Gastroenterology 2019;156:2208–2216

CLINICAL AT

Efficacy of Injection of Freshly Collected Autologous Adipose Tissue Into Perianal Fistulas in Patients With Crohn's Disease

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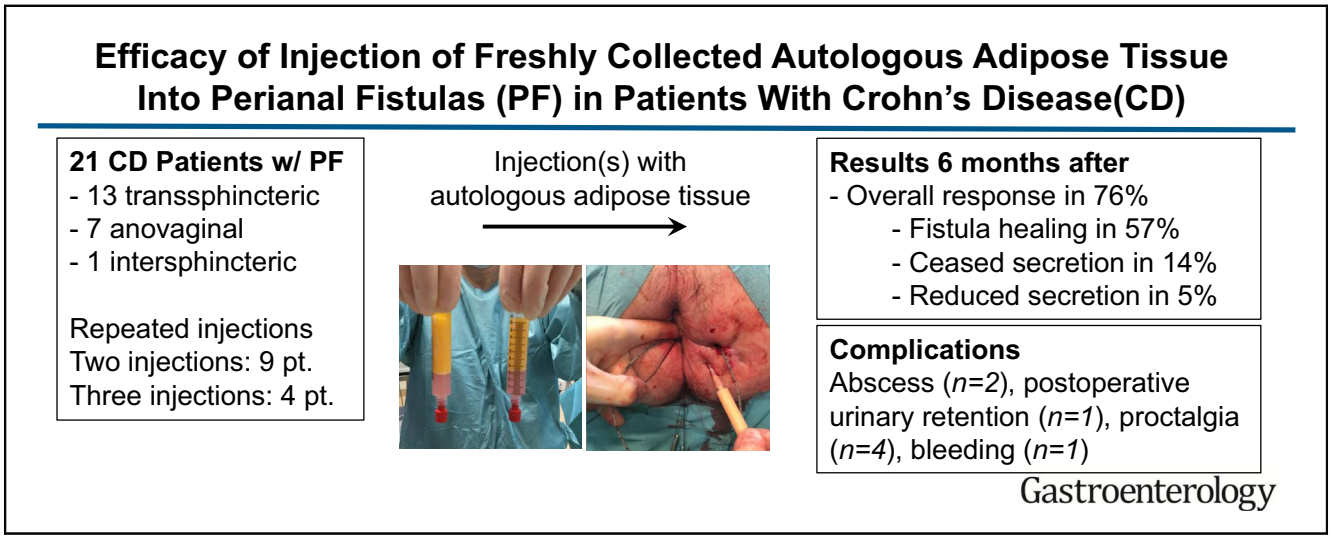

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BACKGROUND & AIMS: Perianal fistulas are common in patients with Crohn's disease (CD). Injections of cultured autologous and allogeneic adipose tissue-derived stem cells have been shown to heal CD-associated fistulas. Unfortunately, this treatment is time consuming and expensive. We investigated the effects of injecting freshly collected autologous adipose tissue into perianal fistulas in patients with CD. **METHODS:** In a prospective interventional study, freshly collected autologous adipose tissues were injected into complex perianal fistulas of 21 patients with CD, from March 2015 through June 2018. The primary endpoint was complete fistula healing (no symptoms of discharge, no visible external fistula opening in the perineum, and no internal opening detected by rectal digital examination) 6 months after the last injection. We performed pelvic magnetic resonance imaging to confirm fistula resolution in patients with intersphincter and transsphincter fistulas who showed complete healing at clinical examination. Patients without complete fistula healing after 6 weeks and those with later relapse were offered additional injections. No control individuals were included. **RESULTS:** Six months after the last adipose tissue injection, 12 patients (57%) had complete fistula healing. Three patients (14%) had ceased fistula secretion, and 1 patient (5%) reported reduced secretion. Among 10 patients with trans-sphincter or intersphincter fistulas, magnetic resonance imaging showed complete fistula resolution in 9 patients and a markedly reduced gracile fistula in the remaining patient. Of the 12 patients with complete fistula healing, 9 (43%) required 1 injection, 2 (10%) required 2 injections, and 1 (5%) required 3 injections. The predominant adverse effect was postprocedure proctalgia lasting a few days. Two patients developed small abscesses, 1 had urinary retention, and 1 had minor bleeding during liposuction. **CONCLUSION:** In a study of 21 patients with CD and perianal fistulas, we found injection of recently collected autologous adipose tissue to be safe and to result in complete fistula healing in 57% of patients. ClinicalTrials.gov, Number: NCT03803917.

Keywords: Crohn's Disease; Perianal Fistulas; Cell- and Tissue-Based Therapy; Treatment.

Abbreviations used in this paper: **CD, Crohn's disease; MRI, magnetic resonance imaging; PBS, phosphate-buffered saline; SVF, stromal vascular fraction.**

Most current article

0016-5085/$36.00
https://doi.org/10.1053/j.gastro.2019.02.005

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Injections of cultured autologous and allogeneic adipose tissue-derived stem cells have shown promising healing rates of Crohn's disease (CD) fistulas. Use of these cells are hampered by either price or demands for laboratory facilities and cell culture.

NEW FINDINGS

A study of 21 patients with CD and perianal fistulas found that injections of freshly collected autologous resulted in complete fistula healing in 57% of the treated patients. The treatment was safe and well tolerated.

LIMITATIONS

The study was not placebo-controlled.

IMPACT

The injection of freshly collected autologous adipose tissue is a promising technique in the treatment of perianal fistulas in patients with CD.

Perianal fistulas are frequently present in Crohn's disease (CD). The cumulative incidence of CD patients who develop perianal fistulas during 20 years of follow-up has been reported as high as 20%–26%, with the greatest risk for developing fistulas occurring in the first 10 years after diagnosis.[1] Despite the introduction of biological treatment, the risk of CD patients developing fistulas has remained stable over the last 2 decades.[2] The presence of fistulas negatively affects a patient's quality of life due to the occurrence of pain, the embarrassment of discharge, and the impairment of both physical and sexual functions.[3] The best approach to achieving fistula healing is a combination of medical and surgical management,[4] resulting in fistula healing in 50% of patients.[3]

Treatment of perianal fistulas with cultured mesenchymal stem cells derived from adipose tissue or bone marrow has shown promising results in both CD[5–11] and non-CD patients.[11,12] The initial studies used cultured, autologous, adipose tissue-derived stem cells and reported a healing rate of up to 64% of treated CD patients.[8] Use of a matrix plug to deliver the autologous adipose tissue-derived stem cells to the fistula tract has also shown promising results, with the healing of fistulas in 83% of the treated CD patients.[6] One small study using bone-marrow–derived stem cells reported that the treatment was safe, but the researchers were unable to demonstrate significant improvement compared with placebo.[10] The use of cultured autologous cells, however, has the disadvantage of being time consuming. In addition to the collection procedure, treatment with cultured autologous cells requires access to laboratory facilities and personnel with expertise in stem cell isolation, as well as weeks of in vitro stem cell expansion. A ready-to-use alternative is in vitro expanded allogeneic stem cells. Allogeneic adipose-derived stem cells were initially reported to induce complete fistula closure in 56% of treated CD patients.[7] Recently, a large, multicenter phase 3 trial reported that treatment of CD fistulas with in vitro expanded allogeneic adipose-derived stem cells resulted in a closure rate of 50%, compared with 34% in the placebo group, at 24 weeks after treatment.[5] This particular treatment effect was maintained after 52 weeks[13] and has recently become commercially available in Europe. Treatment with allogeneic adipose-derived stem cells, however, is expected to be costly and may become a major expense for health care systems.

Freshly collected adipose tissue may represent a faster and more cost-effective alternative to in vitro expanded autologous or allogeneic adipose-derived stem cells. The non-adipocyte fraction of freshly collected adipose tissue, termed stromal vascular fraction (SVF), contains numerous living stromal (mesenchymal) stem cells. Thus, stromal (mesenchymal) stem cells constitute 10%–50% of the cells in SVF.[14–16] Therefore, freshly collected adipose tissue may represent an easily accessible alternative to in vitro expanded autologous or allogeneic adipose-derived stem cells. Recently, we reported encouraging results of injection with freshly collected autologous adipose tissue in a mixed cohort of patients with anovaginal fistulas.[17] Others have reported similar results of injection using freshly collected autologous adipose tissue in non-CD patients with complex transsphincteric fistulas.[18]

In this present study, we report treatment results obtained from the use of injections of freshly collected autologous adipose tissue in 21 CD patients with complex fistulas that had previously been resistant to standard medical and surgical interventions. Our primary objective was complete clinical fistula healing. We also report all secondary treatment outcomes, such as reduced or ceased fistula secretion, as well as any complications resulting from treatment injections.

Methods

Study Design and Patients

We prospectively registered symptoms, complications, and results of treatment in 21 CD patients who had treatment with freshly collected autologous adipose tissue for perianal fistulas between March 2015 and June 2018. Inclusion criteria consisted of the following: the presence of complex CD fistulas refractory to standard surgical intervention, including long-term seton and regular curettage, and/or medical treatment. Furthermore, the fistula had to be assessed as suitable for a final closing procedure by the treating surgeon. The treatment was offered as a less-mutilating alternative to other surgical procedures for fistula closure, and it was also applicable to patients excluded from flap surgery because of the location of the internal fistula opening. The diagnosis of CD had been made according to clinical, endoscopic, histological, and biochemical criteria.[19]

Pelvic magnetic resonance imaging (MRI) was performed to describe the location and extent of the fistula. We did not perform an endoscopy before treatment. Only patients who exhibited normal rectal mucosa or mild proctitis, as determined by anoscopy, were offered treatment.

Patients receiving immunosuppressants or biological treatment continued their specific course of treatment after

adipose tissue transplantation. As a precautionary measure, the treatment was paused from 2 weeks before transplantation to 3 weeks after. None of the patients received corticosteroids.

Patients who developed fistulas after proctectomy were excluded.

Surgical Procedure

A loose seton was placed as a first-step procedure, and repeated curettage was performed every 6–8 weeks until the secretory activity of the fistula was reduced. The fistula was deemed suitable for treatment when some fistula shrinkage had occurred and only limited secretion was observed.

The collection and injection of autologous adipose tissue was carried out in 1 surgical step under general anesthesia. Prophylactic antibiotics (1.5 g cefalotin,1.5 g metronidazole, and 1 g of dicloxacillin) were administered intravenously. A mixture of 400 mg of lidocaine and 1 mg of adrenaline was added to 1000 mL of Ringer's solution. Then, 200–300 mL of this mixture was injected into the subcutaneous adipose tissue of the lower abdomen. Approximately 100 mL of adipose tissue was collected via manual liposuction with a standard 3.5-mm liposuction cannula with side holes. The suspension was centrifuged for 3 minutes at 1000 revolutions per minute (Figure 1*A*), and the aqueous fraction was subsequently expelled. The remaining adipose tissue (approximately 80 mL) was then collected in 10-cm^3 syringes and homogenized by shifting the adipose tissue between 2 syringes connected to each other by a Luer-Lok connector (Figure 1*B*). The microfragmented adipose tissue was transferred to 2- cm^3 syringes and injected around the fistula tract via a blunt Coleman 2.5-mm injection cannula or a sharp 1.2-mm needle.

The patient was placed in the lithotomy position to clean the fistula tract and to close the internal opening. The epithelium and granulation tissue was removed with a curette, and a thin catheter was placed through the fistula to maintain orientation of the fistula tract during the procedure (Figure 1*C*). The epithelium of the internal opening in the anal canal was removed, and the mucosal edges were lifted before the internal opening was closed, including the internal anal sphincter and the mucosa, with Vicryl absorbable sutures (Novosyn 2-0, 5/8c; A.M.I. Ltd, Feldkirch, Austria) (Figure 1*D*).

Two small incisions were made at each side of the external opening of the fistula, and the adipose tissue graft was injected transperineally around the internal opening and around the fistula tract, creating a doughnut form. The adipose tissue was injected via blunt Coleman 2.5-mm injection cannula or a sharp 1.2-mm needle at different levels by using multiple passes from the internal to the external openings until there was firm swelling surrounded the fistula tract (Figure 1*E*). The amount of injected adipose tissue varied between 18 mL and 104 mL (median, 46 mL), depending on the length and size of the fistula tract. With the catheter in the fistula tract used as a guide, the fistula tract was cut transversely with a sharp Coleman fat-injection cannula. This procedure was performed to release fibrotic scar tissue surrounding the fistula, thereby allowing the adipose tissue to be interposed between the cut ends. Ciprofloxacin (500 mg $\times$ 2) was given orally for 5 days, and 1000 mg ascorbic acid was administered daily for 6 weeks. Postoperative pain was treated with paracetamol and ibuprofen. Patients were discharged the following day. If clinical healing of the fistula was not obtained 6 weeks after the first treatment, patients were offered a second adipose injection within 2 weeks. A third injection was offered to patients who did not have complete healing within 6 weeks of the first 2 injections.

Clinical Evaluation of Treatment Effects

Clinical examinations of the patients were performed at intervals of 6 weeks, 3 months, and 6 months after adipose tissue injection. The primary endpoint was complete fistula healing at clinical examination 6 months after the last injection. A fistula was considered completely clinically healed if 1) the patient had no symptoms of discharge, 2) there was no visible external fistula opening in the perineum, and 3) no internal opening could be palpated with rectal digital examination. Secondary endpoints included either reduced or ceased fistula secretion. Pelvic MRI was performed before adipose tissue injection to assist with the surgical procedure and to assess the presence of abscess. To investigate whether treatment had resulted in complete fistula resolution and not only closure of the internal and external openings, pelvic MRI was repeated in all patients with transsphincteric and intersphincteric fistulas who, on clinical examination, showed complete healing at 6 months after the last injection. The MRIs of the anal canal were performed with T2-weighted sequences (both with and without fat saturation), which are very sensitive to fluid identification. An active or nonhealed fistula conducts fluid. Complete fistula healing on MRI was defined as no signs of a fluid-conducting tract at the location of the former fistula. Patients exhibiting evidence of a fluid-containing tract, a fluid-containing defect, or a cavity corresponding to the internal opening of the former fistula were classified having as incomplete healing on MRI, regardless of clinical findings or symptoms.

Fistula resolution of anovaginal fistulas was not confirmed with pelvic MRI because resolution of these short, unbranched fistulas can be easily evaluated through clinical examination. Complications to the treatment were registered in each patient's medical chart and graded according to the Clavien-Dindo classification of surgical complications.[20]

Cellular Characterization of the Adipose Tissue Obtained From Liposuction

Aspirated adipose tissue from a single additional CD patient with a perianal fistula was analyzed. This patient was not included in the original study cohort of 21 patients, but the adipose tissue was handled in a similar fashion. Cells were isolated according to the protocol of Yu et al[21] with a few modifications. Adipose tissue was washed 2 times in phosphate-buffered saline (PBS), followed by collagenase digestion (Invitrogen Col type I, catalog no. 17018-029 [Invitrogen, Waltham, MA], at a concentration of 0.1%, in 1% bovine serum albumin and PBS with calcium and magnesium) for 45 minutes to 1 hour at 37°C in a shaking water bath, followed by centrifugation, red blood cell lysis, filtration through 70-μm mesh, and washing with PBS.

Cell concentration was determined with a cell chamber, and cell viability was determined by flow cytometry with 7-aminoactinomycin D (7-AAD) (BD Biosciences, San Jose, CA; catalog no. 559925) staining of isolated cells according to the manufacturer's instructions. For determining the proportions of cell types among the isolated cells, the cells were immunophenotyped with a panel of fluorescence-conjugated

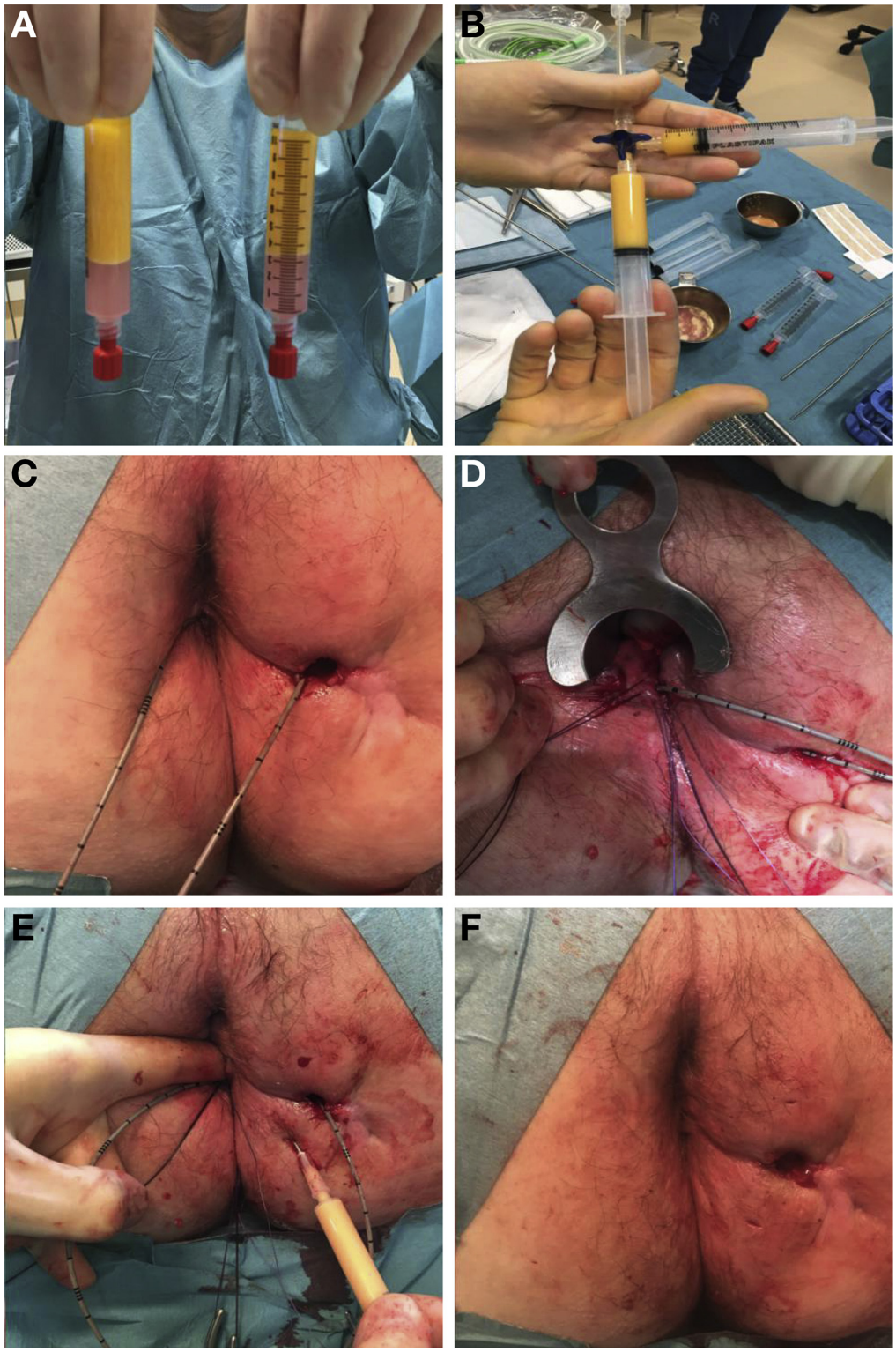

Figure 1. The surgical procedure. Adipose tissue was collected via liposuction. (*A*) Collected adipose tissue was centrifuged, and the aqueous fraction was subsequently expelled. (*B*) The remaining adipose tissue was homogenized by shifting it between 2 connected syringes. (*C*) Epithelium and granulation tissue were removed by curettage, and a thin catheter was placed through the fistula. (*D*) The mucosal edges were lifted before closing the internal opening, including the internal anal sphincter and the mucosa. (*E*) The adipose tissue was injected around the internal opening and around the fistula tract, creating a doughnut shape, until a firm swelling surrounded the fistula tract. (*F*) The perianal area and external opening after the procedure.

antibodies: PE-conjugated-anti-CD44 (Beckmann Coulter Life Sciences Indianapolis, IN; catalog no. A32537), PE-conjugated-anti-CD90 (Beckmann Coulter Life Sciences, catalog no. IM3600U), PE-conjugated-anti-CD105 (Beckmann Coulter Life Sciences, catalog no. A07414), FITC-conjugated-anti-CD271 (BioLegend, San Diego, CA; catalog no. 345104), FITC-conjugated-anti-CD31 (BD Biosciences, catalog no. 555445), PE-conjugated-anti-CD34 (BD Biosciences, catalog no. 555822), PE-conjugated-anti CD14 (BD Biosciences, catalog no. 555398), and antigen-presenting cell–conjugated-anti-CD45 (BD Biosciences, catalog no. 555485). The cells were incubated with Fc-blocking solution (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by incubation with specific antibodies according to the manufacturer's recommendations. After a 30

Table 1. Baseline Demographics and Result of Injections With Freshly Collected Autologous Adipose Tissue

Patient number	Sex	Type of Fistula	Duration of Fistula, *mo*	Medical Treatment	Injections	Result
1	Female	Transsphincteric	84	None	1	No improvement
2	Female	Anovaginal	12	Azathioprine	2	Ceased secretion
3	Female	Anovaginal	72	Infliximab, azathioprine	3	Complete healing
4	Female	Transsphincteric	15	Infliximab	1	No improvement
5	Female	Anovaginal	15	Adalimumab	3	Reduced secretion
6	Female	Anovaginal	36	Infliximab, mercaptopurine	2	Complete healing
7	Female	Transsphincteric	120	Adalimumab, azathioprine	2	Complete healing
8	Female	Transsphincteric	12	Infliximab, azathioprine	1	Complete healing
9	Male	Transsphincteric	26	None	2	No improvement
10	Female	Transsphincteric	48	Infliximab, azathioprine	1	Complete healing
11	Male	Transsphincteric	25	Azathioprine	1	Complete healing
12	Female	Anovaginal	13	Infliximab, azathioprine	3	No improvement
13	Female	Anovaginal	8	Infliximab	3	No improvement
14	Female	Transsphincteric	116	Infliximab, azathioprine	2	Ceased secretion
15	Female	Anovaginal	85	Infliximab, azathioprine	1	Ceased secretion
16	Male	Transsphincteric	56	Infliximab	1	Complete healing
17	Female	Transsphincteric	69	Infliximab, Methotrexate	1	Complete healing
18	Male	Transsphincteric	47	None	1	Complete healing
19	Female	Transsphincteric	34	Infliximab, azathioprine	1	Complete healing
20	Male	Transsphincteric	55	Vedolizumab	1	Complete healing
21	Male	Intersphincteric	23	Adalimumab, azathioprine	1	Complete healing

minutes of incubation in the dark at 4°C, cells were washed and analyzed with a BD LSR II (BD Biosciences). The flow cytometry analysis was performed by Kaluza Analysis Software, version 1.1. The coexpression of CD44/CD271, CD31/CD34, and CD14/CD45 was used for the identification of stromal (mesenchymal) stem cells,[22] endothelial cells, and macrophages, respectively, among the isolated cells. Flow cytometry gating was defined based on relevant isotype controls (as exemplified in Supplementary Figure 1*B*)

Ethics

The study was registered at the Danish Data Protection Agency. According to the Regional Committee on Health Research Ethics, the study did not require formal ethical approval because it was considered quality-improvement related. The study is registered as ClinicalTrials.gov (NCT03803917). All authors had access to the study data and reviewed and approved the final manuscript.

Results

Study Population

Fifteen women and 6 men with CD fistulas received treatment with freshly collected autologous adipose tissue injection. The fistulas were transsphincteric (n = 13), anovaginal (n = 7), and high intersphincteric (n = 1). A single patient had developed the transsphincteric fistula after the inappropriate removal of a CD skin tag. The fistulas had been present for a median of 36 months (interquartile range, 15–69 months) at the time of treatment.

Fifteen patients received treatment with anti–tumor necrosis factor-α antibodies at the time of injection of freshly collected autologous adipose tissue (12 infliximab, 3 adalimumab). Of these, 11 received concomitant treatment with immunosuppressants in the form of azathioprine (n = 9), mercaptopurine (n = 1) or methotrexate (n = 1). Two patients received monotherapy with azathioprine, and a single patient was treated with vedolizumab. Patient characteristics are presented in Table 1.

Effects of Autologous Adipose Tissue Injection

The results of the autologous adipose tissue injections are shown in Figure 2 and Table 1.

Results From a Single Autologous Adipose Tissue Injection. Six weeks after the first autologous adipose tissue injection, 7 (33%) of the 21 total patients not only had complete healing of the fistula on clinical examination but also had a total cessation of symptoms. An additional 6 patients (29%) experienced marked reduction in fistula secretion. Four of these 6 had total cessation of symptoms and had complete healing of the fistula on clinical examination 10–26 weeks after the injection. Two of the 7 patients with initial complete healing 6 weeks after the injection had a recurrence of the fistula before the final 6-month follow-up examination. Thus, 9 patients of the total 21 patients (43%) had complete healing of the fistula 6 months after a single injection. All 9 patients had transsphincteric (n = 8) or intersphincteric (n = 1) fistulas. Pelvic MRI showed complete fistula resolution in 8 of the patients. In the remaining patient, a gracile fistula was still visible, although there was marked improvement compared with the baseline pelvic MRI.

One of the 12 patients who did not achieve complete fistula healing from a single injection with freshly collected autologous adipose tissue experienced ceased secretion 6 weeks after the injection and did not consent for additional treatment. This cessation in secretion was maintained 6 months after the injection.

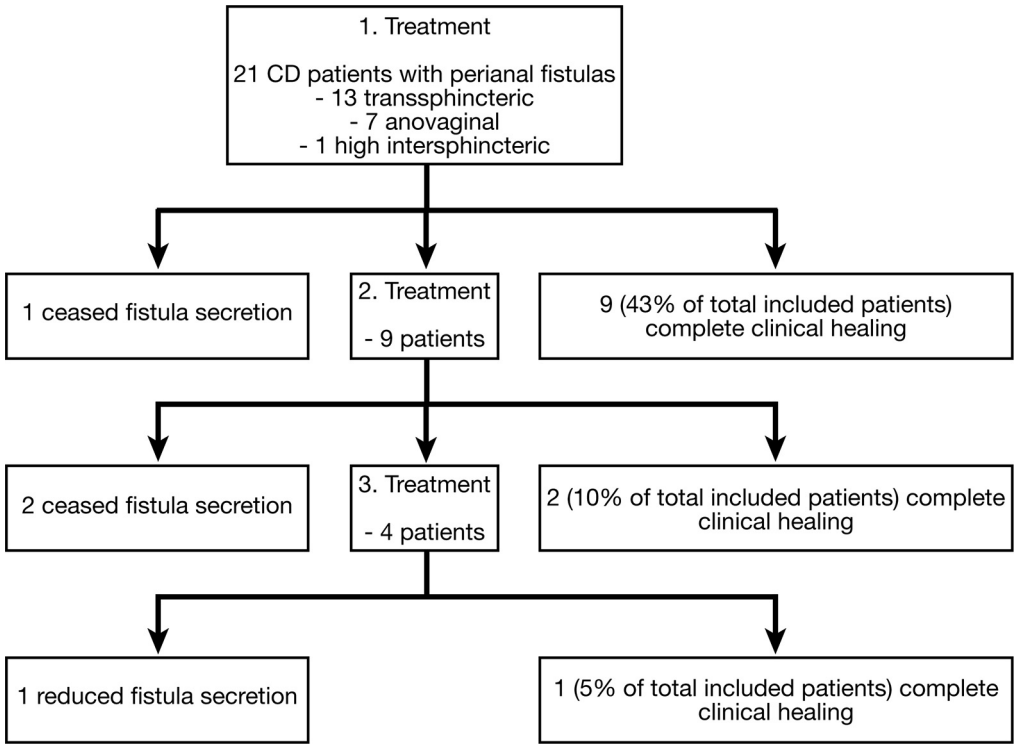

Figure 2. The effects of freshly collected autologous adipose tissue injections. Of the 21 treated patients, 12 achieved complete fistula healing, and 4 patients experienced marked improvement with ceased or reduced fistula secretion. Five patients did not experience any improvement from injections with adipose tissue (1 treatment: n = 2; 2 treatments: n = 1; 3 treatments: n = 2).

Results From Repeated Autologous Adipose Tissue Injections. Among the 12 (57%) patients who did not experience complete healing of the fistula after the first injection, 9 elected to receive a second adipose tissue injection. In 2 of these 9 patients, this second injection resulted in complete fistula healing by 6 weeks, and this response was maintained through the 6-month follow-up. Pelvic MRI showed complete resolution of the treated transsphincteric fistula in 1 of these 2 patients. The other patient had achieved complete healing of an anovaginal fistula. An additional 2 of the 9 patients who received a second adipose tissue injection experienced cessation of fistula secretion by 6 months after the second injection. A third injection was given to 4 (19%) of the total 21 patients. This third injection resulted in the complete healing of an anovaginal fistula in 1 patient and reduced secretion in another patient.

Overall Results From Single and Repeated Injections With Freshly Collected Autologous Adipose Tissue. In total, 12 (57%) patients had complete fistula healing on clinical examination 6 months after 1 (n = 9), 2 (n = 2) or 3 (n = 1) injections with adipose tissue. Among these, 10 had transsphincteric (n = 9) or intersphincteric fistulas (n = 1), and pelvic MRI confirmed complete fistula healing in 9 of these 10 patients. The remaining patient had a gracile fistula tract, which was markedly reduced in size and fluid content compared with the baseline pelvic MRI results. Patients with transsphincteric and intersphincteric fistulas responded much more successfully to the treatment than patients with anovaginal fistulas. Of the 14 total patients with transsphincteric or intersphincteric fistulas, 10 (71%) achieved complete fistula healing. Of the 7 patients with anovaginal fistulas, 2 (29%) patients achieved complete fistula healing.

Moreover, 3 patients (14%) of the total 21 patients reported ceased fistula secretion, and 1 (5%) patient reported decreased fistula secretion 6 months after the final injection. A total of 5 patients did not experience any improvement from injections with adipose tissue.

Complications

The predominant adverse event was proctalgia for a few days after the injection. Minor pain was experienced by the patients after the liposuction procedure. In all but 1 patient, pain was sufficiently treated with paracetamol and ibuprofen. The patient with more pronounced pain required an epidural analgesia. Clinical examination 5 days later showed that the adipose tissue had been injected into the labia major, resulting in the development of an abscess. This patient had a transsphincteric fistula and had received a large volume (104 mL) of injected adipose tissue. The abscess was drained, resulting in the prompt relief from pain. Another patient with an anovaginal fistula developed an ischiorectal abscess after the second adipose tissue injection, thus requiring a surgical incision. This patient had also received a large volume (64 mL) of injected adipose tissue. One male patient experienced urinary retention after the procedure, but bladder function normalized by the following day. In a single patient with sparse subcutaneous abdominal fat, liposuction was discontinued because of minor bleeding, which did not require blood transfusion. Complications and severity grades are given in Table 2.

Table 2. Complications of Injections With Freshly Collected Adipose Tissue

Complication	Patients, n	Classification[a]
Proctalgia	4	I
Abscess	2	IIIb
Bleeding (liposuction)	1	I
Urinary retention	1	I

[a]According to the Clavien-Dindo classification.

Cellular Viability and Characterization of the Adipose Tissue Obtained From Liposuction

The yield of cells obtained from the liposuction was 416,000 cells/mL of aspirate. Flow cytometry analysis showed a viability of 99% among the isolated cells (Supplementary Figure 1*A*). Flow cytometry analysis confirmed that the cells were predominantly of stromal origin, because we observed that 49%–57% of the cells expressed the stromal (mesenchymal) cell markers CD44, CD90, and CD105 (Supplementary Figure 1*B*). Furthermore, flow cytometry analysis showed the presence of 8% stromal (mesenchymal) stem cells ($CD44^+CD271^+$), 20% endothelial cells ($CD31^+CD34^+$), and 1% monocytes/macrophages ($CD14^+CD45^+$) in the total cellular population of the collected adipose tissue (Supplementary Figure 1*C*).

Discussion

To our knowledge, our study is the first to report the effects of injection with freshly collected autologous adipose tissue as a treatment for fistulas in a cohort of CD patients. The treatment was safe and well tolerated. Furthermore, treatment resulted in complete clinical fistula healing in 57% of patients, ceased fistula secretion in 14%, and reduced fistula secretion in 5%, resulting in an overall response to treatment in 76% of the patients. Compared with other emerging treatment modalities for fistulas in CD, the procedure used in our study is extremely simple and inexpensive.

The observed overall healing rate of 57% of patients from the injection of autologous adipose tissue in our present study is comparable to outcomes after the injection of cultured autologous and allogeneic adipose tissue-derived mesenchymal stem cells.[5–9]

However, the results are difficult to compare because different criteria for defining fistula healing were applied in the studies. We used very strict criteria for complete fistula healing, because we did not consider a fistula to be completely healed unless we observed the following: 1) complete reepithelization of the external opening; 2) no internal opening palpable during rectal digital examination; and 3) no discharge. Our study differs from the other studies,[6,7] including the large, placebo-controlled study with allogeneic adipose-derived stem cells by Panes et al,[5] in that they used less stringent criteria to consider a fistula healed. These less rigorous criteria were the absence of secretion by gentle compression with a finger and no collections of more than 2 cm on pelvic MRI. Only the study by Lee et al[8] used criteria comparable to the stringent criteria in this present study. We used pelvic MRI to investigate whether treatment had resulted in complete fistula resolution and not only closure of the internal and external openings in the 10 patients with transsphincteric and intersphincteric fistulas in whom a clinical examination 6 months after the last treatment injection showed complete fistula healing. Pelvic MRI showed complete fistula resolution in 9 out of these 10 patients and marked improvement in the remaining patient. We did not perform an MRI to confirm the healing of anovaginal fistulas, because healing of these short, unbranched anterior fistulas is easily evaluated by clinical examination. One could argue that sufficient assessment of closure of the internal fistula opening of intersphincteric and transsphincteric fistulas includes an examination under anesthesia. However, this would expose the patients for general anesthesia solely for this purpose, which we found unjustifiable from an ethical point of view. Furthermore, regarding the healing of these fistulas, one can always assume that if the external opening persists, so does also the internal opening.

Another issue that makes it difficult to compare study results is the number of treatments each patient received to achieve the reported results. In the large study with allogeneic adipose tissue-derived stem cells by Panes et al[5] and in the study by Dietz et al[6] using autologous adipose-derived stem cells applied in a bioabsorbable matrix, patients were given only a single treatment. As in the studies by Lee et al[8] and de la Portilla et al[7], our patients were offered an additional 1 or 2 treatments if fistula healing was not achieved from a single treatment. In hindsight, we would have preferred a longer period than 6 weeks to observe whether patients achieved fistula closing after the first injection. Because the second injection was preceded by curettage of the fistula tract, it abolished the potential for a late-healing effect of the first adipose tissue injection. Four patients who had almost complete healing of the fistula and total symptom relief at the 6-week clinical examination did not consent to a second injection. These 4 patients showed complete healing at clinical examination within the 6-month follow-up period.

Our method for injection of adipose tissue and the closing of the internal fistula opening is comparable to the methods used in the clinical studies with allogeneic mesenchymal stem cells and the studies using cultured, autologous, adipose tissue-derived stem cells. However, the use of freshly collected autologous adipose tissue has several advantages compared with the use of cultured autologous or allogeneic adipose-derived stem cells. First, the freshly collected adipose tissue is ready to use and easily available in most patients, and the procedure is without significant expense. Second, there is no need for laboratory facilities and time-consuming cell culture, as with the use of cultured autologous adipose-derived stem cells. Our study design is similar to the one reported in a recently published study by Naldini et al,[18] reporting excellent results using freshly collected adipose tissue for the treatment of non-CD fistulas. They applied essentially the same technique as in the present study, although the collected adipose tissue was

processed by a Lipogems filter (Lipogems International S.p.A, Milano, Italy) to obtain microfragmented adipose tissue with intact stromal niche and mesenchymal stem cells with high regenerative potential. The use of the non-adipocyte fraction of adipose tissue (SVF) is also being investigated as a treatment option in a variety of other conditions such as osteoarthritis,[23] cardiomyopathy,[24] chronic wounds,[25] and systemic sclerosis.[26]

Our study design did not directly address the mechanism of healing. However, our finding that injections with freshly collected autologous adipose tissue have a similar effect to the effects reported in studies using either autologous or allogeneic adipose tissue–derived stem cells suggests that the functional cells mediating the response are stromal (mesenchymal) stem cells present in the adipose tissue. Cellular characterization of the adipose tissue from a patient with CD showed that the tissue, in addition to the mature adipocytes, contained a mixture of living stromal cells, stromal (mesenchymal) stem cells, endothelial cells, and immune cells. The challenge of comparing the cell products among different studies is that the composition of the SVF depends on the source of the adipose tissue and the type of surgical procedure used to obtain the fat tissues, that is, needle biopsy, liposuction, or plastic surgery removal. However, most sources indicate that stromal (mesenchymal) stem cells vary between 10% and 50% of the SVF obtained from liposuction. Endothelial cells (mature and progenitors) can represent 7%–30% of the SVF.[14,15,27] The surgical procedure itself may also contribute to fistula closure, as indicated by the relatively high placebo effect observed in the clinical trials with expanded allogeneic adipose-derived mesenchymal stem cells[5] or other placebo-controlled studies.[10,12] However, future studies will be necessary to discriminate whether the observed effects of injections with autologous adipose tissue in CD can be ascribed to harbored stem cells, factors produced by these stem cells, regenerative factors produced by the adipose cells themselves, the attached stromal cells, and/or the procedure itself.

The applied volume of adipose tissue may also be of importance. We found that the injection of larger volumes of tissue was associated with 2 cases of abscesses and reduced rates of healing. As a result, we have reduced the tissue volume injections to 30–40 mL in our daily clinical practice. Injections with adipose tissue had a more pronounced effect in the treatment of transsphincteric and intersphincteric fistulas than in the treatment of anovaginal fistulas. Thus, 10 of 14 patients (71%) with transsphincteric or intersphincteric fistulas achieved complete fistula healing, whereas 2 of 7 (29%) patients with anovaginal fistulas achieved complete fistula healing. We have no obvious explanation for this difference in healing responses.

The present study has some important limitations. The most important limitation is that the study did not include a control group. Furthermore, the clinical follow-up was performed unblinded, without using standardized disease activity scores, such as the Perianal Disease Activity Index. Finally, we included only 21 patients, and pelvic MRI was not applied to confirm the complete healing of anovaginal fistulas observed in 2 patients.

In conclusion, injection of freshly collected autologous adipose tissue holds great potential for the future treatment of perianal fistulas in CD. The treatment may be an easily accessible alternative to cultured autologous adipose-derived stem cells and more cost effective than treatment with commercially available allogeneic stem cells.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2019.02.005.

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Received September 18, 2018. Accepted February 8, 2019.

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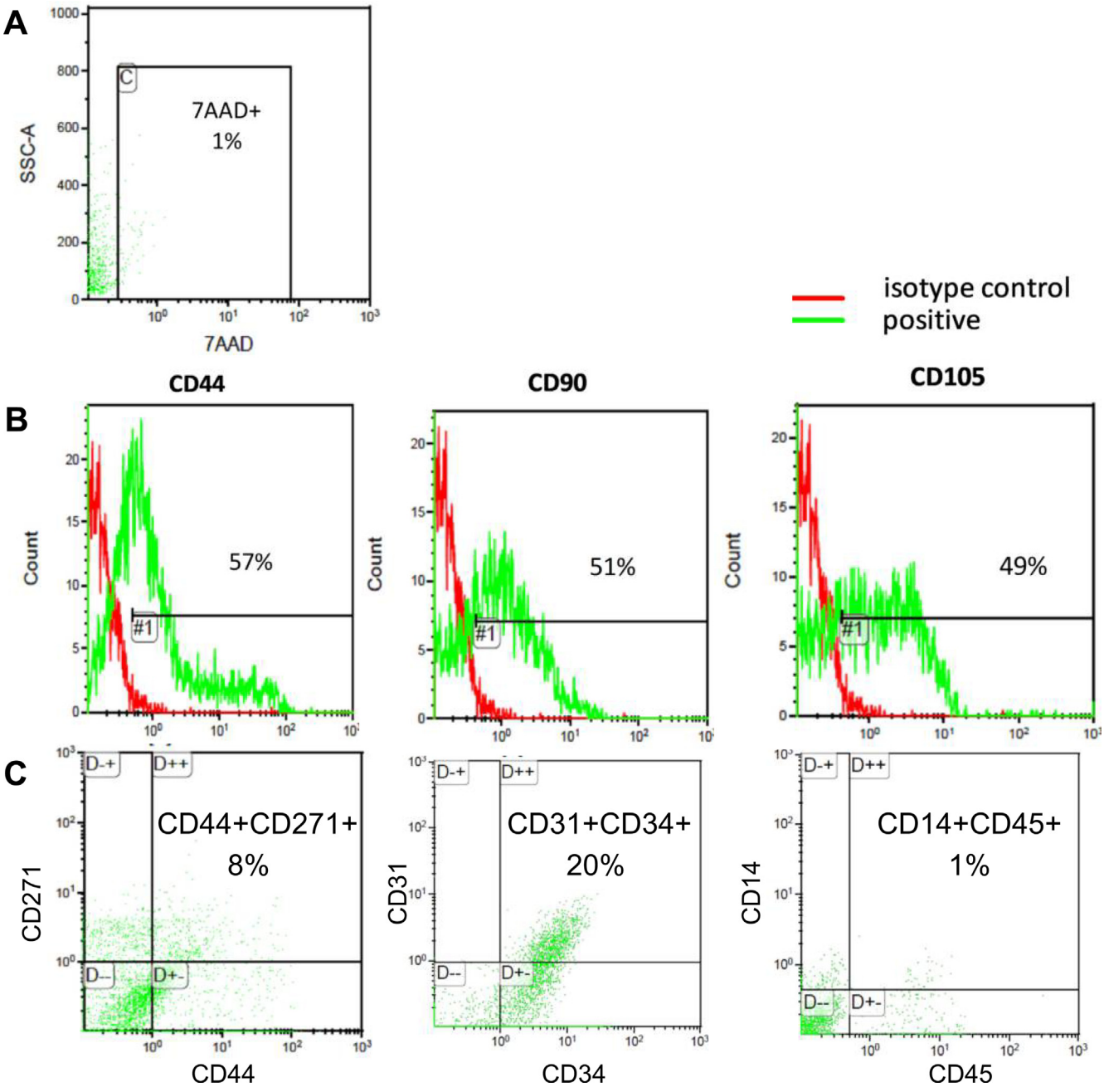
Acknowledgments
Author contributions: Anders Dige: acquisition of data, analysis and interpretation of data, drafting of the manuscript; Helene Tarri Hougaard: study concept and design, acquisition of data, critical revision of the manuscript for important intellectual content; Jørgen Agnholt: study concept and design, analysis and interpretation of data, critical revision of the manuscript for important intellectual content; Bodil Ginnerup Pedersen: acquisition of data, analysis and interpretation of data, drafting of the manuscript; Moustapha Kassem: acquisition of data, analysis and interpretation of data, drafting of the manuscript; Michaela Tencerova: acquisition of data, analysis and interpretation of data, drafting of the manuscript; Klaus Krogh: study concept and design, analysis and interpretation of data, drafting of the manuscript; Lilli Lundby: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript.

Conflicts of interest
The authors disclose no conflicts.



Supplementary Figure 1. Flow cytometry analysis of viability and cellular content in aspirated adipose tissue. Cells were isolated by enzymatic digestion of aspirated adipose tissue. Cells were stained for the examination of viability and their expressions of CD markers. (*A*) The viability of SVF cells measured by 7AAD. (*B*) Stromal cell markers (CD44, CD90, CD105). (*C*) Stromal (mesenchymal) stem cells identified as $CD44^{+}CD271^{+}$, endothelial cells as $CD31^{+}CD34^{+}$, and monocytes/macrophages as $CD14^{+}CD45^{+}$. Percentages refer to the total cellular population of the aspirated adipose tissue. 7AAD, 7-aminoactinomycin D.