widespread use for preclinical pharmacological studies for the treatment of fistulizing
44 Crohn's disease might not be feasible.

45 In 2019, Flacs *et al.* developed an experimental model of perianal fistulizing Crohn's disease by
46 inserting a surgical thread for 28 days across the anal sphincter of rats with 2,4,6-
47 trinitrobenzenesulfonic acid (TNBS)-induced chemical colitis [109], with biweekly instillations of
48 TNBS to maintain inflammation. Persistence of the fistulous tract 1 week post thread removal was
49 proven by MRI in all 9 animals, and the radiopathological findings strongly resembled those observed
50 in perianal fistulizing Crohn's disease. The main limitation of this model, besides those classically
51 associated with the TNBS colitis model [110,111], is the lack of study of the persistence of the fistula
52 tract beyond one week post thread removal. Nevertheless, provided these results can be validated in
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1 further studies, this straightforward fistula induction protocol and these high fistula persistence rates at
2 1 week render it very interesting for the investigation of new therapies for Crohn's fistulae.

3 Finally, a mouse model of enterocutaneous fistula was established in 2019 by the subcutaneous
4 xenotransplantation of fetal human gut in immunodeficient mice, which resulted in spontaneous fistula
5 development between the transplant and the skin in 17% of the cases, associated with lymphocytic
6 enteritis and histopathological features close to those of fistulizing Crohn's disease [112]. The
7 relatively low fistulization rate and the ethical and practical concerns raised by the use of human fetal
8 gut are the two main drawbacks to the widespread use of this model. However, it remains the only
9 preclinical model of enterocutaneous Crohn's-associated fistulae described to this day.

10 To the best of our knowledge, only one study investigated the potential of stem/stromal cell
11 therapy in an animal model of fistulizing Crohn's disease [106]. This open-label, uncontrolled, single-
12 center clinical trial included 6 dogs with canine anal furunculosis resistant to standard therapy
13 (cyclosporine). Its main endpoints were the number and depth of fistulae at 6 months post
14 stem/stromal cell therapy and the average dose of cyclosporine necessary to keep the dogs in
15 remission. The dogs were treated with a single local injection of 20 million human embryonic stem
16 cells (hESC)-derived mesenchymal stem/stromal cells and fibrin glue. No adverse event was reported
17 except one mild perianal erythema, and no open fistula or ulcer was observed in any of the dogs at 3
18 months post treatment. Two recurrences were reported at 6 months of follow-up and the mean dose of
19 cyclosporine received by the dogs did not decrease significantly. Overall, this study demonstrated the
20 safety and efficiency of the mentioned approach as an add-on therapy to immunosuppressive drugs for
21 the treatment of perianal fistulizing Crohn's disease in a large animal model. Its main limitations
22 reside in the low number of animals included, the lack of a control group, and the use of SCs derived
23 *in vitro* from hESC which might hamper any eventual clinical translation.

30 5.2 Stem/stromal cell therapy for the treatment of fistulizing Crohn's disease – Evidence from 31 clinical trials

32 Since the first case report in 2003 showing the potential of local autologous AdSCs injection for
33 the repair of recurrent Crohn's-related rectovaginal fistula [113], a number of clinical studies have
34 investigated the safety and efficacy of stem cell therapy in fistulizing Crohn's disease (Table 1,
35 [102,103,114–136]). Notably, in 2009, a multicenter, open-label, controlled phase II clinical trial on
36 49 patients with perianal fistulae either Crohn's-related (n=14) or of cryptoglandular origin (n=35)
37 investigated the potential of autologous AdSCs as an add-on therapy to fibrin glue and demonstrated a
38 better healing in the AdSC group (71% in the Crohn's disease subset) than in the control group (14%
39 in the Crohn's disease subset) 8 weeks after treatment, with a recurrence rate of only 17.6% at one
40 year [115]. An uncontrolled multicenter phase II study from another group confirmed those
41 encouraging results in 2013 with complete healing observed in 28 patients out of 42 (intention-to-treat
42 analysis, 66.7%) at 8 weeks [122] with no recurrence at 2 years in 83.3% of the cases [124]. Other
43 trials explored the local injection of autologous AdSCs in conjunction with serum [136] or platelet-
44 rich plasma [131,132] instead of fibrin glue (Table 1). Interestingly, the combination of cell
45 administration with another sphincter-preserving technique for Crohn's-associated fistula repair has
46 also been studied: indeed, Dietz *et al.* and Lightner *et al.* reported the use of autologous AdSC-coated
47 fistula plugs in phase I clinical trials, for the repair of perianal and rectovaginal fistulizing Crohn's
48 disease (Table 1, [102,103]).

49 The use of autologous SVF or AdRCs, both obtained from adipose tissue without the need for cell
50 expansion, and/or of autologous adipose tissue injection for fistulizing Crohn's disease has also been
51 described [116,129,133–135,137–139].

52 In 2011, a pilot phase I/II clinical trial of multiple BM-SC injections for the treatment of perianal
53 and enterocutaneous Crohn's-related fistulae on 10 patients evidenced complete healing at 1 year in 7
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1 patients and a decrease of >50% of fistula drainage in 3 [118]. Allogeneic BM-SCs injections have
2 **also been investigated in refractory perianal fistulizing Crohn's disease in a double-blind, placebo-**
3 **controlled, dose-escalating clinical trial [126], and were found to induce a significantly better healing**
4 **at week 6 at a dose of 30 million cells compared to placebo (though the low numbers of patients**
5 **included impeded the generalization of this difference to subsequent endpoints).**

6 Two phase I/IIa clinical trials have been conducted on patients with perianal [121] or rectovaginal
7 [128] **fistulizing Crohn's disease to study the safety and efficacy of allogeneic AdSCs local injection**
8 **without fibrin glue adjunction.** For perianal fistulae, at 24 weeks post injection, a total of 9 patients
9 (56.3% of the full analysis population) demonstrated closure of the external openings with combined
10 remission – defined as the complete re-epithelialisation of external orifices, the absence of fistula
11 drainage, either spontaneous or upon compression, and the absence of collections above 2cm
12 communicating with the treated fistula tract at MRI – found in 6 patients (30% of the full analysis
13 population) [121]. For rectovaginal fistulae, the final efficacy rate was of 60% (3 patients healed out of
14 5 at 52 weeks of follow-up), however the reliability of those results can be questioned since half of the
15 included patients dropped out of the study before completing the follow-up period due to a flare-up of
16 luminal disease and/or the need for biological medication [128]. A pilot, dose-escalation trial from
17 another group in 2016 investigated the safety and efficacy of allogeneic AdSCs local injection with
18 fibrin glue for perianal **fistulizing Crohn's disease.** However, the limited number of patients included
19 in each group precluded them from drawing any firm conclusion on the effectiveness of the 2 different
20 concentrations of cells they tested [127].

21 The first results from the pivotal ADMIRE-CD trial (n°NCT01541579), a large, phase III,
22 randomised, double-blind, placebo-controlled, multicenter (49 different centers in 8 countries) clinical
23 trial evaluating the potential of allogeneic AdSCs **for the treatment of refractory complex Crohn's-**
24 **associated perianal fistula** were published in 2016 [130]. 212 patients were included, and received a
25 single local injection of 120 million allogeneic AdSCs (n=107) or an equivalent volume of saline
26 (24mL, n=105). The primary endpoint of combined remission at week 24 was achieved in 50% (53 out
27 of 107 patients) in the AdSC group versus 34% of the control group (36 out of 105 patients, p=0.024)
28 in the intention-to-treat analysis. Higher clinical remission rates (53% versus 41%) and clinical
29 response rates (66% versus 53%) were observed by week 24 in the AdSC group than in the control,
30 however, the difference was not significant. 131 patients from the phase III trial completed a long-term
31 follow-up study (70 in the treatment group and 61 in the placebo group). This study evidenced a lower
32 recurrence rate in the AdSC group than in the control group at 52 weeks of follow-up [140]. These
33 **positive efficacy results for Crohn's-associated fistulae** have mainly been attributed to the
34 immunomodulatory properties of SCs, and notably to indoleamine 2,3-dioxygenase-dependant
35 regulation of T-cell activity[141,142].

36 Most studies have reported a good safety profile of local stem/stromal cells administration for the
37 treatment of **fistulizing Crohn's disease (Table 1,[102,103,114–136]).** In particular, the ADMIRE-CD
38 trial reported treatment-related adverse events in 18 out of 103 patients included in the safety analysis
39 in the AdSC group (17%) versus 30 out of 102 in the control group (29%), including 5 cases of serious
40 treatment-related adverse events in the AdSC group against 7 in the control, by week 24 [130]. After
41 52 weeks of follow-up, a total of 21 treatment-related adverse effects had been described out of 103
42 patients (20.4%) in the AdSC group versus 27 out of 102 (26.5%) in the control, including 7 cases of
43 serious treatment-related adverse events in each group [140]. One of the greatest concerns with
44 stem/stromal cell therapy is neoplasia development. Biopsies taken from the injection area of 5
45 patients included in a phase II clinical trial of autologous AdSC and fibrin glue injection for the
46 treatment of complex perianal fistulae [115] showed no evidence of neoplastic tissues up to 2 years
47 after treatment [143], and similarly, no signs of cytologic transformation were observed in biopsies
48 taken at 7 months and 1 year from treatment with autologous AdSCs of, respectively, an

1 enterocutaneous fistula and a rectovaginal fistula in the initial phase I trial of 2005 [114]. However,
2 following allogeneic BM-SCs injections for the treatment of refractory perianal fistulizing Crohn's
3 disease, 2 cases of neoplasia were reported: a cecal adenocarcinoma with peritoneal carcinomatosis
4 >15 months after the intervention [126] and a rectal Epstein-Barr virus-associated B-cell
5 lymphoproliferative disease 4 years after the intervention [144]. In the first case, a family
6 predisposition was suggested while for the rectal lymphoproliferative disease, it was imputed to
7 **Crohn's disease and prolonged immunosuppressive treatments without totally excluding a potential**
8 contribution of allogeneic BM-SC injection. Recently, a case series gave some first encouraging
9 insights on the safety profile of SC therapy in terms of fertility and pregnancies [145].

10 Several systematic literature reviews and meta-analyses in the past 3 years underlined the
11 acceptable safety profile of **SC therapy for perianal fistulizing Crohn's disease** [146–149]. For
12 instance, a meta-analysis from 3 controlled trials by Lightner *et al.* reported an absence of significant
13 increase of either adverse events (OR = 1.07, 95% confidence interval [0.61;1.89], p=0.81) or serious
14 adverse events (OR= 0.53, 95% confidence interval [0.28;0.98]; p=0.04) after treatment with SCs
15 [148].

16 Those positive results in terms of efficacy and security led to the market approval by the
17 European Medicines Agency of expanded allogeneic AdSCs for the treatment of refractory complex
18 **Crohn's-associated perianal fistulae** in 2018 (market authorization n° EU/1/17/1261/001).

24 5.3 Extracellular vesicles therapy for fistulizing Crohn's disease – A future direction?

25 Evidence from small animal models tends to indicate a potentially beneficial effect of systemic,
26 intra-peritoneal or local stem cells-derived EVs administration for luminal Crohn's disease [150–152].
27 Thus, in view of emerging experimental data on the use of EVs for post-surgical gastrointestinal
28 fistula treatment [57,58], we consider that EV therapy for perianal and non-perianal fistulizing
29 Crohn's disease could constitute an interesting future preclinical research axis.

33 6. Limits, challenges and perspectives for clinical translation

34 6.1 Defining the optimal cell source, formulation and administration protocol – An ongoing 35 challenge

36 As of today, the question of the optimal formulation and
37 administration protocol for stem/stromal cell therapy
38 products for post-surgical and/or Crohn's-associated
39 digestive fistula treatment remains to be solved. Indeed,
40 though clinical trials have investigated a variety of potential
41 cell sources (adipose-derived versus bone marrow-derived,
42 autologous versus allogeneic, with or without expansion,
43 etc), no controlled trial has formally compared them with
44 one another to investigate potential differences in terms of
45 safety and/or efficacy. In the absence of such evidence,
46 other considerations, such as availability, number and cost
47 of procedures, should be taken into account for clinical
48 studies or compassionate use programs. AdSCs can usually
49 be obtained at high yields through liposuction [14];
50 however, this might not be feasible in some patients with
51 insufficient levels of adipose tissue. The procedure to
52 harvest BM-SCs, requiring bone marrow aspiration, is classically more invasive [14]. While
53 autologous stem/stromal cells are usually considered safer, results from clinical trials of allogeneic
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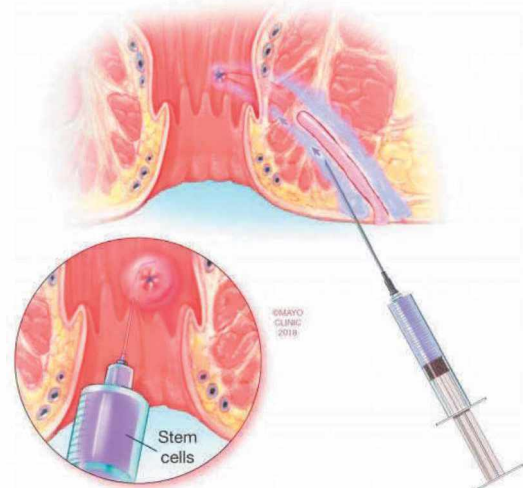


Figure 4: Stem cell application to the internal orifice and fistula tract walls according to standardized protocols. Reprinted with permission from Springer, [153] ©2018

ones administrated **in perianal fistulizing Crohn's disease demonstrated an acceptable safety profile** [121,126–128,130,140]. Although some patients developed donor-specific antibodies directed against HLA-I, it was not correlated with therapeutic efficacy [153]. The use of an allogeneic cell source might therefore present several advantages, such as immediate, off-the-shelf availability, avoiding potential age-related or disease-related effects on stem/stromal **cells' therapeutic potential**, and limiting the number of interventions performed on the patient [121,126]. The use of autologous SVF/AdRCs avoids the need for cumbersome, expensive expansion procedures, while permitting re-implantation in the same day, thus limiting the therapeutic delay observed with expanded autologous cells. Moreover, their isolation can be performed in completely closed systems, which will further decrease the risk of sample contamination [134,135,154].

The question of the best administration protocol to maximize therapeutic efficacy while limiting complications and invasiveness constitutes yet another fundamental consideration. Of note, all trials so far have focused on a local administration route for fistula treatment. Indeed, a local administration would seem more relevant in this indication, by limiting off-target effects [155] and enabling the delivery of larger numbers of cells without additional risks of thrombus formation and pulmonary embolism, as sometimes observed after systemic high dose cell treatment [36,156]. Several clinical trials have investigated a concomitant use of tissue sealing agents such as fibrin glue. Fibrin glue consists in a solution of human fibrinogen, which will form a clot upon adjunction of thrombin and calcium chloride [157]. It has shown a good safety profile but a limited efficacy as a stand-alone treatment for gastrointestinal fistulae [3,73,87,158]. In stem/stromal cell therapy trials, both its use solely as a fistula sealing agent after local cell injection in the perifistular tissues [114,115,120] or as a scaffold to retain part of the cells within the fistula tract [101,116,122,124,127] have been described. However, the added benefits of these approaches to stem/stromal cell therapy alone are yet to be investigated. The administration of AdSC-coated fistula plugs has been described for several different fistula types to permit sustained local retention of high concentrations of cells [102–104]. Though the theoretic rationale behind this approach is certainly attractive, caution is advised in regard of the limited efficacy results reported with fistula plug placement as a treatment for gastrointestinal fistulae [87,158], mostly imputable to early plug extrusion. Of note, this adverse effect, which would lead to a complete loss of all cells administered in this manner prior to fistula healing, has already been reported in the early phase trials of AdSC-coated fistula plugs [103,104]. The question of the benefits of multiple administrations and their intervals, which would seem relevant due to the chronicity of gastrointestinal fistulae, has already been raised [115,118,119,121,122,124,125,128] but never properly studied. However, in view of the costs linked with cell therapy and the demonstration of long-term efficacy in the ADMIRE-CD phase 3 randomized controlled trial after a single dose [130,140], it would seem more relevant to consider a repeat administration only in case of a failure of the first one, as was done in the aforementioned trials. In spite of the variety of administration protocols described in clinical trials, adequate prior seton drainage [102,103,115,119,126,130–132,134–136,140], thorough tract curettage and irrigation [101–103,114–116,118–122,124–128,130–132,134–136,140], and internal opening closure through sutures and/or an advancement flap [114,115,119–122,124,126–128,130–132,134–136,140] constitute commonly accepted preliminary steps, to, respectively, control sepsis, destroy the granulation tissues or neo-epithelialized tissues lining the fistula tract, and prevent the passage of gastrointestinal luminal contents through the tracts. In the **case of perianal fistulizing Crohn's disease, some efforts have been made towards more standardized administration protocols** [155,159]. Both articles stressed the importance of the aforementioned steps, along with the necessity to thoroughly map and identify the fistula tract and openings prior to treatment for an optimal administration. A precise injection protocol was advocated, with injection of half of the cells around the closed internal orifices and of the other half in the perifistular tissues along the fistula walls (**Figure 4**). On the other hand, injection of the cells directly within the fistula lumen

was strongly discouraged in both articles since it would result in their being washed out of the tract. Other important considerations notably included favoring preoperative antibiotherapies, anesthesia techniques and antiseptic agents that will not affect the viability and therapeutic potential of the cell therapy product [155]. A more widespread use of such standardized protocols, validated in clinical trials, would enable an easier comparison of efficacy results between subsequent studies. They may also constitute the first golden standards to serve as a reference for studies aiming at optimizing the administration strategy.

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Table 1: Stem cell therapy trials for Crohn's-associated fistulae. Case reports, case series which do not present a detailed protocol, and studies focusing on the sole administration of adipose tissue were not included. Only the adverse events (AE) and serious adverse events (SAE) that were originally reported as related or possibly related to treatment (stem cell administration or control treatment) are accounted for. Except when stated otherwise, efficacy data are given with regard to the number of patients and not to the number of fistulae. Partial responses (*i.e.* a decrease in fistula output) are not reported here. Recurrence is defined as the reopening of a previously healed fistula. **RCT**: randomized controlled trial; **RVF**: rectovaginal fistulae; **PF**: perianal fistulae; **ECF**: enterocutaneous fistulae; **AdSC**: adipose-derived stromal cells; **SVF**: stromal vascular fraction; **BM-SC**: bone marrow-derived stromal cells. **N.A.**: not applicable; **FG**: fibrin glue. *: the safety and efficacy data and follow-up periods presented between brackets correspond to subsequent long term follow-up studies prolonging the original trial. **: these results correspond to the whole study population, which also included PF of cryptoglandular origin. ***: Half of the patients were excluded during the follow-up period due to severe luminal disease flare-up.

Study	Fistulae	Treatment	Source	Administration	Dose of cells	Treatment-related AE	Treatment-related SAE	Complete healings	Recurrences	Follow-up	Reference(s)
Phase I	RVF/PF/ECF	AdSC (n=4)	Autologous	Local ± FG (1×)	3-30×10 ⁶	None	None	75% of the fistulae	N.A.	12-30 months	[114]
Phase II*	PF	AdSC (n=7)	Autologous	Local + FG (1-2×)	2×10 ⁷ ± 4×10 ⁷	None	None	5 out of 7	17.6%** (5 out of 12**)	1 year (approx. 40 months)	[115,119]
		Control (n=7)	N.A.		N.A.	6**	1	1 out of 7	0 (1 out of 3**)		
2 case series	ECF	AdSC (n=2)	Autologous	Local + FG (1×)	3.5-30×10 ⁶	None	None	75%	0	1 year	[116]
		SVF (n=4)			5.5-176×10 ⁶			25%	0		
Phase I/II*	PF/ECF	BM-SC (n=10)	Autologous	Local (2-5×)	1.5-3×10 ⁷	None (none)	None (none)	7 out of 10	0 (63% after 2 years)	1 year (5 years)	[118,125]
Phase I	PF	AdSC (n=10)	Autologous	Local + FG (1×)	1-4×10 ⁷ /mL	None	None	3 out of 10	0	8 weeks ± 6 months	[120]
Phase I/IIa	PF	AdSC (n=24)	Allogeneic	Local (1-2×)	2×10 ⁷ ± 4×10 ⁷	5	2	9 out of 24	N.A.	24 weeks	[121]
Phase II*	PF	AdSC (n=42)	Autologous	Local + FG (1-2×)	3-6×10 ⁷ /cm fistula length ± 1.5× as much	None (none)	None (none)	28 out of 42	3 out of 26 (4 out of 24)	8 weeks up to a year (2 years)	[122,124]
RCT	PF	BM-SC (n=15)	Allogeneic	Local (1×)	1×10 ⁷	None	None	6 PF out of 9	0	24 weeks	[126]
					3×10 ⁷	None	None	6 PF out of 7	0		
					9×10 ⁷	None	None	2 PF out of 7	0		
					N.A.	None	None	3 PF out of 9	0		
Pilot	PF	AdSC (n=6)	Allogeneic	Local + FG (1×)	1-3×10 ⁷ /mL	None	None	3 out of 6	0	8 months	[127]
Phase 3 RCT*	PF	AdSC (n=107)	Allogeneic	Local (1×)	1.2×10 ⁸	18 (21)	5 (7)	53 out of 107	0 (13/52)	24 weeks (52 weeks)	[130,140]
		Control (n=105)	N.A.		N.A.	30 (27)	7 (7)	36 out of 105	0 (15/34)		
Phase I/IIa	RVF	AdSC (n=10)	Allogeneic	Local (1-2×)	2×10 ⁷ ± 4×10 ⁷	None	None	3 out of 5***	N.A.***	52 weeks	[128]
Phase I	PF	AdSC (n=12)	Autologous	Local on a fistula plug (1×)	2×10 ⁷	None	None	10 out of 12	0	6 months	[102]
Pilot	PF/pouch-vaginal	AdSC (n=9)	Autologous	Local + platelet-rich plasma (1×)	1-2×10 ⁸	None	None	10 fistulae out of 11	0	21-37 months	[131,132]
Phase I	PF	SVF (n=10)	Autologous	Local + adipose tissue (1×)	1.09-4.78×10 ⁷	5	1	6 out of 10	0	48 weeks	[134,135]
Phase I	RVF	AdSC (n=5)	Autologous	Local on a fistula plug (1×)	3.5×10 ⁷	None	None	0 out of 5	0	6 months	[103]
RCT	PF	AdSC (n=11)	Autologous	Local + serum (1×)	5-10×10 ⁶ /cm length	None	None	10 out of 11	3 out of 10	1 year	[136]
		Control (n=11)	N.A.	N.A.	N.A.	11	None	6 out of 11	0		

6.2 *Towards a more widespread use – Darvadstrocel and stem/stromal cell therapy for fistula treatment in clinical reality*

To this day, allogeneic AdSC administration as described in the ADMIRE-CD phase III randomized controlled trial [130,140] constitutes the only stem/stromal cell therapy product to have received marketing authorization for gastrointestinal fistulae treatment, as a second line treatment for complex Crohn's-associated perianal fistulae refractory to conventional therapeutic agents and/or biotherapies, under the designation "darvadstrocel" (Alofisel). This relatively restrictive indication constitutes one of the major obstacles towards a more widespread use of darvadstrocel and stems directly from the very specific patient populations enrolled in the ADMIRE-CD trial. Indeed, this trial, like most clinical studies so far, has solely focused on perianal fistulizing Crohn's disease, thus there is not enough clinical evidence yet to recommend the use of this cell therapy for other types of gastrointestinal fistulae outside of clinical studies or compassionate use programs. Moreover, no results have been published to this date in the paediatric population, thus the use of darvadstrocel is currently only indicated for adult patients. Additionally, like most innovative therapies involving cells, darvadstrocel remains very expensive. The reported price for a single dose containing 120 million cells reaches 54,000€. Thus, the feasibility of routine use in clinical practice will depend on the possibility of reimbursement and funding, which may not be approved in some countries.

For instance, while the French National Authority for Health has considered darvadstrocel sufficiently beneficial to be eligible for reimbursement by the Social Security, the National Institute for Health and Care Excellence has not given a positive recommendation for funding in the United Kingdom. The medical community nevertheless seems rather favorable to the possibility of allogeneic AdSC injections in this indication. It was indeed already described as a potentially safe and efficient alternative in the most recent guidelines by the European Crohn's and Colitis Organization, although the need for longer term follow-up studies has been emphasized [87]. In this regard, several clinical trials have been launched to answer remaining issues: a multicenter single group open-label trial of single-dose darvadstrocel injection in adult patients with complex refractory perianal fistulizing Crohn's disease is currently being conducted in Japan with a follow-up of safety extending over 3 years (trial n°NCT03706456). A second phase III randomized controlled trial is currently recruiting to confirm the safety and efficacy data of darvadstrocel at a wider scale (trial n°NCT03279081). Finally, an open-label phase 4 study is planned to investigate the long-term safety of repeated administration of darvadstrocel (trial n°NCT04118088).

6.3 *EVs for fistula treatment – a promising research axis despite ongoing translational hurdles*

Beyond their therapeutic potential and the emerging preclinical evidence of their efficacy for fistula treatment, EVs present several advantages which justify ongoing research in spite of the drastically more advanced results already obtained with stem/stromal cell therapy. First and foremost, as a cell-free therapeutic modality, EVs could alleviate most of the remaining safety concerns associated with cells, such as the risks of proliferation and inadequate differentiation; while offering an easier storage and limiting the complex handling issues linked with viable stem cell therapy products [57,160,161]. Additionally, EVs can be loaded with drugs, nanoparticles or other agents for controlled drug delivery or imaging purpose [38]. Specifically, in the field of fistula therapy, it could be envisioned to load EVs with anti-bacterial agents to help control sepsis or with immunomodulators in the case of fistulizing Crohn's disease. Of note, their structure comprises a bilayered phospholipidic membrane, allowing local drug delivery through possible fusion with the cell membrane, and protecting their contents from potentially aggressive extracellular environments [38] – here, a fistula tract exposed to gastrointestinal secretions containing enzymes, feces, etc. Moreover, they are produced physiologically and, as such, are less likely to be immunogenic than artificial vectors [38]. In this regard, stem/stromal cell-derived EVs may present an immune-privileged status potentially compatible with their use in allogeneic settings, thus providing off-the-shelf availability which would tremendously facilitate any eventual clinical translation [57]. However, several technical challenges still remain to be overcome before their use in clinical trials (not to mention routine clinical practice) can be envisioned. On the one hand, most

EV production methods, like parental cell culture in hollow-fiber bioreactors, or under serum starvation, remain very time-consuming, and do not permit to obtain high yields of EVs [38]. Though emerging methods based on the use of physical triggers such as shear stress have drastically improved the yields [38], exposing cells to intense physical stresses might alter their viability and integrity, with a potential impact on the quality of produced EVs, which should therefore properly be characterized. The development of optimized production processes, to obtain high yields of EVs, implementable at a wide scale and compatible with the good manufacturing practices, therefore constitutes a necessary first step to launch wide scale preclinical studies or clinical trials. The same considerations should apply to subsequent isolation steps (which classically include ultracentrifugation, tangential flow filtration, and/or chromatography as of today) [161], while keeping in mind that many different factors throughout the fabrication process – from the choice of parental cells to the final isolation and concentration steps – may contribute to alter the EVs’ contents and therefore their therapeutic potential, thus stressing the need for extensive characterization all throughout the development of the product [40]. On the other hand, ensuring patients’ safety during early phase clinical trials constitutes a major concern for such an exploratory treatment. Beyond the collection of extensive preclinical safety data, a consensus should be reached on the best characterization assays and quality controls to perform to check batch quality and sterility. These assays should ideally associate low cost, straightforward protocols, and not be overly time-consuming to remain compatible with clinical practice. Overcoming such hurdles would enable a smooth clinical translation, both for fistula therapy and in other indications.

7. Conclusion

Innovative stem/stromal cell-based therapies have provided a major hope for digestive fistulae management. Especially encouraging results were obtained both in terms of safety and efficacy for perianal fistulizing Crohn’s disease, where local allogeneic AdSC injection constitutes a clinically accepted therapeutic alternative for patients who are refractory to standard treatment. Though some tentative steps have been made towards the development of a standardized administration protocol, optimizing delivery strategies may tremendously affect treatment efficacy. The cost of such therapies remains one of the major obstacles towards a widespread use in clinical practice. Recently, emerging preclinical evidence has stressed the therapeutic interest of EVs for post-surgical digestive fistulae. While further exploratory studies are warranted, both for post-surgical and Crohn’s-associated fistulae, the development of adequate production, isolation and characterization processes still constitutes a necessary first step before clinical translation can be envisioned.

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Declaration of interest

Florence Gazeau, Amanda Karine Andriola Silva, Claire Wilhelm and Gabriel Rahmi are co-founders of the spin-off Evora Biosciences. Amanda Karine Andriola Silva and Claire Wilhelm are co-founders of the spin-off EverZom. The other authors have no conflicts to declare.

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