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**Traitement endoscopique et médecine régénérative
utilisant des vésicules extracellulaires dans la prise en
charge des fistules digestives**

Par Arthur Berger

Thèse de doctorat de **biologie cellulaire**

Dirigée par M. le Professeur Gabriel Rahmi

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Résumé

Les fistules post-opératoires sont des complications fréquentes, responsables d'une morbi-mortalité élevée. La prise en charge de ces fistules est complexe. Les vésicules extracellulaires (VEs) initialement dénommées « poussières cellulaires » représentent un outil innovant et prometteur de la médecine régénérative. L'objectif de ce travail de thèse est : 1/ d'évaluer l'effet régénératif des VEs issues des cellules stromales du tissu adipeux (AdSC) et leur voie d'administration dans un modèle connu de fistules colo-cutanées chez le rat ; 2/ de développer des nouveaux modèles de fistules post-opératoires gastro-cutanées post-sleeve gastrectomie chez le petit et le gros animal ; 3/ d'évaluer l'efficacité un gel de pluronic F127 combiné ou pas à des VEs dans la cicatrisation de ces fistules chez le petit animal (rat) et validation des résultats chez le gros animal (porc).

Méthodes : Nous avons évalué l'effet des VEs sur la fermeture des fistules digestives, sur trois modèles animaux de fistules digestives. Nous avons utilisé un modèle de fistules colo-cutanées déjà connu chez le rat, où nous avons réalisé une étude de biodistribution des VEs par TEP-IRM, et créé un modèle de fistules gastro-cutanées sur sleeve gastrectomie chez le rat puis chez le porc. Les modèles de fistules gastro-cutanées étaient fidèles aux fistules post-opératoires vues chez l'homme. Après la création du trajet de fistules, les animaux ont été randomisés en plusieurs groupes de traitement (contrôle, gel seul, gel + VEs), et dans le modèle de fistules gastro-cutanées chez le rat, nous avons également testé deux autres groupes de traitement (AdSC, VEs seules).

Résultats : Dans le modèle de fistules colo-cutanées chez le rat, les résultats de biodistribution des VEs par TEP-IRM ont montré une meilleure distribution des VEs administrées localement surtout dans le gel. Nous avons montré l'efficacité thérapeutique de cette approche. Dans le modèle de fistules gastro-cutanées chez le rat, les meilleurs résultats

thérapeutiques ont été observés pour les groupes AdSC, VEs et gel + VEs pour la cicatrisation des fistules par rapport au gel seul et au contrôle. Les résultats obtenus dans le modèle porcin ont validé ceux du modèle de rat.

Conclusion : Ces études sur le petit et gros animal confirment que les VEs présentent des propriétés régénératives. En effet, l'application de VEs induisait une fermeture accélérée des fistules associée à une diminution de l'inflammation et de la fibrose. Ces résultats prometteurs nous incitent à évaluer ce traitement dans le cadre d'une étude clinique de phase I/II.

Summary

Post-operative fistulas are frequent complications, responsible for a high morbidity and mortality. The management of these fistulas is complex. Extracellular vesicles (EVs), represent an innovative and promising tool in regenerative medicine. The aims of this thesis were 1/ Evaluated healing effect of EVs derived from adipose tissue stromal cells (AdSC) and biodistribution of EVs in rat model of colo-cutaneous fistulas; 2/ To develop new models of post-sleeve gastrectomy gastro-cutaneous fistulas in small and large animals; 3/ To evaluate the efficacy of pluronic F127 gel combined or not with EVs in the healing of these fistulas in small animals (rats) and validation of the results in large animals (pigs).

Methods: We evaluated the effect of EVs on digestive fistula closure in three animal models. First, we used an existing rat model of colocutaneous fistulas, we performed a PET-MRI biodistribution study of EVs. Second, we created a gastrocutaneous sleeve gastrectomy model in rats and then in pigs for validation. The gastrocutaneous fistula models were similar to post-operative fistulas observed in humans. After surgery, animals were randomised into several treatment groups (control, gel alone, gel + EVs). In the rat model of gastro-cutaneous fistula we also tested two other treatment groups (AdSC, EVs alone).

Results: In the rat model of colo-cutaneous fistulas, the results of bio-distribution of EVs by PET-MRI showed a better distribution of locally administered EVs, especially in the gel. We then showed the therapeutic efficacy of this approach. In the rat model of gastrocutaneous fistula, the best therapeutic results were observed for the AdSC, EVs and gel + EVs groups for fistula healing compared to gel alone and control. The results obtained in the porcine model validated those of the rat model.

Conclusion: These studies on small and large animals confirm that EVs have regenerative properties. Indeed, the application of EVs induced a significant closure of

fistulas associated with a decrease in inflammation and fibrosis. These promising results encourage us to evaluate this treatment in a phase I/II clinical trial.

Mots clés :

Fistules post-opératoires, médecine régénérative, Vésicules extracellulaires, thérapie combinée, hydrogel

Key words:

Post-surgical fistulas, regenerative medicine, extracellular vesicles, combine therapy, hydrogel

Abréviations

AdSC: Adipose Derived Stem Cells

ADK: Adénocarcinomes

CE: carcinome épidermoïde

CS: cellule stromale

CSM: cellules stromales mésenchymateuses

CT: computed tomography

EV: extracellular vesicles

FAD: Food Drug Administration

FDG: fluoro-D-glucose

IGF-1: insulin growth factor-1

IMC: Indice de masse corporel

LAP : lésions ano-périnéales

LPS: lipopolysaccharides

MC : Maladie de crohn

MEC : Matrice extra-cellulaire

MICI : maladie inflammatoire chronique de l'intestin

NO : oxyde nitrique

OR : Odds Ratio

PDGF : platelet-derived growth factor

POD : post-operative day

SC: stromal cell

SG: sleeve gastrectomie

SUV: standardized uptake value

TBWL: total body weight loss

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Avant-propos

Les **fistules digestives** sont des entités inflammatoires mutilantes définies par une communication anormale entre deux viscères (fistule interne) ou un viscère et la peau (fistule entéro-cutanée). Les principales causes de fistules digestives sont la maladie de Crohn (MC) et la chirurgie digestive. En chirurgie digestive, la fistule post-opératoire est une complication fréquente. La chirurgie œsogastrique, la chirurgie bariatrique, la chirurgie pancréatico-biliaire et la chirurgie colo-anale sont les chirurgies les plus à risque. Les fistules post-opératoires sont responsables d'une morbi-mortalité élevée [1,2]. La prise en charge des fistules est complexe, associant un drainage et une cicatrisation dirigée. En cas d'échec, une nouvelle intervention chirurgicale peut être proposée, même si elle est le plus souvent mutilante et associée à un taux de mortalité élevé [3].

Le traitement à base de cellules stromales mésenchymateuses (CSM) donne des résultats encourageants grâce à leur effet régénératif mais surtout à leur effet paracrine [4,5]. Il existe actuellement un changement d'orientation en **médecine régénérative** en proposant un passage de la thérapie cellulaire à la thérapie vésiculaire basée sur des **vésicules extracellulaires** (VEs). Elles sont constituées de lipides, de protéines, et d'ARNm [6–8]. Les VEs jouent un rôle direct dans la restauration de la fonction tissulaire et l'homéostasie en modulant le recrutement cellulaire, la prolifération et la différenciation [9–11]. L'utilisation de VEs pourrait réduire les contraintes de production, d'acheminements, de stockage, de délivrance et de coût par rapport à la thérapie cellulaire par CSM [12–15]. Le statut immunitaire des VEs (dépourvues de complexe MHC41), permet leur utilisation en tant que

traitement allogénique [14,16]. Les VEs apparaissent comme une alternative aux CSM, grâce à des propriétés régénératives et paracrines similaires. Le potentiel effet des VEs a déjà été largement étudié pour nombreuses maladies comme l'insuffisance cardiaque, la NASH, l'arthrose (etc) [17–21].

Dans ce travail de thèse, nous nous sommes intéressés au rôle régénératif des VEs combinées à un gel de pluronic F127 dans la prise en charge des fistules post-opératoires. Dans un premier temps, nous avons évalué la voie d'administration la plus adaptée des VEs, en reproduisant un modèle de fistules colo-cutanées chirurgicales chez le rat. Nous avons ensuite mise en place un nouveau modèle de fistules gastro-cutanées après sleeve gastrectomie chez le rat et le porc. Nous avons ensuite évalué l'efficacité des VEs dans la cicatrisation des fistules gastro-cutanées post-sleeve gastrectomie chez le petit animal (rat) et validé les résultats chez le gros animal (porc). Les résultats favorables que nous avons obtenus nous ont incités à évaluer ce traitement chez l'homme et nous avons réalisé une première étape qui était l'utilisation du gel de pluronic F127 seul chez un patient. L'utilisation des VEs chez l'homme est plus complexe en raison d'une réglementation qui n'est pas encore complètement lisible et nous détaillerons les différentes étapes à franchir avant un éventuel essai clinique [22,23].

Chapitre 1 : Fistules digestives

1. Définition

Les **fistules digestives** sont des entités inflammatoires mutilantes définies par une communication anormale entre deux viscères (fistule interne) ou un viscère et la peau (fistule entéro-cutanée) (Figure 1 [24]). Les principales causes de fistules digestives sont la MC et la chirurgie digestive. Les fistules sont secondaires à une chirurgie dans 75 à 85% des cas [25,26].

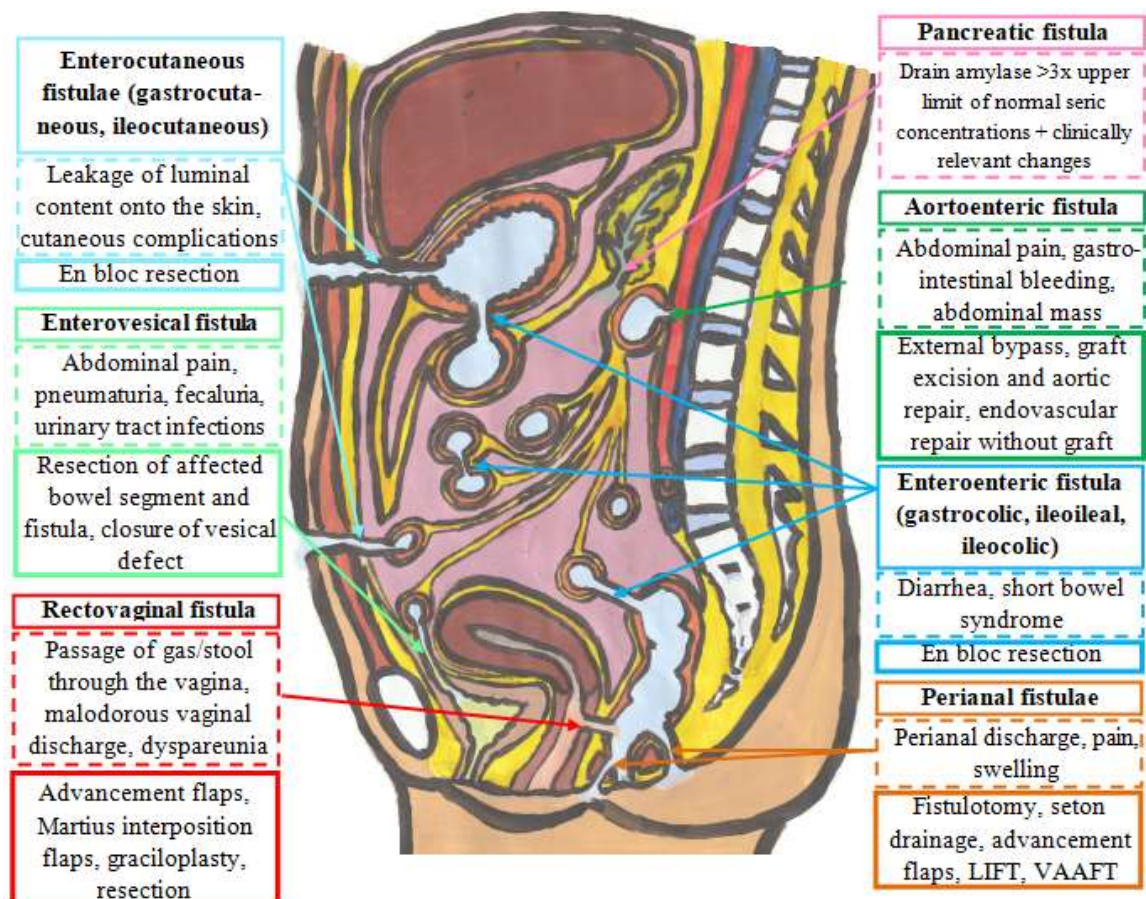


Figure 1 : Fistules digestives : symptômes les plus courants et méthodes de traitement chirurgical définitif. Cadres en pointillés : présentation commune. Cadres en gras : principales stratégies de fermeture chirurgicale définitive. LIFT : Ligature du tractus de la fistule intersphinctérienne.

VAAFT : Traitement vidéo-assisté des fistules anales (Figure issue de la publication de Sebbagh A, et al),

1.1. Fistules post-opératoires

La chirurgie œsogastrique, la chirurgie bariatrique, la chirurgie pancréatico-biliaire et la chirurgie colo-anale sont les chirurgies les plus à risque. Après une chirurgie oeso-gastrique, le taux de fistules post-opératoires est d'environ 10% [27,28]. Les fistules post-opératoires sont responsables d'une morbi-mortalité élevée [1,2].

Les fistules post-chirurgicales sont le plus souvent liées à un défaut de l'anastomose ou à des plaies du grêle en per-opératoire [26,29,30]. Cela peut entraîner des complications infectieuses graves, potentiellement mortelles, comme des abcès ou des péritonites. L'extension progressive d'un abcès par érosion des tissus de voisinage peut entraîner la formation d'une fistule [31]. Un défaut de l'anastomose peut être secondaire à une dévascularisation entraînant une ischémie locale, induit par une ligne de sutures trop tendues et des facteurs locaux (infections péri-anastomotiques, utilisation de segments malades du tractus gastro-intestinal) [29]. D'autres facteurs de risques liés aux comorbidités peuvent influencer le risque de fistules comme la présence d'une insuffisance respiratoire chronique (Odds Ratio (OR) 1,97), d'une hypo-albuminémie (OR 1,66), d'un syndrome d'apnée du sommeil (OR 1,52), d'une hypertension artérielle (OR 1,36) et d'un diabète (OR 1,18) [32].

1.1.1. *Fistules après chirurgie bariatrique*

a) Généralités

L'obésité est une pathologie chronique complexe, avec un coût sociétal estimé à 20 milliards d'euros en France en 2012. La prévalence de l'obésité est d'environ 17% dans la population

adulte française (données Inserm et HAS 2019). Elle est définie par un indice de masse corporelle (IMC) supérieur à 30 kg/m². L'obésité est responsable de complications métaboliques, cardio-vasculaires, respiratoires, rhumatologiques, oncologiques et digestives. La prise en charge multidisciplinaire doit permettre de modifier les habitudes de vie, de dépister et prendre en charge précocement les risques de complications de l'obésité. La chirurgie bariatrique ou chirurgie de l'obésité est à la fois curative et préventive des complications de l'obésité, et est actuellement le seul traitement qui a prouvé une réduction de la morbi-mortalité à long-terme [33,34]. Le traitement chirurgical de l'obésité améliore la survie des patients à long terme, avec une mortalité globale de 13,8% à 10 ans versus 23,9% sans chirurgie bariatrique [33]. Le traitement chirurgical de l'obésité présente une meilleure efficacité à long terme que la prise en charge médicale seule avec une perte de poids totale de 18,6% après sleeve gastrectomie (SG) et de 23,2% après bypass gastrique [35]. La chirurgie bariatrique est actuellement recommandée pour les patients bien préparés (entre 6 et 12 mois de préparation multidisciplinaire – recommandation HAS), qui présentent une obésité de grade III (IMC \geq 40 kg/m²) et/ou une obésité de grade II (IMC \geq 35 kg/m²) avec des comorbidités susceptibles d'être améliorées par la chirurgie (exemple : diabète, HTA, syndrome d'apnées obstructives du sommeil, etc). Le nombre de procédure est en augmentation en France ces dernières années (rapport IGAS n°2017-059R d'après DREES). Plusieurs interventions sont proposées aux patients en fonction du terrain, et de la balance bénéfice/risque. La chirurgie bariatrique a débuté dans les années 1960 et le bypass gastrique est actuellement considéré comme la méthode de référence. Le développement de la SG commence à supplanter le bypass gastrique, malgré une efficacité légèrement inférieure car cette procédure chirurgicale est plus simple avec une diminution de la morbi-mortalité. Chaque année, environ 60.000 patients bénéficient d'une prise en charge chirurgicale de

l'obésité en France. Malgré son efficacité, seulement 1% des patients éligibles à la chirurgie bariatrique sont opérés [36].

b) Morbi-mortalité

La chirurgie bariatrique est plus efficace sur la perte de poids que la prise en charge médicale ou endoscopique, mais elle est associée à une morbi-mortalité plus importante. La morbidité globale après chirurgie bariatrique varie de 0 à 17,5 % et la mortalité globale de 0 à 1,2 % [37–41]. De plus, le taux de reprises chirurgicales peut aller jusqu'à 13% . La fistule digestive est l'une des principales causes de reprise chirurgicale. Après la SG, le taux de fistules est d'environ 2 à 3% [32,41,43–47]. La fistule gastrique peut être à l'origine de complications locorégionales (abcès, péritonite) et systémiques (maladie thromboembolique et embolie pulmonaire, SIRS, défaillance multi-viscérale). La prise en charge de ces fistules nécessite des soins médicaux lourds avec des délais de cicatrisation entre 4 et 6 mois. En cas de péritonite post-opératoire, le taux de mortalité de cette fistule atteint 20%. Il s'agit d'une complication grave, lourde de conséquences chez des patients jeunes, opérés pour une pathologie ne menaçant pas le pronostic vital à court terme.

c) Facteurs de risque de fistules post-opératoires

La plupart des fistules se produisent à l'emplacement proximal de la ligne d'agrafes sous la jonction oeso-gastrique dans 90% des cas et peuvent être plus ou moins complexes (Figure 2) [44]. Cette localisation est probablement favorisée par une hypoperfusion vasculaire de la partie haute de la ligne d'agrafes. Les variations de l'artère gastrique gauche peuvent expliquer l'ischémie des tissus de la partie haute de la ligne d'agrafage après la section des vaisseaux courts dans le ligament gastro-splénique. En effet, dans environ 45% des cas, les estomacs présentent une faible densité de vaisseaux entre les branches cardio-tubérositaire et terminale antérieure de l'artère gastrique gauche [48]. De plus, la section gastrique avec

conservation du pylore participe à augmenter la pression gastrique. Ceci a été démontré par Yehoshua et al. en 2008, avec des pressions de base à 43 mmHg après sleeve gastrectomie contre 34 mmHg chez les contrôles, avec $p < 0,05$ [49]. En cas d'agrafage non optimal, la ligne d'agrafes peut être vrillée et entraîner une sténose de la partie moyenne de la sleeve, entraînant une augmentation de la pression du cardia, favorisant les fistules [50]. Une méta-analyse reprenant les essais randomisés de SG ne retrouve aucun bénéfice des techniques (surjets, colles biologiques...) de renfort des lignes d'agrafes per-opératoires [51]. D'autres facteurs de risque liés aux comorbidités peuvent influencer le risque de fistules [32].

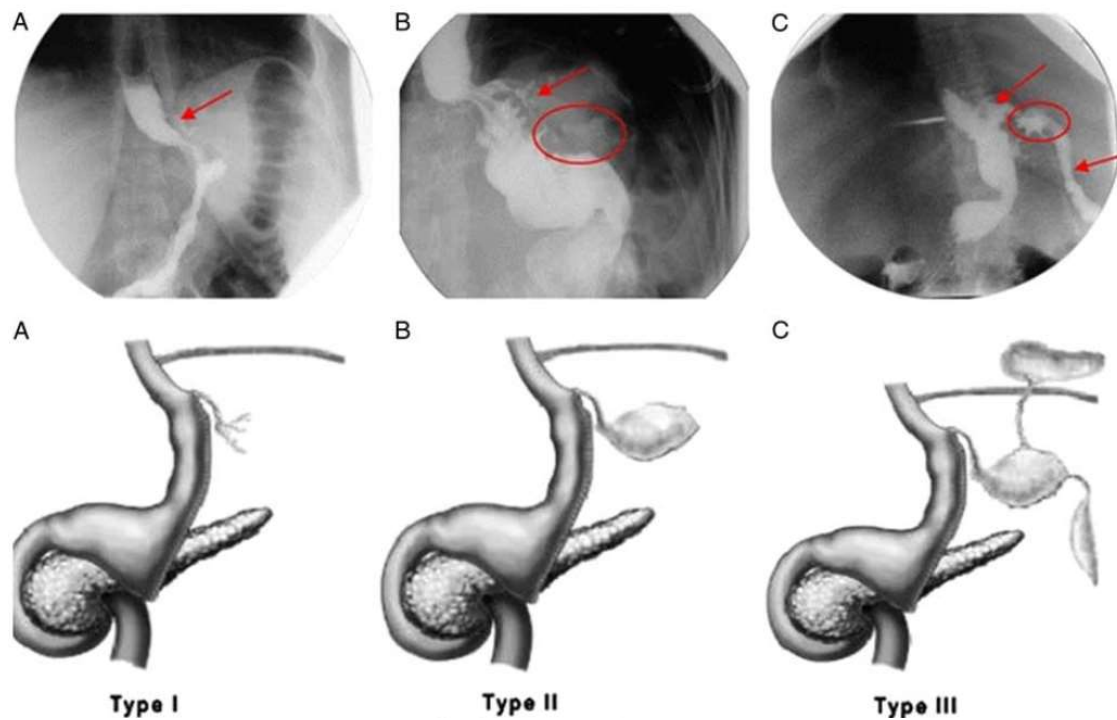


Figure 2 : Trajet de fistule post-sleeve gastrectomie ; Type I, trajet de fistule sans collection au contact ; Type II, trajet fistule avec collections inique au contact en intra-abdominal ; Type III, trajet de fistule complexe avec collection intra-abdominale et sus-diaphragmatique [52].

1.1.2. *Fistules après chirurgie oesogastrique*

Environ 455 800 nouveaux cas de cancer de l'œsophage et 400 200 décès dus au cancer de l'œsophage ont eu lieu en 2012 dans le monde [53]. Le carcinome épidermoïde (CE) est le type histologique prédominant de cancer de l'œsophage, sauf dans les pays occidentaux où la majorité des cas sont des adénocarcinomes (ADK) de l'œsophage [53]. L'œsophagectomie avec curage des ganglions lymphatiques est le traitement de référence pour les tumeurs de l'œsophage et/ou du cardia invasive (\geq T2). Plusieurs techniques chirurgicales sont possibles en fonction de la localisation de la tumeur œsophagienne. En cas de tumeur du 1/3 inférieur de l'œsophage, l'intervention de référence est le Lewis Santy, qui consiste à remonter l'estomac en trans-thoracique et à faire une anastomose oeso-gastrique thoracique. En cas de lésions œsophagiennes cervicales ou d'antécédent de gastrectomie, une coloplastie peut également être réalisée afin de reconstruire l'œsophage retiré. L'œsophagectomie est associé à des taux de morbidité et de mortalité élevés [54]. Dans une étude de cohorte internationale incluant 2 247 patients, le taux de fistules anastomotiques post-œsophagectomies était de 14,2%, avec une mortalité de 11,3% en cas de fistules [55]. En cas de fistules nécessitant une reprise chirurgicale le taux de mortalité était d'environ 25% [55,56].

1.1.3. Fistules après chirurgie colorectale

La chirurgie colorectale est l'une des chirurgies les plus à risque de fistules. Le risque de fistules est d'autant plus important que l'anastomose digestive est basse. Le taux de fistule colo-rectale varie de 3 à 19% en fonction de la localisation (Tableau 1).

Tableau 1 : Taux de fistules digestives après chirurgie digestive iléo-colo-rectale en fonction de la localisation.

| Localisation anastomose digestive | Taux de fistule (%) | ref |
|-----------------------------------|---------------------|---------------|
| Grêlo - grêlique | 1 à 2 | [57,58] |
| Iléo-colique | 1 à 4 | [57,59–62] |
| Colo-colique | 2 à 3 | [59,60,62,63] |
| Iléo-rectal | 3 à 7 | [57,60] |
| Colo-rectal/anale | 5 à 19 | [57,59,62,64] |
| Iléo-anale | 4 à 7 | [65,66] |

Les fistules digestives après chirurgie colo-rectale sont liées à une désunion entraînant la formation d'un abcès. Une ré-intervention est parfois nécessaire pour drainer l'abcès et éventuellement protéger l'anastomose par la création d'une stomie. De plus, en cas de complications post-opératoires après une chirurgie colo-rectale, le risque de complications fonctionnelles (troubles sexuels, incontinence) et de récurrences oncologiques est augmenté [67].

1.2. Fistules sur maladie de crohn

Dans 15 à 25% des cas, les fistules gastro-intestinales se développent spontanément, notamment au cours de la MC . La MC, maladie inflammatoire chronique de l'intestin (MICI), est caractérisée par une atteinte de l'ensemble du tube digestif, de la cavité buccale à la sphère ano-périnéale où elle est source de plusieurs manifestations.

Les lésions ano-périnéales (LAP) peuvent être multiples (ulcérations, fissures) mais l'abcès de la marge anale et la fistule ano-périnéale restent les manifestations les plus fréquentes. En cas de LAP on observe une altération de la qualité de vie [68]. On estime que 25% des patients

atteints de MC développeront une LAP fistulisante, évoluant sous forme de poussées. Le risque cumulé augmente avec la durée d'évolution de la maladie. Eglinton *et al.* ont déterminé dans leur étude que la fistule sur LAP pouvait atteindre 29,5% des patients à 10 ans d'évolution, et jusqu'à 42,7% des patients 20 ans après le diagnostic [69]. Une autre étude a illustré l'importance de la durée d'évolution de la maladie dans la survenue d'une LAP, avec un taux de patients développant une maladie fistulisante de 12% à 1 an, 15% à 5 ans, 21% à 10 ans, et 26% après 20 ans d'évolution [70]. Les LAP sont fréquemment rencontrées dans les formes coliques (41%) et rectales (92%) de la maladie, alors qu'elle s'associe peu aux formes iléales pures (15%) [71]. Les LAP sur MC sont difficiles à traiter ; ainsi à 1 an le taux de guérison des fistules simples est de 88,2%, alors que celui des fistules complexes n'est que de 64,6% [72]. A 10 ans, le taux de guérison sans récurrence de ces fistules complexes n'est plus que de 37% dans la MC [73].

2. Physiopathologie

La physiopathologie des fistules digestives reste encore mal connue. Les fistules post-chirurgicales sont souvent liées à un échec anastomotique ou à des entérotomies par inadvertance, entraînant une fuite du contenu de la lumière en dehors de la lumière intestinale [26,29,30]. Il existe des facteurs de risque induits par la chirurgie responsables de défauts au niveau de l'anastomose [74]. D'autres facteurs liés au terrain peuvent favoriser la survenue de fistules post-opératoires, comme la présence d'une super-obésité, d'un apnée du sommeil, d'un diabète, etc. [74].

Lors de la formation d'un trajet de fistules, il existe un déséquilibre dans les étapes de la cicatrisation et de la régulation entre inflammation et fibrose. Il existe une réponse inflammatoire inadaptée, induite par des traumatismes ou infections (mécanismes exogènes), et/ou du fait d'une réponse immunitaire incontrôlée comme dans la MC (mécanisme

endogène). On distingue trois grandes étapes dans la cicatrisation : l'inflammation, la réparation et le remodelage (Figure 3 [75]). L'inflammation peut devenir chronique, et entrainer la formation de tissus de fibrose. Au-delà de 12 semaines d'évolution, on parle de fistules chroniques.

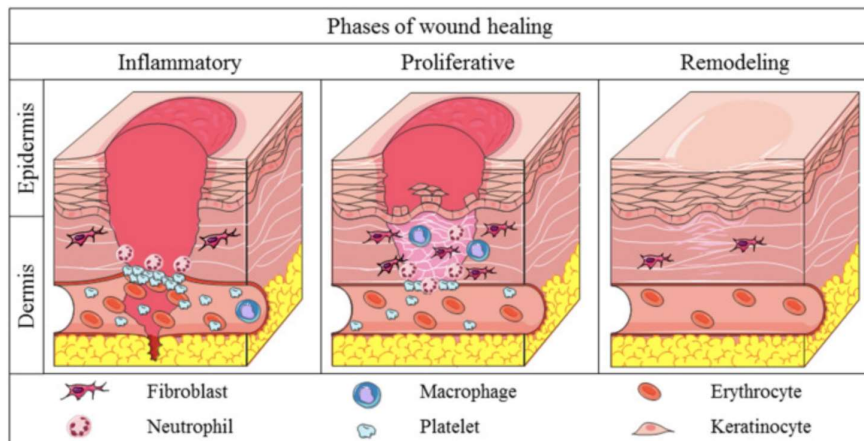


Figure 3 : Schéma de la cicatrisation physiologique incluant 3 phases ; inflammatoire, prolifération, remodelage [75].

2.1. Phase de l'inflammation

L'objectif de cette première phase est d'isoler les éléments étrangers, d'éliminer les débris cellulaires et de préparer la réparation tissulaire. Les macrophages contenus dans le tissu jouent le rôle de première barrière en isolant précocement d'éventuels microbes. Les plaquettes vont libérer des cytokines pro-inflammatoires comme l'IL-1 β (interleukine-1 β), le TNF- α (facteur de nécrose tumorale- α), le FGF (facteur de croissance des fibroblastes) et le PDGF qui vont attirer les leucocytes sur le site lésionnel. Dans cette première phase, une sécrétion d'oxyde nitrique (NO) va entrainer une vasodilatation locale permettant un recrutement local de médiateurs de l'inflammation et de l'immunité [76]. Les neutrophiles vont libérer du VEGF (vascular endothelial growth factor) et l'IGF-1 (insulin growth factor-1) qui activent la prolifération locale des fibroblastes, des kératinocytes et des cellules endothéliales [77,78].

Tableau 2 : Cytokines et facteurs de croissance activant les voies de l'inflammation d'après Gushiken et al [75].

| Cytokines / | | |
|--------------------------------|--|---|
| Facteurs de croissances | Origine cellulaire | Effets biologiques |
| IFN- γ | Neutrophile, Macrophage, et lymphocyte T | Sécrétion de MMPs, diminution de synthèse de collagène et activation des macrophages |
| IL-1 β | kératinocyte, neutrophile, macrophage, | Secrétions des MMPs et activation des kératinocytes et fibroblastes |
| IL-6 | kératinocyte, neutrophile, macrophage, | Prolifération des fibroblastes, recrutement des neutrophiles et des macrophages. |
| IL-8 | fibroblaste, macrophage, neutrophile | Synthèse de collagène, recrutement des neutrophiles et des macrophages. |
| TNF- α | Neutrophile, Macrophage, | Secrétion de MMPs, apoptose des neutrophiles et macrophages |
| EGF | kératinocyte, macrophage | Prolifération des kératinocytes et des fibroblastes |
| FGF-2 | Cellule endothéliale, fibroblaste | Prolifération des kératinocytes et des fibroblastes |
| IGF-1 | Cellule endothéliale, fibroblaste, neutrophile | Prolifération des kératinocytes, des fibroblastes |
| KGF | fibroblaste | Migration des kératinocytes |
| PDGF | Macrophages/Platelets | Prolifération des kératinocytes, cellule endothéliale, activation des kératinocytes et neutrophiles |

| | | |
|----------------|---|---|
| TGF- β 1 | Cellule endothéliale, fibroblaste, neutrophile, plaquette, macrophage, kératinocyte | Prolifération des fibroblaste, angiogénese, remodelage de la matrice extra-cellulaire |
| VEGF | Cellule endothéliale, neutrophile, plaquette | Angiogénese |

2.2. Phase de la réparation

La phase de réparation permet la création d'un nouveau tissu de granulation qui se forme en même temps que d'autres processus de cicatrisation, notamment la réépithélialisation, la néo-vascularisation et l'immuno-modulation (Figure 4). Le tissu de granulation est principalement formé par des fibroblastes activés qui synthétisent une nouvelle matrice extra-cellulaire (MEC), stimulés par la sécrétion de TGF β par les macrophages de type M2. La transformation des fibroblastes en myofibroblastes va entraîner un rapprochement des berges. L'angiogénèse est favorisée par la sécrétion de VEGF et PDGF suite à l'hypoxie tissulaire, permettant la formation de nouveaux capillaires [79].

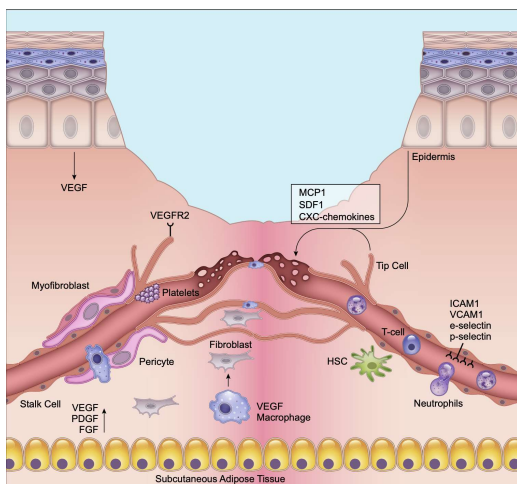


Figure 4 : Angiogénèse pendant la cicatrisation, d'après Rodrigues et al, 2019 [80]

2.3. Phase du remodelage

Lors de cette étape, il y a une diminution du tissu de granulation et une substitution de la matrice extracellulaire provisoire [81]. Les fibroblastes sont stimulés par le TGF- β 1 pour se différencier en myofibroblastes [82]. Ainsi, les fibroblastes du tissu de remodelage synthétisent du collagène de type I, de l'élastine et d'autres composés de la matrice extracellulaire permanente, ce qui se traduit par une résistance et une flexibilité accrues du tissu régénéré [78].

2.4. Cicatrisation pathologique et fibrose

La MEC est régulée par des mécanismes de production (fibrinogène) et de dégradation (fibrinolyse). Un état d'inflammatoire chronique peut-être responsable d'un retard de cicatrisation [83]. En raison d'un déséquilibre entre les médiateurs pro- et anti-inflammatoires, il existe un recrutement exacerbé de neutrophiles et de macrophages, avec une surexpression des cytokines pro-inflammatoires [84]. Il existe deux voies d'activation des macrophages. La voie M1 des macrophages dit pro inflammatoire, qui vont sécréter du NO, et des cytokines pro inflammatoires, ayant pour effet de réduire la réplication cellulaire et d'augmenter la destruction cellulaire (figure 5) [85,86]. A l'inverse, la voie M2 tend à réguler l'activité des macrophages vers un rôle anti-inflammatoire (Figure 5) [85,86].

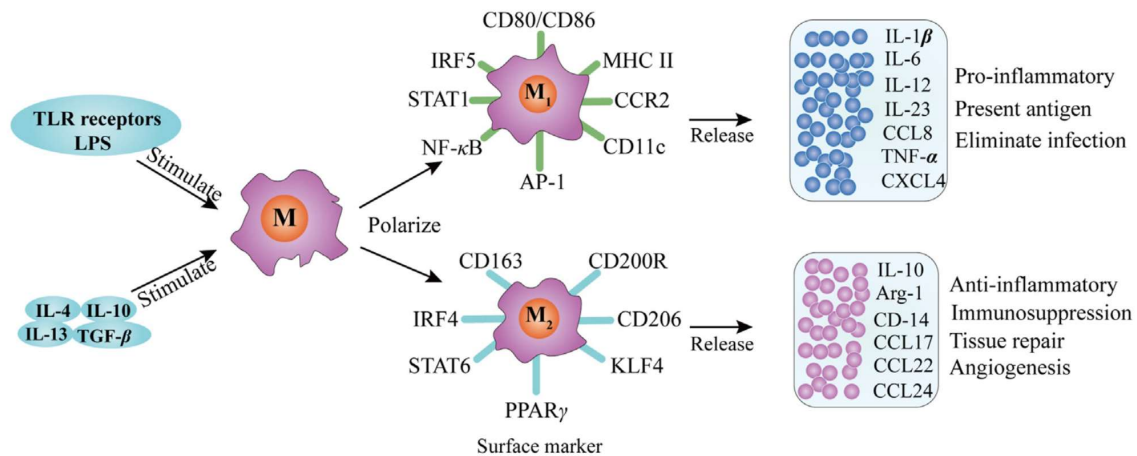


Figure 5 : Voie d'activation des macrophages. Il existe deux voies d'activation via les récepteurs lipopolysaccharides (LPS) et Toll-like receptor (TLR) qui vont activer les macrophages pro-inflammatoires. A l'inverse, les cytokines l'IL-4, l'IL-10, l'IL-13 et le TGF- β poussent les macrophages à adopter des phénotypes M2, ce qui amène à un sous-type de macrophages avec des effets anti-inflammatoires [85].

L'augmentation des cytokines pro-inflammatoires affecte les mécanismes de cicatrisation, en altérant la prolifération/migration cellulaire et la matrice extracellulaire. En cas d'inflammation chronique il existe un déséquilibre entre les facteurs pro- et anti-angiogéniques qui favorise la diminution de la néovascularisation et du flux sanguin, entraînant une altération des mécanismes de remodelage [87]. En cas de défaut de la phase de réparation lié à une agression chronique, on observe un dépôt de la MEC et la formation de la fibrose. Les mécanismes physiopathologiques de la fibrose sont complexes et peuvent intervenir lors de la phase initiale d'inflammation et/ou lors du remodelage tissulaire [88]. Les myofibroblastes semblent jouer un rôle majeur dans la création de ce tissu de fibrose pathologique. En effet, la différenciation des fibroblastes pro-fibrosants en myofibroblastes est favorisée par la sécrétion de cytokines pro-inflammatoires (PDGF, le TGF β 1 et l'IL13) qui sont sécrétées par les plaquettes et les macrophages [88,89]. En cas d'inflammation chronique, il y a un défaut de régulation des fibroblastes pro-fibrosants, entraînant la

formation d'un tissu de fibrose pathologique qui va déformer la structure de l'organe et altérer le fonctionnement normal de l'organe touché comme dans la cirrhose, ou l'infarctus du myocarde... Plus la fibrose devient ancienne plus le tissu de collagène devient important et la proportion d'éléments cellulaires comme les leucocytes devient faible [88].

Au total, les mécanismes de la cicatrisation font intervenir différentes phases allant de l'inflammation au remodelage tissulaire. En cas d'inflammation chronique, l'activation des facteurs pro-inflammatoires va entraîner un déséquilibre entre la production et la dégradation de la MEC pathologique. L'activation des fibroblastes pro-fibrosants va entraîner la formation d'un tissu de fibrose à la suite d'une agression répétée. La thérapie cellulaire peut recréer une homéostasie normale, notamment en jouant sur l'activation des fibroblastes pro-fibrosants en myofibroblastes (Figure 6).

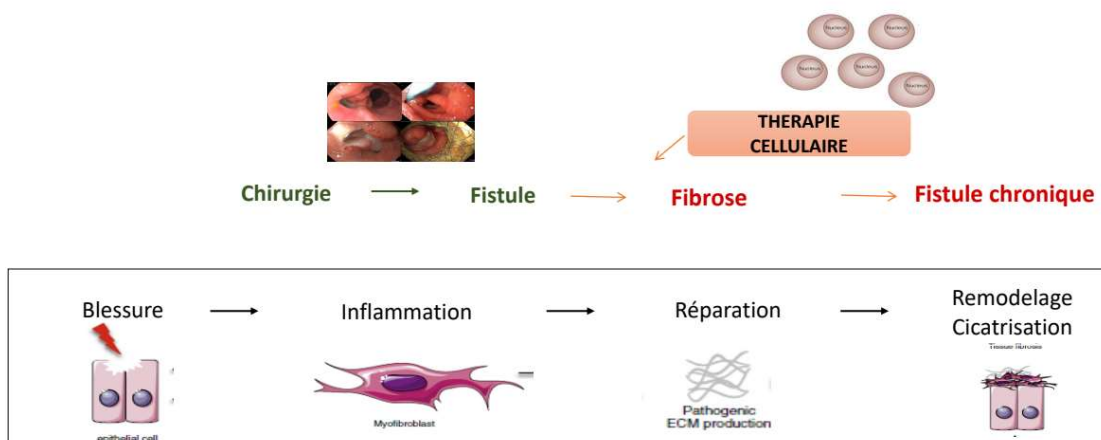


Figure 6 : Schéma des phases de la cicatrisation vers un remodelage pathologique et utilisation potentielle de la thérapie cellulaire.

2.5. Cas particulier des fistules dans la maladie de Crohn

La pathogenèse des fistules dans la maladie de Crohn est complexe (Figure 7). La physiopathogenèse des fistules **dans la MC** fait intervenir deux mécanismes principaux :

- le processus de transition épithélio-mésenchymateux (epithelial to mesenchymal transition, EMT) [90]
- Remodelage enzymatique de la MEC [91]

Il existe des mécanismes de cicatrisation altérés, liés notamment à une diminution du potentiel migratoire des fibroblastes [92,93]. Ces mécanismes de cicatrisation altérés pourraient déclencher la formation d'un EMT induite par le TGF-B, le TNF alpha [94], ainsi que l'IL-13 [95]. Cela va activer la formation de myofibroblastes qui pénètrent ensuite dans la muqueuse intestinale [93,96]. Ce processus est encore facilité par le remodelage de la matrice extracellulaire grâce à l'expression accrue de métalloprotéinases matricielles telles que MMP-3 et MMP-9 [93,97]. Kirkegaard et al en 2004 [98] ont déterminé que l'activité des métalloprotéases est augmentée dans les trajets fistuleux, et notamment la MMP3. D'autres facteurs tels que le rôle du microbiote et la présence d'une dysbiose ont été proposés [99]. Certaines prédispositions génétiques ont été identifiées telles que les mutations de PUS10, allèle c du gène CDK AL1, ou l'absence du gène NOD2. Toutes ces manifestations augmentent le risque de développer une atteinte ano-périnéale complexe au cours de la MC [100,101].

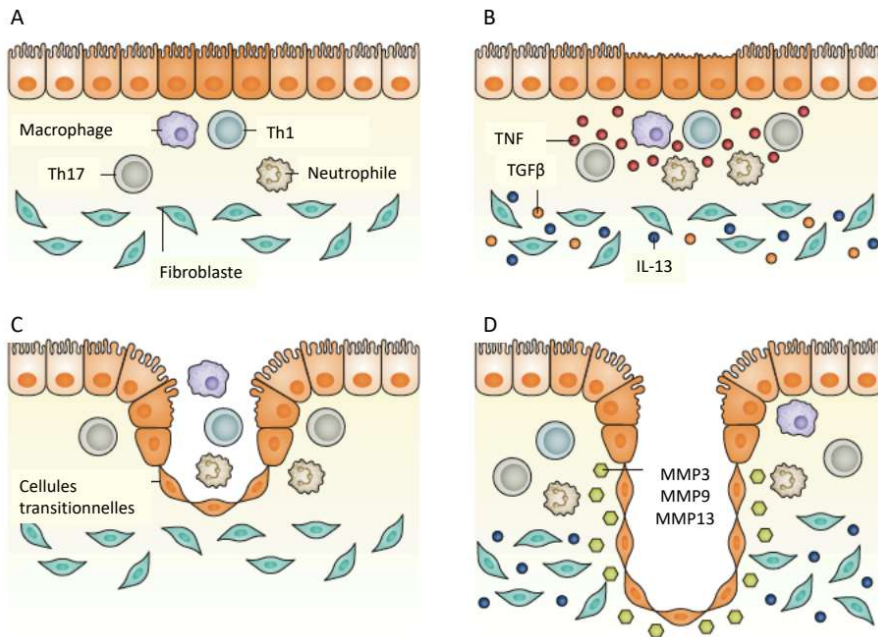


Figure 7 : Mécanismes physiopathologiques d'une fistule ano-périnéale dans la maladie de Crohn, d'après Siegmund et al. [91]

3. Prise en charge des fistules

3.1. Fistules post-opératoires

La prise en charge de ces fistules est complexe, associant un drainage et une cicatrisation dirigée (Figure 8). Une approche médico-chirurgicale pluridisciplinaire est nécessaire, associant le plus souvent un support nutritionnel afin d'aider la cicatrisation. Les trajets de fistules sont souvent associés ou secondaires à un abcès, dans la phase initiale. La prise en charge de ces abcès nécessite l'utilisation systémique d'antibiotiques et la réalisation d'un drainage qui peut être soit interne par endoscopie, soit externe par voie radiologique ou chirurgicale.

Il existe plusieurs types de fistules post-opératoires, en fonction du délai d'évolution.

- La première semaine après la chirurgie : fistule aiguë
- Entre 1 et 6 semaines : fistule précoce

- Entre 6 à 12 semaines : fistule tardive
- Au-delà de 12 semaines : fistule chronique.

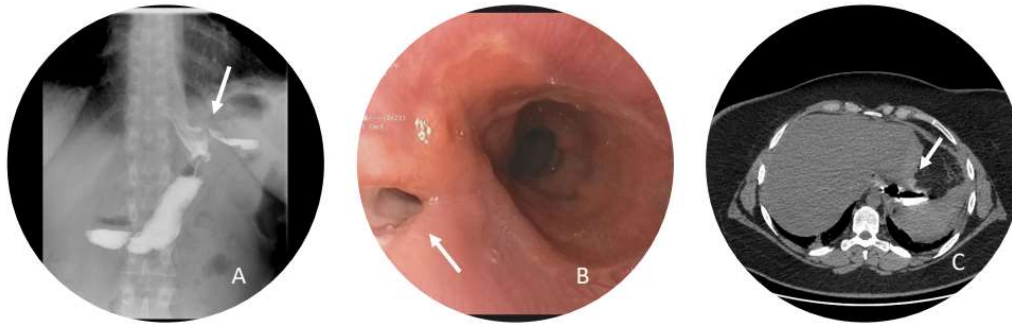


Figure 8: Fistule gastrique après une sleeve gastrectomie ; A, trajet de fistule visualisé sur un transit œsophagien ;B, orifice interne de la fistule gastrique situé au sommet de la ligne d’agrafes ; C, collection en continuité avec le trajet de fistule, opacifié après ingestion de produit de contraste, visualisé en coupe axiale (TDM).

Les traitements ne sont pas les mêmes pour toutes les fistules en fonction du délai d’apparition, de l’état clinique du patient, et de la localisation [102,103] . Habituellement, on admet que pour les fistules oeso-gastriques (Figure 9):

- **Les fistules aiguës** relèvent d’un drainage (+/- lavage) chirurgical au contact de la fistule.
- **Les fistules précoces et tardives** relèvent plutôt d’un traitement par endoscopie, tel que la pose d’un drain dans l’orifice fistuleux (nasogastrique, queue de cochon), ou prothèse gastrique (diversion pour exclure le trajet fistuleux) ou pose de clips type OVESCO.
- **Les fistules chroniques** ne relèvent pas d’une prise en charge codifiée, et font l’objet de traitements chirurgicaux lourds, de la gastro-jéjunostomie à la gastrectomie totale.

Les options chirurgicales conservatrices sont préférées à l’instar de la gastro-jéjunostomie, à réaliser en centre expert [104]. Cependant, il est possible de réaliser une gastrectomie totale avec confection d’une anastomose oeso-jéjunale sur anse en Y en dernier recours [105].

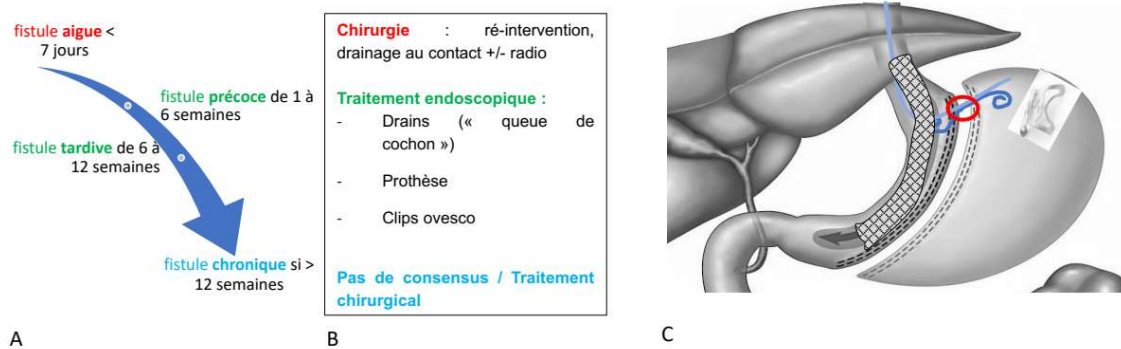


Figure 9: Fistule post-opératoire sur sleeve gastrectomie ;A, chronologie des fistules ; B, type de prise en charge ; C, exemples de traitement endoscopique : drain naso-kystique, drain en double queue de cochon, macro-clip de type OVESCO.

3.1.1. Prise en charge endoscopique des fistules post-opératoires

Méthode de diversion par pose de prothèse métallique couverte : Cette technique a pour objectif d’exclure l’orifice de fistules, elle peut être proposée en l’absence de collection et/ou après drainage. La procédure consiste à mettre en place une prothèse afin de couvrir la fistule. L’exclusion de l’orifice primaire de la fistule permet la cicatrisation de ce dernier en réduisant le contact avec les facteurs agressants locaux (aliments, liquide acide gastrique). Le taux de succès clinique est variable entre 44% et 70% au moment du retrait de la prothèse [106–108]. Les taux de complications sont également aussi variables ; la complication principale étant la migration de la prothèse. Le taux de migration peut aller jusqu’à 53%, avec dans 3% des cas

la nécessité une laparoscopie pour récupérer la prothèse [107]. La mise en place d'une prothèse peut également entraîner un risque d'érosion de la paroi digestive et d'hémorragie digestive qui peut être fatale en cas de fistules aorto-oesophagiennes [107,109].

La deuxième technique consiste en la mise en place de prothèses plastiques en double queue de cochon au travers de l'orifice primaire de la fistule, et au sein du trajet fistuleux, constituant ainsi un drainage interne de la fistule. L'intérêt en phase précoce est de permettre une organisation du trajet fistuleux d'une part et de drainer une collection formée à l'extrémité distale du trajet fistuleux, voire de prévenir son apparition ou son infection. Pour se faire, un cathéter muni d'un fil guide est utilisé afin de « canuler », sous contrôle fluoroscopique, l'orifice de fistules et de déterminer la longueur du trajet fistuleux et la présence ou non, par opacification d'une collection. Puis, le fil guide sert à l'insertion de prothèses double queue de cochon, classiquement deux prothèses, de 7Fr de diamètre et d'une taille variable généralement de 5 cm (selon la longueur du trajet fistuleux). Cette technique a montré des résultats intéressants dans le cadre de fistules post-chirurgies bariatriques avec un taux de fermeture d'environ 84% [110–113]. Le taux de complications rapporté dans une méta-analyse de type hémorragie, migration des prothèses, perforation est de 13,7% [111].

Thérapie par VAC endoscopique de type Endosponge : L'utilisation d'un système de VAC par voie endoscopique peut être utilisé pour la prise en charge des fistules post-opératoires oeso-gastriques ou colo-rectales. Ce traitement consiste à la mise en place endoscopique d'une éponge reliée à un système d'aspiration continue permettant une cicatrisation dirigée progressive. Le taux de fermeture rapporté dans une méta-analyse récente est de 89.4% dans le traitement des fistules digestives hautes [114]. Des résultats similaires ont été rapportés dans la prise en charge des fistules colo-rectales. Cette technique est efficace dans la prise en charge précoce des fistules post-opératoires liées à une désunion de l'anastomose [115].

Cependant elle nécessite un changement de l'éponge tous les 2 à 3 jours le plus souvent sous anesthésie générale et la prise en charge dure parfois plusieurs semaines.

Technique de fermeture endoscopique : Il existe actuellement deux techniques de fermeture endoscopique, soit par macro-clip, soit par suture endoscopique. Les techniques de fermeture endoscopique sont réservées aux cas de fistules sans collection après un drainage efficace et avec un trajet fin. Le système de macro-clip est formé d'un capuchon positionné à l'extrémité de l'endoscope. L'orifice interne du trajet de fistules est capturé dans le capuchon à l'aide d'une pince. Le clip est ensuite largué, les mors du clip vont capturer la paroi digestive autour du trajet de fistules et le refermer. Cette technique est limitée aux fistules ayant un diamètre inférieur à 10 mm. En effet, au-delà de 10 mm, il y a un risque de ne pas pouvoir capturer entièrement l'orifice interne du trajet de fistules. Le taux de fermeture varie de 70% à 80% [112,116,117]. Un autre système consiste à utiliser un dispositif de sutures endoscopiques. Les taux de fermeture varient entre 75% et 83% [118–121]. Cependant le taux de récurrence à long terme reste important avec environ 65% de réouverture du trajet de fistules à 6 mois [119]. La ré-épithélialisation de l'orifice et du trajet de fistule interne favorise probablement la réouverture tardive des trajets après fermeture endoscopique. Thomson et al, proposaient de réaliser une destruction de la muqueuse autour de l'orifice interne de fistules par dissection sous muqueuse endoscopique pour favoriser la cicatrisation de l'orifice de fistules [122].

D'autres techniques de fermeture par utilisation de biomatériaux, tels que des « plugs », ou de la colle biologique ont été étudiées. L'efficacité de ces traitements est modérée. L'utilisation de colle biologique en cas de drainage incomplet expose au risque de complications infectieuses à type d'abcès [123].

3.1.2. En synthèse :

La prise en charge endoscopique des fistules digestives post-opératoires est un traitement efficace de première intention. Les différentes techniques endoscopiques décrites ci-dessus sont le plus souvent associées. La première étape consiste en général à drainer par voie endoscopique en cas de collection ou à exclure le trajet de fistules liée à la désunion par la mise en place d'une prothèse métallique couverte. Les techniques de fermeture sont en général utilisées en deuxième intention. La prise en charge endoscopique nécessite en général entre trois à quatre procédures endoscopiques sous anesthésie générale [112]. En cas d'échec, une nouvelle intervention chirurgicale peut être proposée, mais elle est le plus souvent mutilante. Une chirurgie de sauvetage est nécessaire lorsque les stratégies mini-invasives échouent et/ou en cas de complications mettant en jeu le pronostic vital [30]. Cette chirurgie est complexe, elle tentera de fermer la fistule et de reconnecter le tractus gastro-intestinal avec un risque de stomie définitive, de syndrome de grêle court, et de récurrence des fistules [26,30,31,124]. Les défis posés par la prise en charge des fistules post-chirurgicales ont récemment motivé l'étude de la thérapie cellulaire ou vésiculaire comme alternative mini-invasive [30,125].

3.2. Traitements des fistules sur maladie de Crohn

3.2.1. Approche médicale

La maladie de Crohn doit faire l'objet d'un traitement pluridisciplinaire médico-chirurgical [126]. En ce qui concerne la stratégie pharmacologique, des antibiotiques, la prise en charge nutritionnelle et des immunosuppresseurs ont été utilisés dans le traitement des fistules sur maladie de crohn [127]. Les fistules ano-périnéales de MC sont difficiles à traiter. Le principal facteur d'échec est la présence d'une rectite concomitante [128]. Les traitements les plus utilisés dans la MC sont les anti-TNF (notamment l'Infliximab). Un essai randomisé

contrôlé en double aveugle montre l'amélioration du taux de fermeture des fistules périnéales après l'induction du traitement [129]. La rémission était obtenue dans 13% des cas avec le placebo contre 55% des cas à 5mg/kg ($p=0,001$) et 38% des cas à 10 mg/kg ($p=0,04$). Par la suite, l'essai ACCENT II a évalué l'Infliximab contre placebo en phase de maintien chez les malades ayant répondu à l'induction thérapeutique. Une rémission était maintenue à la semaine 54 chez 19 des 99 patients sous placebo (19,2%) contre 33 des 96 patients sous Infliximab (34,4%) (RR : 1,79 IC95% 1,10 - 2,92) [130]. Ces données ont été confirmées dans différentes cohortes observationnelles. La thérapie systémique par anti-TNF- α s'est avérée limitée sur la fermeture des fistules et associée à un sur-risque de cancers et d'infections opportunistes. Pour les autres biothérapies il existe pour l'instant peu de données qui sont souvent contradictoires. En l'absence de meilleure alternative les Anti TNF sont recommandés par ECCO en traitement médical de première intention [132].

3.2.2. Approche chirurgicale

Plusieurs procédures chirurgicales existent en fonction des besoins du patient, et de l'expérience locale de l'opérateur [126]. Les techniques les plus couramment utilisées sont : Le drainage des abcès et la mise en place de sétos pour permettre une cicatrisation dirigée et une exclusion progressive du trajet de fistules [133]. Le **lambeau rectal d'avancement**, qui est la méthode la plus utilisée. Soltani et al ont montré dans une méta-analyse un taux de fermeture de 64% [134]. La réalisation d'une exérèse complète du trajet de fistules et la ligature trans-sphinctérienne (technique du L.I.F.T) présente des taux de guérison similaire de 67% à 12 mois [135]. La colostomie d'amont peut être proposée de façon transitoire quand l'atteinte pelvienne est sévère et non contrôlée. Cette technique ne permet pas la fermeture de la fistule, et doit être réalisée en complément d'une prise en charge médicale adaptée. Dans ce cas, le taux de stomie définitive reste élevé chez 17% des patients [136].

3.2.3. Approche régénérative

a) Colles biologiques :

Des traitements à base de colles biologiques faites de fibrine et de thrombine ont été évalués. Elles servent de matrice pour les fibroblastes et le collagène. Le taux de fermeture après traitement était de 38%, mais une récurrence était observée dans 25% des cas [137]. L'approche régénérative via la thérapie cellulaire et vésiculaire sera traitée dans le chapitre suivant.

Chapitre 2 : Médecine régénérative

2. Thérapie cellulaire

La thérapie cellulaire représente actuellement un espoir pour le traitement des fistules complexes en échec des traitements conventionnels. Les CSM sont des cellules adultes, non hématopoïétiques et multipotentes qui peuvent se différencier en plusieurs types de cellules. Les travaux actuels portent sur des cellules stromales (CS) pluripotentes, et plus particulièrement des CSM. Les CS peuvent être récoltées à partir de liposuction ou lipoaspiration de tissus adipeux humains de type AdSC [138–140]. Les AdSC sont plus facilement accessibles que les CSM provenant de la moelle osseuse ou de tissus néonataux [141]. Au départ, la communauté scientifique pensait que l'action de ces cellules provenait de leurs capacités à se différencier, en remplaçant les tissus lésés cibles. Cependant, l'intérêt des CS pour la médecine régénérative est rapidement allé au-delà de leur capacité à se différencier en plusieurs types de cellules, avec la démonstration préclinique de leur propension à favoriser la cicatrisation et l'angiogenèse, ainsi que leurs propriétés immunomodulatrices et leur effet anti-fibrotique (Tableau 3 et Figure 10) [142–145]. De plus, les résultats de plusieurs études précliniques semblaient remettre en considération l'effet direct des CS sur la régénération des tissus. Il a été démontré que très peu de CS se greffaient durablement dans le tissu cible [15,146]. En présence d'un milieu trop inflammatoire, les CS ont une durée de vie limitée, et meurent prématurément [146]. L'effet thérapeutique des CS semble se produire dans un intervalle de temps trop court pour être attribué à leur différenciation cellulaire [15]. Ces résultats ont incité un changement de paradigme dans la thérapie cellulaire vers des mécanismes d'action principalement paracrines [147].

Tableau 3. Propriété anti inflammatoire et immunomodulatrice des CS.

| Propriété anti inflammatoire | Propriété immunomodulatrice |
|--|---|
| Diminution : | Action par : |
| <ul style="list-style-type: none">- IL 12- IL 6- TNF alpha | <ul style="list-style-type: none">- Lymphocytes T- Cellules dendritiques- Lymphocytes B |
| Élévation : | <ul style="list-style-type: none">- Lymphocytes NK |
| <ul style="list-style-type: none">- IL 10- TGF bêta (favorise la voie des lymphocytes T-régulateurs et inhibe les Th1). | |

Figure 10 : Mécanismes d'action des cellules souches stromales pour la thérapie des fistules digestives. Tiré de l'article Sebbagh A et al, *Advanced Drug Delivery Reviews* 2021.

2.1. Le traitement des fistules ano-périnéales réfractaires : le Darvadstrocel

Actuellement, le seul traitement à base de CSM dérivées du tissu adipeux, ou « Adipose Derived Stem Cells » (AdSC) dans le traitement des fistules ayant une AMM est le Darvadstrocel (Alofisel®) dans le cas des MC avec fistules ano-périnéales réfractaires. Ce

traitement est une suspension de cellules souches allogéniques dérivées du tissu adipeux de donneurs sains (Cx601) dont les bénéfices, le profil de sécurité et de tolérance ont été publiés dans le Lancet par Panès et al en 2016 [148]. Ce traitement (120 millions de cellules) permet d'avoir des taux de cicatrisation dans le cas de maladies réfractaires de 50% dans le groupe Alofisel® contre 34% avec le placebo ($p=0,024$) à 6 mois, avec un effet maintenu à 1 an (56,3% de guérison contre 38,6% avec le placebo ($p=0,01$)). Le traitement été injecté dans le trajet et/ou en périphérie du trajet de fistules (Figure 11), dans les deux groupes un traitement standard été associé. Ce traitement permet un taux de guérison non atteint par les autres traitements conventionnels disponibles, ce qui illustre l'importance de la thérapie cellulaire.

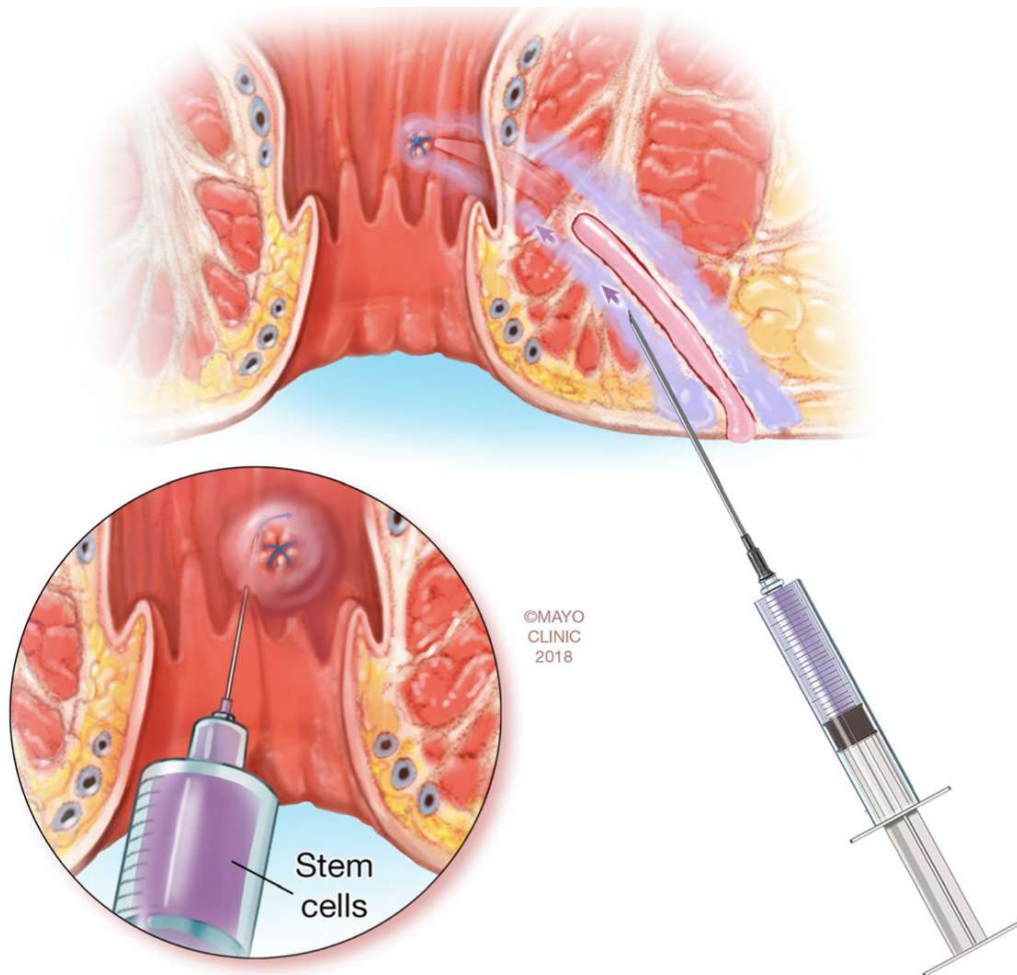


Figure 11: Méthode d'injection de l'Alofisel® dans le traitement des fistules ano-périnéales de MC [149].

2.2. La thérapie cellulaire dans la prise en charge des fistules digestives post-opératoires.

En raison d'une morbi – mortalité importante, la thérapie cellulaire représente un espoir dans le traitement des fistules post-opératoires chroniques [150]. Si la thérapie cellulaire par Alofisel est aujourd'hui commercialisée pour les fistules sur LAP, les données sur les fistules post-opératoires sont toujours en phase de recherche. Afin d'étudier les mécanismes d'action et l'effet de la thérapie cellulaire en médecine régénérative plusieurs modèles animaux de fistules digestives ont été mis en place (annexes et résultats article 2).

Des modèles de fistules entéro-cutanées traitées par thérapie cellulaire ont été étudiés avec des résultats probants chez l'animal [151–154]. Le potentiel de la thérapie cellulaire par CSM a été étudié dans des modèles expérimentaux de fistules entéro-cutanées post-opératoires [151–154]. Une première étude, portant sur un modèle de fistules entéro-cutanées chez le rat, après réalisation d'une fistule entéro-cutanée de type caecostomie, a montré une amélioration de la fermeture de l'orifice externe avec injection locale de CSM humaines [155]. Une étude réalisée par notre équipe, a évalué le potentiel des feuillets de SC humaines issues de la moelle osseuse dans un modèle murin de fistules colo-cutanées de type caecostomie [152]. Deux semaines après la transplantation, l'évaluation clinique, l'IRM, l'analyse histologique et les résultats de l'endomicroscopie confocale à sonde ont indiqué une meilleure guérison dans les groupes traités par feuilles par rapport au groupe témoin. Les feuillets ont permis une réduction de l'inflammation, de fibrose, une meilleure microvascularisation, une surface de fistules plus faible et une diminution de la taille maximale de l'orifice fistuleux. Le mécanisme d'action des feuilles cellulaires a été étudié par endomicroscopie confocale qui retrouve une augmentation de la densité de néo-vaisseaux, favorisée par l'augmentation de facteur VEGF (Figure 12). Les CSM ont permis un effet paracrine par sécrétion de facteurs anti-inflammatoires et anti-fibrosants.

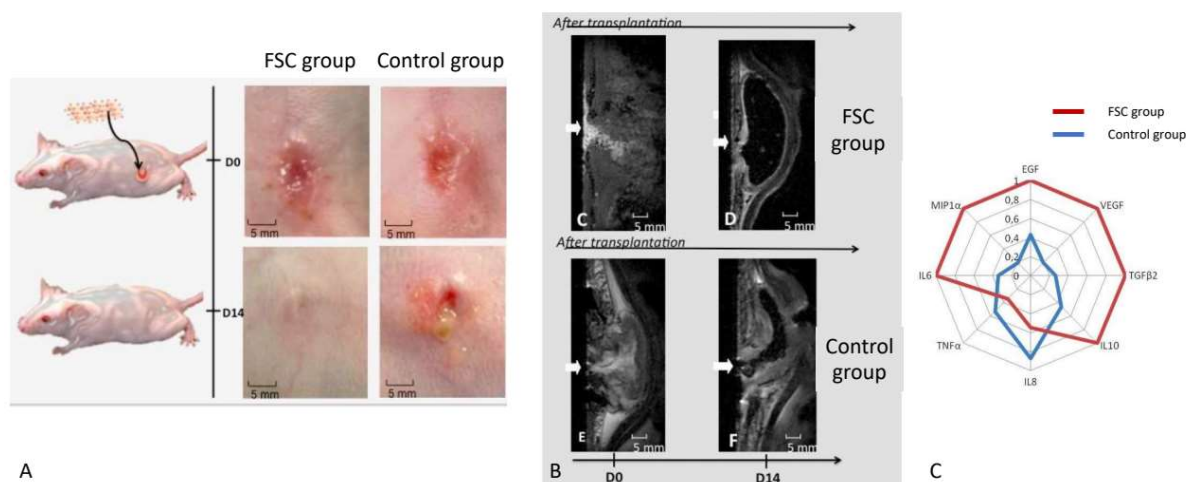


Figure 12 : Modèle de fistules colo-cutanées chez la souris traitée par feuillet de cellules souches (FSC) humaines issues de la moelle osseuse. A, évaluation clinique ; B, évaluation par IRM ; C, évaluation par biologie moléculaire de la proportion de facteur pro/anti-inflammatoires dans le trajet de fistules à la fin du suivi en fonction du groupe de traitement [152].

Une troisième étude a évalué les bénéfices de l'injection locale d'AdSCs allogéniques de rats (4 millions de cellules) dans un modèle similaire de fistules colo-cutanées chez le rat [154]. Un mois après le traitement, six fistules sur 16 s'étaient refermées dans le groupe AdSC contre 1 sur 16 dans le groupe témoin ($p=0,033$).

Dans un autre modèle de fistules chroniques gastro-cutanées chez le lapin traité par une fraction vasculaire stromale autologue (SVF, contenant des AdSC) a été injectée dans la fistule [153]. Trois semaines après le traitement, l'examen des orifices externes et l'analyse histologique du trajet de fistules ont mis en évidence une guérison presque complète dans le groupe SVF.

2.3. Thérapie cellulaire en prévention des fistules

D'autres études ont cherché à monter l'intérêt de la thérapie cellulaire dans la prévention des fistules post-opératoires. L'utilisation de feuilles de CS de type AdSC ou dérivées de moelle osseuse ont été déposées en per-opératoire sur les anastomoses a été étudiée pour prévenir le développement de fistules pancréatiques post-chirurgicales [156–158]. Des modèles de fistules pancréatiques post-chirurgicales chez le rat ont été établis par transection du canal pancréatique splénique [156,157] ou par division des canaux pancréatiques gastriques et spléniques avec pancréatectomie distale et splénectomie [158]. Le traitement par feuilles de cellules a permis de réduire la fuite pancréatique au début de la période postopératoire, comme en témoigne le volume de liquide intra-abdominal [158] et les concentrations d'amylase et de lipase pancréatiques ascitiques [156,157]. Elle a également entraîné moins de dommages intra-abdominaux associés [156,157], moins d'infiltration de cellules inflammatoires [156–158], une régénération tissulaire accrue [158] et une meilleure survie à un mois [156]. Dans l'ensemble, ces trois études tendent à montrer des effets bénéfiques des feuilles de cellules pour la prévention du développement de fistules pancréatiques post-opératoires.

Dans l'ensemble, les preuves actuelles provenant de petits modèles animaux soulignent le potentiel de l'administration locale de CS comme traitement curatif des fistules colo-cutanées. Cependant, des études expérimentales supplémentaires sont justifiées dans des modèles animaux de grande taille et plus proche des problématiques de fistules post-opératoires chez l'homme. Parallèlement une évaluation multimodale des paramètres de cicatrisation, et des mécanismes d'action des CS dans la cicatrisation des fistules est nécessaire avant une transposition chez l'homme.

2.4. La thérapie cellulaire dans les fistules post-opératoires chez l'homme

À ce jour, très peu d'essais cliniques évaluant le potentiel de la thérapie par cellules souches pour le traitement des fistules post-chirurgicales ont été réalisés.

Un premier essai clinique ouvert de phase I a étudié la sécurité et l'efficacité des AdSC pour les fistules entéro-cutanées post-chirurgicales [159]. Les AdSC autologues ont été isolées à partir d'échantillons de tissus adipeux sous-cutanés fessiers en utilisant des systèmes de lipo-aspiration de type Celution®. Six patients présentant des fistules entéro-cutanées réfractaires persistantes secondaires à une chirurgie gastro-intestinale ont été inclus. Tous les patients présentaient une maladie intestinale inflammatoire sous-jacente (quatre cas de colite ulcéreuse, dont un avec un carcinoïde rectal concomitant, et deux cas de MC), et ont développé des fistules entéro-cutanées à la suite d'une coloproctomie avec anastomose iléale-anale, ou d'une résection antérieure basse, ou d'une résection iléo-cæcale (avec ou sans résection antérieure). La durée de la fistule était de 5 à 44 mois. Le traitement a été administré localement, la moitié des cellules étant injectée dans les tissus voisins (par voie endoscopique et percutanée), et l'autre moitié mélangée à de la colle de fibrine et injectée dans le tractus fistuleux. La dose était comprise entre 9,6 et 142 millions d'AdSC. La fermeture de la fistule a été définie comme l'arrêt du drainage sans récurrence d'un abcès ou d'une fistule. Cinq patients sur six ont obtenu la fermeture de la fistule à quatre semaines, qui a persisté jusqu'à la fin de la période de suivi (24 semaines), associée à la fermeture de l'iléostomie chez quatre patients et à la possibilité de reprendre une alimentation orale chez un patient. Un autre essai clinique de phase 1 de la Mayo clinic est actuel en cours (NCT02807389). Dans l'ensemble, cet essai clinique pilote révèle des résultats préliminaires encourageants en termes de sécurité et d'efficacité du traitement local par AdSC.

Les données actuelles, bien que rares, restent prometteuses pour la prise en charge des fistules post-opératoires.

3. Thérapie vésiculaire

L'effet paracrine a été rapporté comme le mécanisme d'action principal dans le cadre de la thérapie cellulaire, notamment via des VEs secrétées par les CS [147]. Les VEs anciennement dénommées « poussières cellulaires » représentent un outil innovant et prometteur en médecine régénérative. Les VEs issues de CSM reproduisent l'effet paracrine des cellules mères sans risque prolifératif ; et plus facilement conservées par congélation [5,160]. Les VEs sont des particules de taille nanométrique (40-5000 nm) libérées par les cellules (Figure 13). Les VEs sont sécrétées par les cellules spontanément ou bien en cas de stress et/ou stimulation physique, chimique ou biologique.

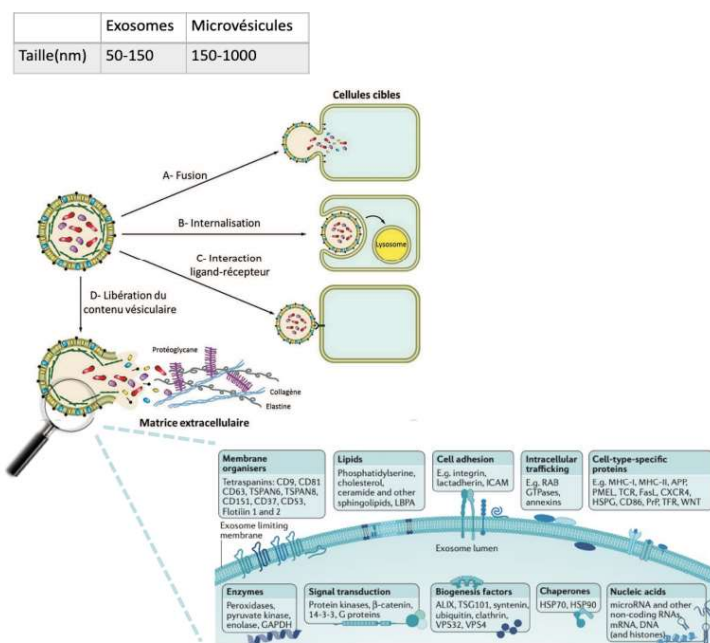


Figure 13 : Vésicules extracellulaires ; mécanisme de communication cellulaire et contenu vésiculaire.

Elles sont constituées de lipides, de protéines, et d'ARNm, leur contenu dépend des cellules qui les sécrètent [6–8]. Les VEs jouent un rôle direct dans la restauration de la fonction tissulaire et l'homéostasie en modulant le recrutement cellulaire, la prolifération, et la différenciation [9–11]. Physiologiquement, les VEs servent de médiateurs pour la communication intercellulaire. (Figure 13). Les VEs dérivées des SC ont démontré des propriétés anti-inflammatoires, immunomodulatrices et anti-fibrotiques [147,161–172]. En activant la voie PI3K/Akt, les VEs augmentent la prolifération cellulaire, tout en diminuant l'apoptose cellulaire aidant ainsi à la cicatrisation (Figure 14) [161,173,174]. L'effet en médecine régénérative des VEs a été démontré dans plusieurs modèles d'études précliniques, comme par exemple : les lésions myocardiales d'ischémie/reperfusion, les lésions rénales aiguës, le diabète sucré de type 2, la fibrose hépatique, la dégénérescence intervertébrale du disque, etc. [21,161,173]. Les VEs semblent également intéressantes dans la prise en charge des fistules digestives [175,176]. Il existe actuellement un changement d'orientation en médecine régénérative en proposant un passage de la thérapie cellulaire à la thérapie vésiculaire. Le statut immunitaire des VEs (dépourvues de complexe MHC41), permet leur utilisation en tant que traitement dit allogénique [16,177].

Figure 14 : Mécanismes d'action des vésicules extracellulaires pour la thérapie des fistules digestives. Tiré de l'article Sebbagh A et al, *Advanced Drug Delivery Reviews* 2021.

3.1. Production des vésicules extracellulaires

La production des VEs est un défi si l'on envisage son utilisation en pratique clinique. Les VEs sont secrétées spontanément par les cellules. Pour passer d'une thérapie cellulaire à une thérapie vésiculaire, on peut considérer que la quantité de CSM utilisée pour le traitement doit être équivalentement pour la sécrétion des VEs [178]. Par exemple le traitement par Alofisel contient environ 120 millions de cellules, il faudra donc envisager d'utiliser la même quantité de CSM pour produire un nombre suffisant de VE, pour faire une dose de traitement par VE. Cela entraîne une problématique d'échelle et de temps. Il faudra environ 50 flacons de 150 cm², et attendre deux semaines pour produire une dose de traitement de VEs [160,179]. Cette approche par culture cellulaire standard en 2D et sécrétion spontanée des VEs n'est pas viable pour une production à grande échelle de VE. Une autre stratégie consiste à augmenter le nombre de VEs sécrété par cellule. En effet, toutes les cellules libèrent spontanément des VEs, le processus peut être amplifié en plaçant les cellules dans un environnement carencé, hypoxique, et/ou de stress physique ou chimique [160].

La technique de production des VEs développée par notre laboratoire utilise un procédé à fort rendement, transposable à grande échelle, applicable à différents types cellulaires et compatible avec une production en bioréacteurs de grade GMP (Good Manufacturing Practices) (brevet déposé : FR1756183 2017 28). Les VEs sont produites à l'aide d'une stimulation mécanique par cisaillement. Cette technique permet une libération massive de VEs par les CSM placées dans un vortex (Figure 15).

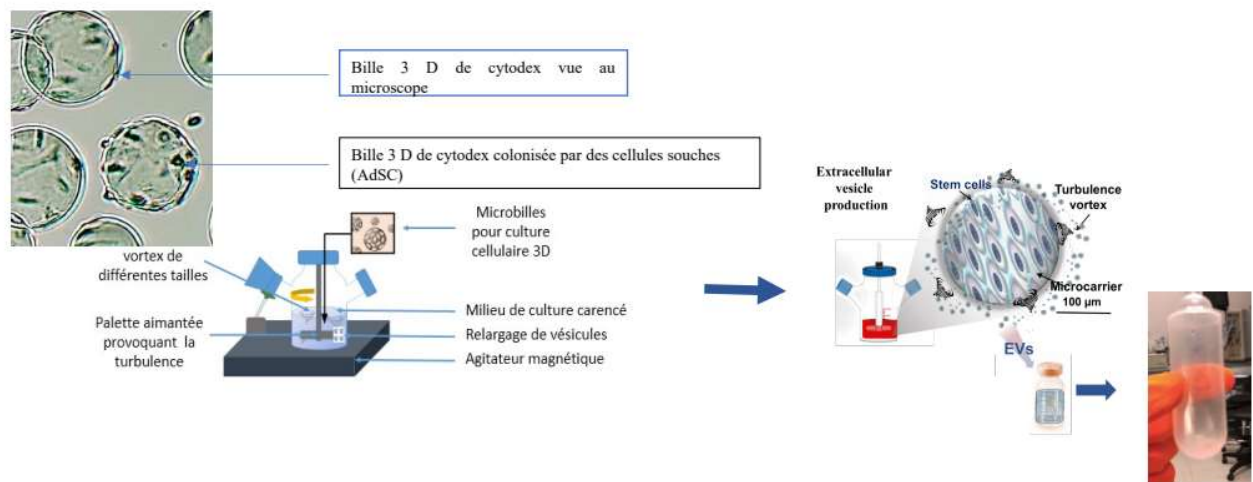


Figure 15: schéma de la production des VEs, selon un principe de vortex.

Une des originalités de la technique est de pouvoir combiner au sein du même bioréacteur à la fois la culture cellulaire et la stimulation mécanique pour l'émission de VEs *via* la génération d'un flux turbulent contrôlé par l'agitation du bioréacteur. La culture 3D repose sur l'ancrage des cellules sur de micro-suppports (200 µm) maintenus sous une rotation permanente *via* une pale rotative à l'intérieur du bioréacteur. Des bioréacteurs du type *spinner flask* avec une capacité de 100 à 1000 ml ont été utilisés pour la culture des CSM humaines. Le contrôle de la vitesse d'agitation permet de modifier les caractéristiques du flux turbulent, et de faire varier la taille du vortex générés. En fonction de la vitesse d'agitation (plus ou moins rapide) on pourra favoriser la culture cellulaire (vortex ~ 100 µm), ou déclencher la vésiculation modérée (vortex ~ 50 µm) ou massive (vortex ~ 35 µm). Rapportée au nombre de cellules, cette méthode permet de produire 10 fois plus de VEs par cellules et 10 fois plus rapidement que la méthode classique de production des VEs *via* la privation de sérum. La taille du vortex a été identifiée comme paramètre de contrôle calculée par l'équation de *Kolmogorov* qui prend en compte les différents paramètres du bioréacteur. Cela permet une transposition des méthodes de production à des bioréacteurs de différentes tailles. Il est possible de déterminer

la vitesse d'agitation à appliquer pour se situer dans les régimes idéaux pour la culture ou pour la stimulation de la vésiculation sur différents bioréacteurs.

3.2. Les vésicules extracellulaires en médecine régénérative

Les vésicules extracellulaires apparaissent comme une alternative aux CSM. Le potentiel effet des VEs a déjà été largement étudié pour un large éventail de maladies (Figure 16 et Tableau 4).

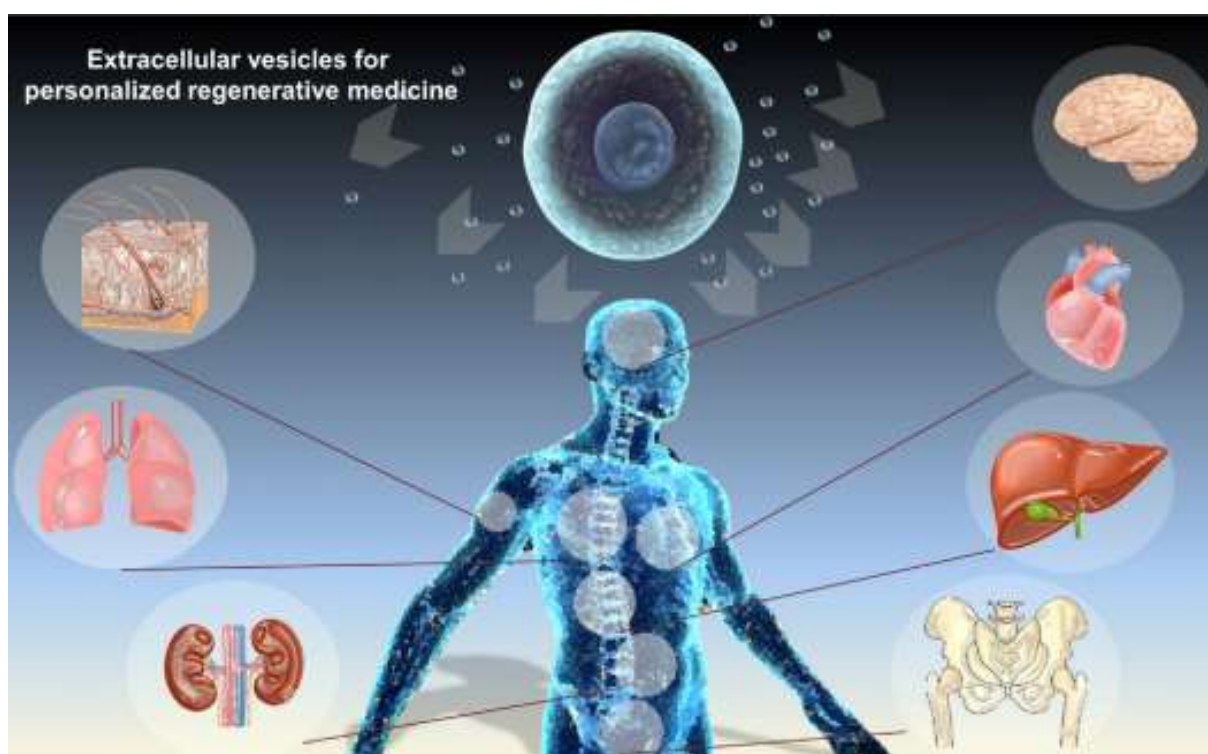


Figure 16 : Impact de la thérapie cellulaire par vésicule extracellulaire en médecine régénérative [160]

Tableau 4 Exemples de modèle animaux utilisant des VEs comme traitement régénératif.

| Référence | Modèle | MSC source | Mécanisme | Effet thérapeutique |
|-----------|-------------------------------|----------------|--------------------------------|---|
| [180] | Souris ; colite inflammatoire | Moelle osseuse | Production IL-10 | Diminution de la réponse inflammatoire |
| [181] | Souris ; Infarctus du | Moelle osseuse | Diminution NF-kB et activation | Diminution inflammation et la destruction des |

| | | | | |
|-------|-------------------------------|-------------------------|--|---|
| | myocarde | | AKT1/AKT2 | cardiomyocytes |
| [182] | Rat ; plaie cutané diabétique | Moelle osseuse | Régulation des voies TLR4/NF-kB/STAT3/ AKT | Réduction inflammation et amélioration de la cicatrisation cutané |
| [183] | Souris ; Asthme | Tissu adipeux | Activation des macrophages M2 anti inflammatoire | Amélioration du remodelage respiratoire |
| [184] | Souris | Cordon ombilical humain | Inhibition de l'activité NLRP3 | Amélioration de la réparation tissulaire |

3.3. Thérapie vésiculaire des fistules digestives

Dans notre équipe, nous avons montré pour la 1^{ère} fois l'intérêt des VEs dans la prise en charge des fistules digestives post-opératoires. Nous avons réalisé une première étude sur un modèle de fistules œsophagiennes chroniques chez le porc, traitées au 30^{ème} jour par $1,3 \cdot 10^{11}$ vésicules dérivées d'AdSC contenu dans du gel de Pluronic F127. Au 45^{ème} jour, le taux de fermeture était de 100% dans le groupe du gel avec vésicules, contre aucune fermeture dans le bras contrôle. L'étude confirme le rôle anti-inflammatoire, pro-angiogénique et antifibrotique des vésicules extracellulaires. Il convient de noter qu'une diminution de l'infiltration de cellules inflammatoires et de la fibrose ainsi qu'une augmentation de l'angiogenèse ont été observées dans les groupes de traitements par rapport au groupe témoin, mais cela n'était significatif que pour le groupe avec VEs. Cette étude a fourni la première preuve in vivo de l'intérêt de l'administration locale de VEs pour le traitement des fistules digestives post-chirurgicales.

Cependant, d'autres études sont nécessaires pour reproduire ces résultats prometteurs avec un plus grand nombre d'animaux et des périodes de suivi plus longues. En particulier, une étude plus approfondie du mécanisme d'action des VEs doit être réalisée avant toute tentative d'application clinique potentielle. En outre, il serait intéressant de faire varier la quantité de VEs administrée afin d'étudier une éventuelle relation dose/réponse, de comparer le potentiel thérapeutique des VEs à celui des cellules souches dans cette indication. De plus, l'effet thérapeutique des VEs en fonction du mode d'administration et leur rétention dans le tractus fistuleux restent pour l'instant inconnus.

Les principaux avantages des VEs sont représentés par leur facilité de stockage, de conservation, de distribution et d'administration en comparaison avec les cellules. En effet, il y a un risque néoplasique plus faible qu'avec les CS, autonomes et capables de se multiplier. Les VEs peuvent représenter un nouvel outil thérapeutique pour l'avenir, présentant des avantages comparables à la thérapie cellulaire par CS, avec une plus grande facilité d'utilisation et un meilleur profil de sécurité.

3.3.1. Vers une thérapie combinée : VEs et biomatériaux

En effet, dans le cadre de trajets de fistules digestives, la zone de traitement est de taille variable, et présentent des trajets plus ou moins complexes. L'administration de VEs constitue un défi ; en effet, la voie systémique a une demi-vie courte et une internalisation rapide par le foie et la rate [185–187]. Cela souligne l'importance de l'administration locale plutôt que systémique pour améliorer la rétention des VEs sur le site d'intérêt [188]. Nos travaux de recherches ont utilisé un gel de poloxamer 20% comme véhicule des VEs afin de permettre l'application des VEs sur l'ensemble du trajet de fistules. L'utilisation du poloxamer 20%, dont la transition sol/gel se produit aux environs de 20°C, permet une injection sous forme liquide à froid pour remplir de manière homogène le trajet de fistules potentiellement irrégulier et une gélification rapide lors

de l'administration pour retenir les VEs dans le trajet de fistules à température corporelle (Figure 17).

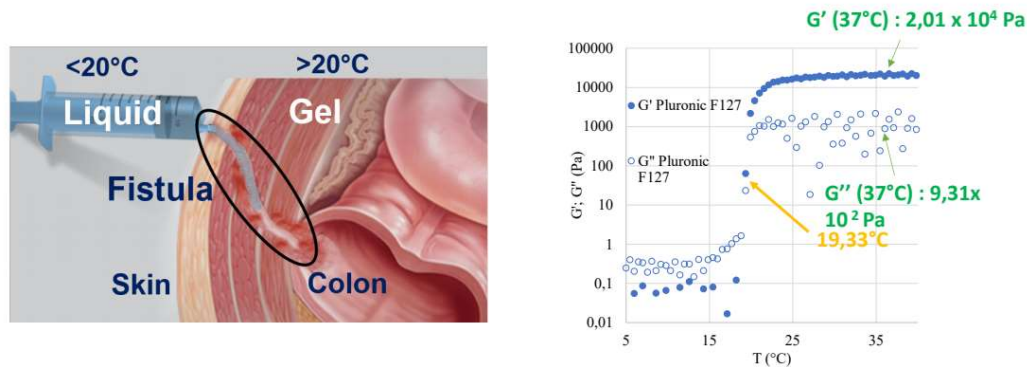


Figure 17 : Schéma d'utilisation du gel de Pluronic F 127, et son évaluation en rhéologique définissant la température de transition entre la forme liquide et gel.

Il s'agit d'une molécule déjà connue, commercialisée et bénéficiant d'un marquage CE et enregistrée auprès de la Food Drug Administration (FDA) aux États Unis. Des travaux actuels se penchent sur ses propriétés cicatrisantes, chez l'animal comme chez l'homme [189]. L'utilisation du gel de Pluronic augmente le temps de contact entre les molécules thérapeutiques et le tissu cible. Ce poloxamère est facilement injectable à travers un cathéter sous la forme liquide (permettant le remplissage de l'intégralité du trajet de la fistule indépendamment de sa taille et de sa morphologie irrégulière). Ce gel thermosensible se gélifie à partir de 19°C dans le corps (permettant la rétention des VEs dans le trajet fistuleux) (Figure 17). Le gel de Pluronic F127 est obtenu à partir d'une poudre de poloxamer qui est diluée dans un solvant (par exemple du PBS ou une solution de NaCl à 0.9%). La dilution de la poudre dans le solvant doit être réalisée à l'aide d'un agitateur magnétique à basse température ($+ 4^{\circ}\text{C}$), pendant 24H. Après obtention d'un gel de Pluronic F127 liquide à $+ 4^{\circ}\text{C}$, on réalise une filtration au filtre $0.2\mu\text{m}$, afin de stériliser le gel avant de le stocker au

réfrigérateur à + 4°C. L'ensemble des étapes de fabrication du gel de Pluronic F 127 est détaillé dans la figure 18.

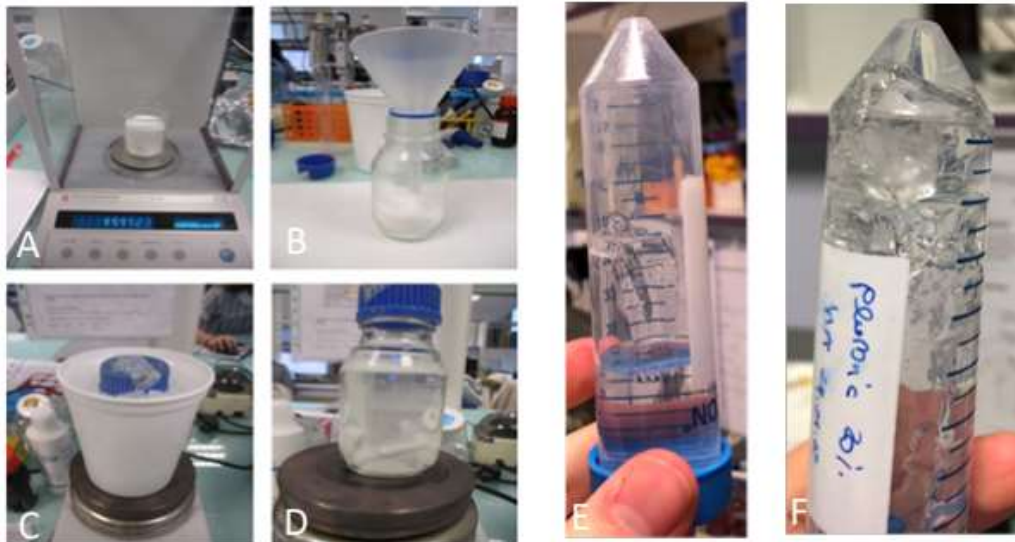


Figure 18: Étapes de fabrication du gel de Pluronic F 127 ; A, poudre de poloxamere de Pluronic F127 pesée pour déterminer la concentration précise du gel. B, bécher de 200 ml permettant de mélanger la poudre avec le solvant (NaCl 0.9%) ; C-D, agitateur magnétique dans la glace à (+ 4°C) ; E, test d'inversion des tubes à + 4°C, afin de confirmer la fluidité du gel ; F, test d'inversion à + 22°C pour confirmer la gélification du gel.

L'emploi d'un tel gel avait déjà été suggéré par des équipes afin d'optimiser le relargage, et d'améliorer la durée de contact entre le médicament (VEs) et le tissu cible, en particulier dans les fistules ano-périnéales dans la maladie MC [149,190]. Dans notre équipe nous avons évalué l'utilisation du gel de Pluronic seul ou avec VEs dans un modèle de fistules œsophagiennes chroniques chez le porc [175]. Cet article montre une amélioration significative du taux de fermeture des fistules avec injection de gel de Pluronic seul versus contrôle. L'ajout de VEs dans le gel, permet d'obtenir une fermeture des trajets de fistules dans 100% des cas versus 67% avec le gel seule [175] (Figure 19). Le gel de pluronic F127

présente des propriétés pro-angiogéniques, anti-inflammatoires et anti-fibrosantes qui sont amplifiées par l'ajout des VEs comme traitement régénératif.

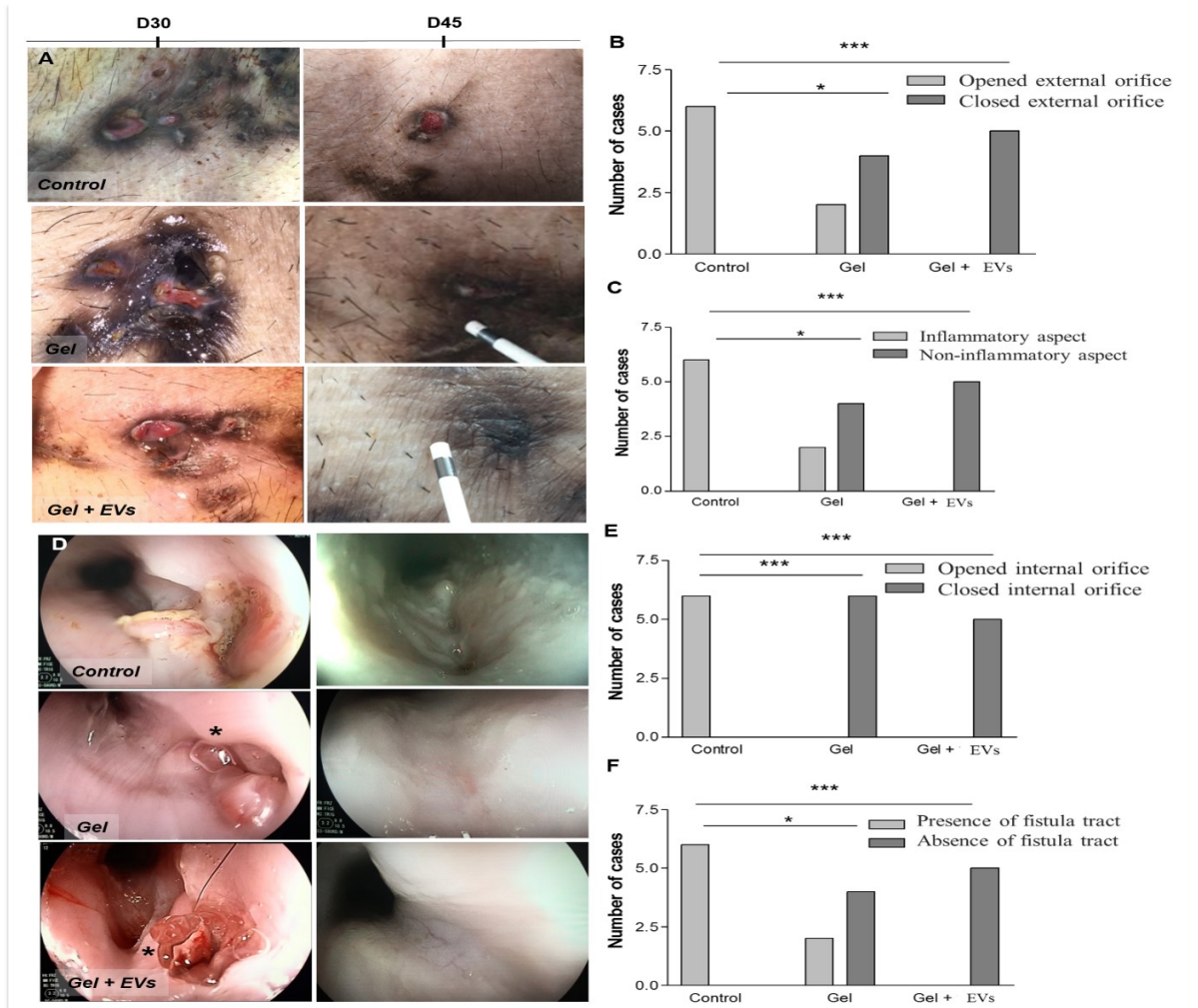


Figure 19 : Résultat de l'article de Amanda K. A. Silva, et al, sur un modèle de fistules oeso-cutanées chez le porc en fonction du groupe de traitement (control, gel seul, et gel + VEs) [175].

OBJECTIFS DU TRAVAIL DE THESE

Les fistules post-opératoires sont des complications fréquentes, responsables d'une morbi-mortalité élevée. La prise en charge des fistules est complexe, associant une prise en charge pluridisciplinaire endoscopique, radiologique, chirurgicale (...). Les premiers temps du traitement consistent à réaliser un drainage et une cicatrisation dirigée si possible par abord mini-invasif endoscopique. En cas d'échec, une nouvelle intervention chirurgicale peut être nécessaire, mais elle est associée à un taux de morbi-mortalité élevée [3].

Des nouveaux traitements à base de CSM donnent des résultats encourageants grâce à leur effet régénératif mais surtout à leur effet paracrine. Il existe actuellement un changement d'orientation en **médecine régénérative** en proposant un passage de la thérapie cellulaire à la thérapie vésiculaire.

L'objectif de ce travail de thèse est :

1/ D'évaluer l'effet régénératif des VEs issu de CSM de souris et leur voie d'administration dans un modèle connu de fistules colo-cutanées chez le rat.

2/ De développer des nouveaux modèles de fistules post-opératoires chez le petit (rat) et le gros (porc) animal, plus proche des fistules post-opératoires chez l'homme.

3/ D'évaluer l'efficacité des VEs issues d'AdSC humaines dans les cicatrisations de fistules gastro-cutanées post-sleeve gastrectomie chez le petit animal (rat) et validation des résultats chez le gros animal (porc).

RESULTATS DU TRAVAIL DE THESE

Article 1

Local administration of stem cell-derived extracellular vesicles in a thermoresponsive hydrogel promotes a pro-healing effect in a rat model of colo-cutaneous post-surgical fistula.

Arthur Berger, Irami Araújo-Filho, Max Piffoux, Alba Nicolás-Boluda et al. Nanoscale, 2021

Article 2

Creation of a gastro-cutaneous fistula model after sleeve gastrectomy in small and pig animal

Arthur Berger, Matthieu Bruzzi, Irami Araújo-Filho, Guillaume Pere et al

En cours de préparation

Article 3

Treatment of chronic gastrocutaneous fistula after sleeve gastrectomy in a rat and PIG models by Extracellular Vesicles embedded with Pluronic gel.

Arthur Berger, Guillaume Pérè, Anna Sebbagh, Matthieu Bruzzi et al.

En cours de préparation

Article 4

Enhancing digestive fistula healing by the off-label use of a thermoresponsive vessel occluder polymer associated with esophageal stent placement: A case report.

Arthur Berger, Eric Caudron, Guillaume Perrod, Imane Boucenna et al. Clin Res Hepatol Gastroenterol. 2021

Article 1: Local administration of stem cell-derived extracellular vesicles in a thermoresponsive hydrogel promotes a pro-healing effect in a rat model of colo-cutaneous post-surgical fistula.

Arthur Berger, Irami Araújo-Filho, Max Piffoux, Alba Nicolás-Boluda et al. Nanoscale, 2021

Introduction :

La prise en charge des fistules digestives post-opératoires est un challenge pour la médecine régénérative. En effet, la prise en charge conventionnelle pluridisciplinaire (endoscopique, radiologique, chirurgicale..), est imparfaite avec une augmentation significative de la morbi-mortalité en cas de fistules digestives post-opératoires. Plusieurs études ont évalué l'effet des CS comme traitement régénératif des fistules post-opératoires chez l'animal. L'utilisation de la thérapie cellulaire par AdSC humaines semble prometteuse dans le traitement des fistules ano-périnéales de MC chez l'homme. L'effet paracrine de la thérapie cellulaire a été rapporté comme le mécanisme d'action principal [147]. Les VEs ou « poussières cellulaires » représentent un outil innovant et prometteur en médecine régénérative. Les VEs issues de cellules souches mésenchymateuses reproduisent l'effet paracrine des cellules mères sans risque prolifératif ; elles sont plus résistantes et facilement conservées. Plusieurs études pré-

cliniques ont montré l'intérêt des VEs comme traitement régénératif dans le cadre d'infarctus du myocarde, insuffisance rénale, de fibrose hépatique... L'effet paracrine des VEs pourrait améliorer la cicatrisation locale des trajets de fistules digestives. Dans l'étude de A. Silva et al, les fistules œsophagiennes chroniques étaient traitées par $1,3 \cdot 10^{11}$ vésicules dérivées d'AdSC incluses dans du gel de poloxamer 407 à 20% afin de retenir les vésicules dans le trajet fistuleux. Cette étude a fourni la première preuve in vivo de l'intérêt de l'administration locale de VEs pour le traitement des fistules digestives post-chirurgicales. Cependant, d'autres études sont nécessaires pour reproduire ces résultats prometteurs dans d'autres modèles animaux. De plus,

l'effet thérapeutique des VEs en fonction du mode d'administration et leur rétention dans le tractus fistuleux restent pour l'instant inconnus.

Dans cette première partie du travail de thèse, nous avons cherché à confirmer les premiers résultats obtenus dans le modèle de fistules oeso-cutanées chez le porc. Nous avons évalué l'effet cicatrisant des VEs dans un modèle connu de fistules colo-cutanées chez le rat. Les VEs utilisées étaient issues de CSM de souris, produites par un procédé innovant à fort rendement par turbulence. Parallèlement, nous avons étudié la bio-distribution des VEs en fonction de la voie d'administration et du véhicule de délivrance grâce à une analyse par TEP-IRM.



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Local administration of stem cell-derived extracellular vesicles in a thermoresponsive hydrogel promotes a pro-healing effect in a rat model of colo-cutaneous post-surgical fistula†

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Extracellular vesicles (EVs), especially from stem/stromal cells (SCs), represent a cell-free alternative in regenerative medicine holding promises to promote tissue healing while providing safety and logistic advantages in comparison to cellular counterparts. Herein, we hypothesize that SC EVs, administered locally in a thermoresponsive gel, is a therapeutic strategy for managing post-surgical colo-cutaneous fistulas. This disease is a neglected and challenging condition associated to low remission rates and high refractoriness. Herein, EVs from a murine SC line were produced by a high-yield scalable method in bio-reactors. The post-surgical intestinal fistula model was induced via a surgical cecostomy communicating the cecum and the skin in Wistar rats. Animals were treated just after cecostomy with PBS, thermoresponsive Pluronic F-127 hydrogel alone or containing SC EVs. A PET-monitored biodistribution investigation of SC EVs labelled with ⁸⁹Zr was performed. Fistula external orifice and output assessment, probe-based confocal laser endomicroscopy, MRI and histology were carried out for therapy follow-up. The relevance of percutaneous EV administration embedded in the hydrogel vehicle was indicated by the PET-biodistribution study. Local administration of SC EVs in the hydrogel reduced colo-cutaneous fistula diameter, output, fibrosis and inflammation while increasing the density of neo-vessels when compared to the PBS and gel groups. This multi-modal investigation pointed-out the therapeutic potential of SC EVs administered locally and in a thermoresponsive hydrogel for the management of challenging post-surgical colon fistulas in a minimally-invasive cell-free strategy.

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Introduction

Extracellular vesicles (EVs) from stem/stromal cells (SCs) are recent players in the evolving landscape of regenerative medi-

cine bringing along new promises and challenges. In the front-line of cell therapy, SCs are known to provide the microenvironment with trophic and survival signals including cytokines released by means of EVs, pointing out EV role in paracrine

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effect.^{1,2} EVs, including exosomes, microvesicles and apoptotic bodies, are sub-cellular membrane-enclosed entities at the nanometer size range (40–5000 nm diameter) shed by cells either in a constitutive or in an inducible basis.^{3–5} Pleiotropic effects have been reported for SC EVs, such as the (i) induction of pro-angiogenesis;⁶ (ii) inhibition of cell apoptosis;^{7,8} (iii) cell proliferation promotion;⁹ (iv) inflammation reduction¹⁰ and (v) fibrosis attenuation.^{10–12} For instance, SC EVs have been shown to induce proliferation and apoptosis resistance in a rat model of renal ischemia/reperfusion injury¹³ and in a rat model of hepatectomy.¹¹ SC-derived EVs also reduced infarct size in a mouse model of myocardial ischemia/reperfusion, mediating a cardioprotective effect.² The regenerative properties of SC EVs were also reported for cutaneous wounds,^{14,15} bone fracture¹⁶ as well as for brain⁶ and lung injuries.¹⁰ The current body of evidence on EV regenerative properties gave rise to a paradigm shift, switching cell therapy to sub-cellular (EV) therapy. The strength of EV therapy is the perspective to mitigate risks of uncontrolled cell replication, differentiation or vascular occlusion, offering storage and shelf-life gains.^{17,18} Another key asset is the immune-privileged status of SC EVs allowing to consider an allogeneic setting¹⁷ as an “off-the-shelf” therapeutic modality.^{19,20}

Going beyond the assets, the other side of the coin concerns the challenges for rendering EV-based regenerative medicine clinically feasible. In this regard, there is namely the demand for high-yield, cost-effective, scalable and standardized EV production complying with regulatory issues and compatible with good manufacturing practice (cGMP) requirements. Another challenge is EV administration. Systemically administered EVs have a short half-life with a rapid uptake by the mononuclear phagocyte system especially in the liver and spleen.^{21–23} This points out the importance of local rather than systemic administration to improve EV retention in the site of interest.^{24,25} EV combination to a biomaterial may represent an eligible strategy in the attempt to extend EV residence time in the target and modulate EV release.^{26,27} In this regard, injectable hydrogels offer the advantages of filling irregular defects and reaching low-accessible areas while enabling the possibility to tune release profile depending on the material choice.²⁸ Thermoresponsive hydrogels that exhibit a lower critical solution temperature (Pluronic F-127 (PF-127), poly (*N*-isopropylacrylamide)) provide the additional advantage of minimally-invasive injection in the liquid state and gelation *in situ* at body temperature, preventing therapy leaking, promoting controlled release and favoring retention at the site of interest.^{29,30}

In the present paper, we tackle EV production and administration challenges by testing, respectively, (i) a high-yield scalable cGMP approach for EV production based on a turbulence-induced stimulation directly in bioreactors and (ii) a local thermo-controlled EV delivery in a thermoresponsive hydrogel. In order to test this approach, we address herein unmet needs in the therapy of post-surgical intestinal fistulas, which are abnormal communication between two organs or organ connection to the skin. Digestive fistulas are challenging con-

ditions associated to low remission rates, high morbidity and refractoriness.^{31–33} Pharmacological approaches based on the systemic administration of antibiotics, somatostatin, and immune-modulators result in poor therapeutic outcome.^{34–36} More than 75% of the digestive fistula cases are related to a postoperative complication.³⁷ Post-surgical fistulas are potentially life-threatening mainly due to septic or hemorrhagic complications and are associated to high morbidity.³⁸ In the USA, complications related to gastrointestinal post-surgical fistulas results in additional length of stay and hospital costs of 7.3 days and \$24 129 per patient, respectively, only considering the first hospitalization.³⁹ The risk of such post-surgical complications in patients undergoing upper gastro-intestinal surgery, for instance, sleeve gastrectomy ranges between 0.5% and 7% (2.5% being considered the overall rate).⁴⁰ Colorectal complications related to post-surgical fistulas occur in 3%–11% of cases (6% being considered the overall rate).^{41–43} The surgical, endoscopic and radiological management has clearly redesigned and improved the management of these fistulas. Fistula care is provided by a team including surgeons, nurses, enterostomal therapists, social workers and nutritionists who will personalize a treatment plan. Patients without life-threatening complications will receive conservative treatment with local management of fistula output, replacement of fluid and electrolyte losses, nutritional support and antibiotic therapy.⁴⁴ Complementarily, endoscopic treatments, with stents or clips have been used.⁴⁵ Rescue surgery is required when minimally invasive strategies fail or in case of life-threatening complications.³⁸ This complex surgery will attempt to close the fistula and reconnect the gastrointestinal tract with a risk of mutilating ileostomy or colostomy. Rescue surgery results in increased morbidity and high mortality rate as well as prolonged hospitalization.⁴⁶ Unmet needs in post-surgical fistulas therapy recently motivated the investigation of cell therapy as alternative. In 2018, the European Commission approved Alofisel® for Crohn’s disease fistula indication becoming the first allogeneic SC therapy (based on adipose tissue-derived SCs – AdSCs), in Europe to receive marketing authorization approval.^{47,48} Although post-surgical fistulas were not listed as a therapeutic indication for Alofisel®, some papers have reported encouraging pre-clinical data based on the administration of AdSCs, associated or not with a biomaterial, for the management of digestive post-surgical complications.^{49–51} A clinical trial on AdSC therapy for gastrointestinal post-surgical fistulas is ongoing by the Mayo Clinic (ClinicalTrials.gov identifier: NCT02807389). In this clinical investigation, cells are administered locally using a synthetic prosthesis biomaterial composed of polymers (polyglycolic acid/trimethylene carbonate) that are gradually absorbed by the body.

The aim of this paper is to investigate a biomaterial-based cell-free alternative to post-surgical fistulas. Our approach relies on turbulence SC EVs embedded in a PF-127 thermoresponsive hydrogel administered percutaneously in colcutaneous fistulas (Fig. 1). The relevance of this approach in terms of biodistribution and therapeutic outcome were evaluated.



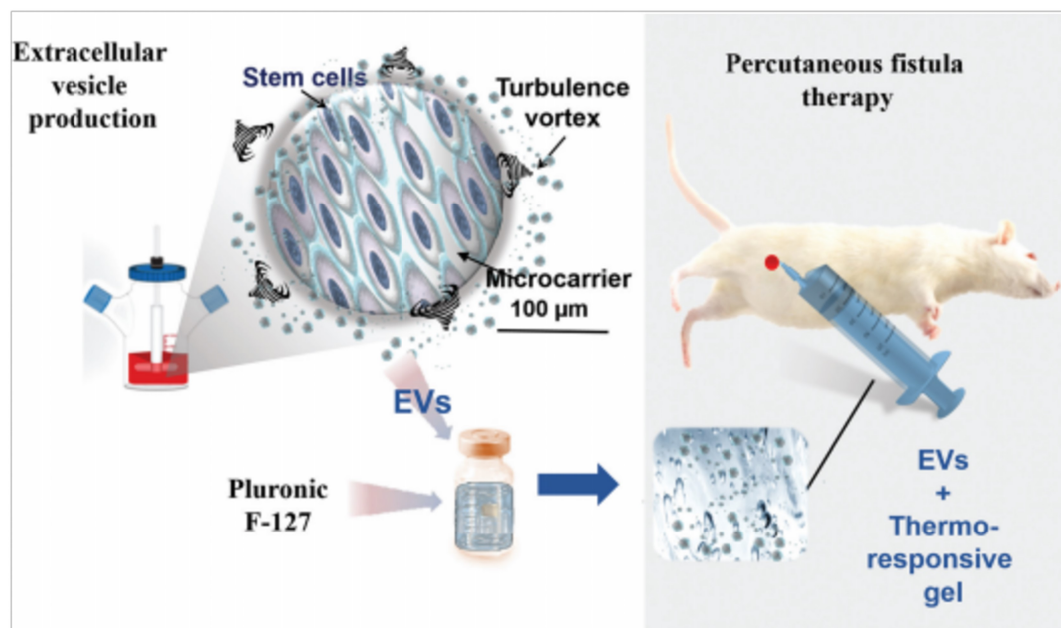


Fig. 1 Schematic representation of the experimental approach based on the production of EVs at high yield by turbulence stimulation from murine stem cells cultured on microcarriers in 1 L bioreactors. EVs were combined to Pluronic F-127 thermoresponsive hydrogel and administered percutaneously at $<19\text{ }^{\circ}\text{C}$ (below gel transition temperature) in a colo-cutaneous fistula model in rats. The formulation gelling *in situ* at body temperature is intended to increase EV residence time at the site of interest.

Experimental section

EVs and gel production and characterization

Cell culture in 2D flasks. Murine C3H/10T1/2, Clone 8 (ATCC®) cells were cultured at $37\text{ }^{\circ}\text{C}$ and 5% CO_2 . Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin was used. Cells were cultured in 150 cm^2 flasks until confluence.

3D cell culture in spinner flask bioreactors. Commercial dextran microcarriers of about $200\text{ }\mu\text{m}$ (Cytodex 1, GE Healthcare) were dispersed in PBS, and autoclaved for sterility. PBS was changed to DMEM without phenol red, and stored at $4\text{ }^{\circ}\text{C}$. Previous to cell seeding, microcarriers were incubated in $37\text{ }^{\circ}\text{C}$ complete medium at a 6 g L^{-1} concentration during 3 h. Cells were seeded with a cell to microcarrier ratio of 5/1 and submitted to 24 cycles of 45 min of rest followed by 3 min of a gentle mixing at 60 rotations per minute (rpm) in 1 L bioreactor to enable homogeneous adhesion of cells on microcarriers. After cell adhesion, microcarriers were diluted to 3 g L^{-1} with complete medium at $37\text{ }^{\circ}\text{C}$ at 60 RPM until reaching confluence on microcarriers (about 7 days). Every 2–3 days, 30 to 70% of the medium was changed according to the cell confluence.

Turbulence-triggered EV production in spinner flask bioreactors. Once cells were confluent on microcarriers, turbulence-triggered EV production was launched. The protocol consisted in changing the complete medium to heated ($37\text{ }^{\circ}\text{C}$) serum-free DMEM media without phenol red with 100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin after 3 rinsing steps.

By controlling the spinner flask stirring, cells on microcarriers were submitted to a turbulent flow featuring a Kolmogorov length of $35\text{ }\mu\text{m}$ during 4 hours to stimulate EV release.⁵² After that, cellular debris of the supernatant were removed by centrifugation at 2000g for 10 min. The supernatant was ultracentrifuged at $110\text{ }000\text{g}$ for 70 min to obtain an EV pellet.

Nanoparticle tracking analysis. EV size distribution and concentration were determined by nanoparticle tracking analysis (NTA) using a Nanosight LM10-HS (NanoSight, UK) with a 405 nm laser. Before measurements, EVs were diluted to an appropriate concentration (between 3×10^8 and 2×10^9 particles per mL) with sterile PBS (confirmed to be particle-free by NTA measurement). For each sample, 5 movies of 30 s were recorded using a camera level of 16. Data were analyzed with NTA Analytical Software.

Cryo-transmission electron microscopy. A total of $2\text{--}7\text{ }\mu\text{L}$ of SC EVs was deposited onto an electron microscopy grid coated with a perforated carbon film (Ted Pella, Redding, CA, USA), the excess liquid was blotted off with a filter paper, and the grid was then quickly plunged into liquid ethane. Analysis was carried out in MET Jeol 2100 (LaB6) at 80 kV .

EV marker analysis by ExoView device. A murine SC EV batch produced by the turbulence method was analysed by ExoView (NanoView Biosciences, USA). All samples were diluted in PBS with 0.05% Tween-20 (PBS-Tw). The samples were incubated on the ExoView Tetraspanin Chip for mouse EVs placed in a 24-well plate for 16 h at room temperature. The chips were washed 3 times with PBS-Tw. Chips were incubated with ExoView Tetraspanin Labelling antibodies that consist of anti-CD81 Alexa-555, anti-CD63 Alexa-488, anti-CD9



Alexa-647 and anti-calnexin Alexa 647. The antibodies were diluted 1 : 5000 in PBS-Tw with 2% BSA. The chips were incubated with 250 μL of the labelling solution for 2 h, washed in PBS-Tw and dried. The chips were imaged with the ExoView R100 reader using the ExoScan 2.5.5 acquisition software. The data were analysed using ExoViewer 2.5.0 with sizing thresholds set to 50 to 200 nm diameter.

In vitro assessment of EV pro-angiogenic properties by HUVEC scratch test

HUVEC (ATCC®) were cultured at 37 °C and 5% CO_2 . DMEM supplemented with 10% FBS and 100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin was used. Cells were cultured in 150 cm^2 flasks until confluence. Pro-angiogenic properties of SC EVs were tested *in vitro* using a scratch test. Monolayers of confluent HUVEC cells in 24-well plates were scratched using a 1000 μL pipet cone and the medium was replaced by fresh one to discard detached cells. HUVEC scratched monolayers were incubated for 9 h with DMEM without serum containing SC EVs. This test was performed with an EV dose set by the producer cell/recipient cell ratio of 1 : 1, 3 : 1 or 10 : 1, corresponding to a minimum of 2.5×10^4 to a maximum of 3×10^6 EV per cell. Serum-free medium and complete medium were used as negative and positive controls, respectively. Pictures of each condition were taken before EV incubation and 9 h later. The percentage of migration was calculated *via* the gap width between wound edges measured using Image J software at 0 and 9 h time points, 100% migration meaning total gap bridging.

Preparation of PF-127 gel. PF-127 was solubilized at a concentration of 20% (weight/weight) in PBS under agitation during 24 hours at 4 °C, followed by a sterilizing filtration (filter 0.22 μm) at 4 °C.

Characterization of PF-127 gel. Rheological studies were performed for the PF-127 hydrogels with a Physica RheoCompass MCR 302 (Anton Paar) using a cone and plate geometry (diameter = 50 mm, cone angle = 1°) and a solvent trap to prevent evaporation. The measurements of G' and G'' , the elastic and viscous moduli, respectively, as a function of temperature were performed. The temperature was gradually increased by 5 °C min^{-1} starting from 5 °C to 40 °C. The sol-gel transition temperature, cross over between G' and G'' , was investigated.

Rat model of colo-cutaneous fistula

All experiments were approved by the animal care and use committee in Brazil and France as well as the Ministry of Higher Education and Research in France. A surgical cecostomy communicating the cecum and the skin was performed in female 11-week old Wistar rats. The animals went through a 7-day acclimatization period with water and food *ad libitum*. The animals were housed in the laboratory animal room, in cages, with regulated temperature, ventilation, and respecting light-dark cycles. Rats received a liquid diet on the day prior to surgery and were fasted overnight before operation. Anesthesia was performed under 2% of isoflurane (Baxter, Maurepas, France). A surgical cecostomy was performed

according to the model described by Bültmann and colleagues.⁵³ Rats were placed in supine position, feet spread apart and their abdomen was shaved. After a midline laparotomy of 3 cm, the distal part of the cecum was passed with a staggered opening through the abdominal muscle and the subcutaneous space, forming a 10 mm-long tract. Four stitches (Vicryl 4/0) were used to attach the cecum to the skin at the site of the incision on the right flank of the rat, creating a colo-cutaneous fistula model. Postoperative analgesia was performed. Animals were isolated in individual cages, one animal/cage, in the pre and postoperative period. Animals were killed, after the experiment by an intracardiac injection of thiopental.

Biodistribution investigation

⁸⁹Zr-oxinate preparation and EV labeling for biodistribution investigation. In a first step, EVs were functionalized with the bifunctional *p*-isothiocyanatobenzyl-desferrioxamine (DFO) *via* lysine-NH₂ groups on EV outer membrane surface proteins following a protocol for antibody functionalization.⁵⁴ Briefly, a 50 mM DFO in DMSO solution was diluted 50× in pH 9 PBS. EVs were resuspended in pH 9 PBS, and centrifuged at 16 200g for 30 min to obtain EVs in 100 μL with a 3 mg mL^{-1} protein concentration. 3 μL of DFO solution were added to EVs, and they were incubated at 37 °C for 30 min. A final washing step (16 200g, 30 min, two times) with normal pH 7.4 PBS with 1% penicillin streptomycin was used to remove DFO. EVs were then frozen until use. [⁸⁹Zr] Zirconium⁵⁵ was produced by a ⁸⁹Y(*p,n*)⁸⁹Zr nuclear reaction^{56,57} in a GE cyclotron PETtrace 880, using a home-made target built in aluminum. A high purity (>99%) [⁸⁹Y] yttrium sheet was bombarded with 12.8 MeV protons, at a 10 μA current, by 180 min. The activated ⁸⁹Y sheet was dissolved in 6 M HCl and [⁸⁹Zr]Zr⁴⁺ was purified by filtration in a ZR resin column (Trisken International, France) and elution was carried out with oxalate (0.1 M). A total of 100 μL of the solution was transferred to an Eppendorf tube, neutralized with 3 × 30 μL of 2 M Na₂CO₃ and incubated at 22–25 °C for 3 min, followed by the addition of 300 μL of HEPES (0.5 M pH 7.17). A total of 250 μL (130 MBq) of the solution of [⁸⁹Zr][Zr(ox)₄]⁴⁻ was transferred to a V reaction vial containing 500 μL 8-hydroxyquinoline (8-HQ) in chloroform (1 mg mL^{-1}), and the vial was shaken for 10 min and the product [⁸⁹Zr][Zr(8-HQ)₄] was recovered from the chloroform phase by evaporation, redissolved in dimethyl sulfoxide (DMSO, 20 μL) and diluted with 0.1 mL of PBS (0.1 M pH 7.4).⁵⁸ Radiochemical purity was checked in ITLC-SG/ethyl acetate chromatography. To these vial was added 50 μL of EVs suspension (a total of 5×10^{12} EVs) and the vial was shaken for 60 min at 37 °C. This suspension was transferred to 10 kDa molecular weight cut-off filters (Amicon®Ultra – 500 μL , Merck Millipore Ltd, Ireland) and volume completed to 300 μL and centrifuged. The residual was washed twice with 300 μL of PBS, and residual ([⁸⁹Zr]Zr-EVs product) was removed from the Amicon®Ultra tube. The [⁸⁹Zr]Zr-EVs product was analysed by ITLC-SG/citrate solution (20 μM – pH 5) chromatography.



Positron emission tomography (PET)/magnetic resonance imaging (MRI) acquisition. A biodistribution study was performed in female Wistar rats (200–250 g, $n = 7$) purchased from the Institute of Biomedical Sciences of the University of São Paulo (ICB/USP). These rats were kept at room temperature of 22 ± 2 °C with light/dark cycle (12:12 h). Food and water were provided *ad libitum*. The rats were allowed to acclimatize for at least seven days. All procedures were conducted according with the guidelines of the National Council for the control of Animal Experimentation (CONCEA, Brazil) and were approved by the Ethics Committee for Animal Research of the Medical school of the University of São Paulo (CEUA-FMUSP/USP, Brazil – protocol number: 904/17). Immediately after surgical cecostomy, the animals were treated with 4 MBq of [^{89}Zr]Zr-EVs by intrafistula administration. One animal was treated with the [^{89}Zr]Zr-EVs into PF-127 gel, another with the [^{89}Zr]Zr-EVs in saline solution and a third one with the [^{89}Zr]Zr-EVs in saline intravenously, then image acquisitions were performed at 1 hour, 24 hours and 7 days.

PET/MR images were acquired with flexible knee coil MRI at the General Electric (GE) Signa PET/MR 3T (HC-FMUSP – Brazil) during the period which patients were not being attended and with all hygiene care. PET image was acquired for 15 minutes simultaneously with T1 weighted MRI with animals under anesthesia with ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}). Emission sinograms were iteratively reconstructed into a single frame of 15 min (OSEM; 4 iterations and 28 subsets), after being corrected for radioactivity decay. Intra-fistula uptake of [^{89}Zr]Zr-EVs of three animals were analyzed in the PMOD 3.4 software (PMODTM Technologies Ltd, Switzerland). Volumes of interest (VOIs) regions were drawn in the PET images fused to the T1 weighted MRI of each animal. The [^{89}Zr]Zr-EVs uptake was analyzed as a standardized uptake value (SUV), which is calculated as radioactivity concentration (kBq cc^{-1}) divided by the ratio between injected dose (kBq) and animal body weight (g) for each time point.

PET acquisition was performed to check the biodistribution of [^{89}Zr][Zr(ox) $_4$] $^{4-}$ or [^{89}Zr][Zr(DFO)] following percutaneous intra-fistula or intravenous administration after 24 hours and 7 days, as a conjugation quality control. These PET images were performed at small-animal PET scanner (TriumphTM – Gamma Medica-Ideas, Northridge, CA, USA). A static image of 25 min (5 min per bed position). Animals were anesthetized with isoflurane mixed with oxygen and 4 MBq of [^{89}Zr][Zr(ox) $_4$] $^{4-}$ or [^{89}Zr][Zr(DFO)] were injected in the tail vein. Emission sinograms were iteratively reconstructed into a single frame of 25 min (OSEM; 20 iterations and 4 subsets), after being corrected for radioactivity decay. The body temperature of the animals was maintained by heating pads and breath rate were monitored.

EV-mediated percutaneous fistula therapy in the hydrogel and follow-up

Therapy. Female Wistar rats (Janvier, 250–300 g) were randomized in three groups and treated just after cecostomy

with 0.6 mL of PBS (control group, $n = 8$), 0.6 mL of thermo-responsive Pluronic F-127 (Sigma-Aldrich) hydrogel at 20% (gel group, $n = 8$) or 0.6 mL of thermo-responsive Pluronic F-127 hydrogel at 20% containing turbulence-triggered EVs at 1×10^{12} EVs mL^{-1} concentration (gel + EV group, $n = 6$). Treatment was repeated once every week during 60 days under 2% of isoflurane anesthesia. In the beginning of the study there were 8 animals per group. However, there were 2 deaths during anesthesia in the group treated with EVs plus gel.

Pre-clinical fistula therapy follow-up. Rats were weekly followed for 60 days after surgery. Animals were anesthetized using isoflurane and fistula diameter was measured by gently inserting the tip of catheters of 6; 4.5; 3; 2; 1 and 0.2 mm of diameter, from the smaller to the largest one. The presence of feces at the fistula orifice, indicative of positive fistula output, was evaluated macroscopically. The absence of feces indicated negative fistula output.

Probe-based confocal laser endomicroscopy. Microvascular network at the fistula was assessed by a probe-based confocal laser endomicroscopy device CellvizioTM (Mauna Kea Technologies, France). This analysis was carried out at day 60th (D60), before sacrifice, under isoflurane anesthesia. The equipment featured a flexible fiber optic miniprobe that functions as a microscope objective (1.8 mm diameter, 3.5 μm spatial resolution, 12 images per second acquisition). The probe was positioned on the external fistula orifice border for image acquisition. The density of the microvascular network was investigated after the intravenous administration of FITC-dextran with an average molecular weight of 500 000 (FD500S, Sigma-Aldrich). MosaicingTM software (Mauna Kea Technologies, France) was used for image analysis and a dedicated module, Vessel DetectionTM (Mauna Kea Technologies, France), enabled to compute the functional capillary density (FCD) and diameter distribution. In order to avoid underestimating small vessels, which play an important role in tissue regeneration, we chose a diameter of interest (DOI) of 4 μm . Each parameter was evaluated on 3 different slices. In order to limit the biases related to the selection of the analysis slice, the mean of the results of 3 slices was used as a reference value for the quantitative variables.

MRI for therapy follow-up. MRI was performed using a 4.7 T preclinical MRI scanner (BioSpec 47/40 USR, Bruker, Ettlingen, Germany). MRI was carried out at D60 just before sacrifice using a T1-weighted axial and sagittal FLASH gradient-echo sequence (TR/TE: 100/2.6 ms; angle: 70°; field-of view: 5 × 5 cm; matrix: 256 × 256; resolution: 195 × 195 microns; T_{acq} : 2 min and 33 s: 11 slices of 1 mm). Animals were placed under isoflurane anesthesia and breathing was monitored. In order to facilitate imaging, a gadolinium contrast agent (Dotarem at 0.5 mM mL^{-1} , Guerbet, France) was administered *via* the external fistula orifice just before image acquisition. The first objective was to evaluate the fistula healing by measuring the area of central fistula orifice with the software, OsiriX Lite V.11.0.2, Pixmeo (Switzerland), for which the area was calculated (mm^2).



Histological analysis. At D60, rats were sacrificed and the fistula site as well as its periphery (1 cm³ specimen) were collected and transferred to a formaline solution. Specimens were embedded in paraffin and sectioned perpendicular to the center of the fistula to obtain thin tissue sections of 7 μm, which were stained with hematoxylin and eosin (HE) and Sirius Red (fibrosis assessment). Slices were analyzed with an optical microscope (Leica DMIL). Two investigators, blinded to treatment allocation, performed histological analyses. All slides were digitally scanned (Digitiser Hamamatsu Photonics®, Massy, France) and analyzed with dedicated software (NDP.view software®, Massy, France). The density of cell type, fibrosis and inflammation were determined by semi-quantitative analysis using a score from 0 to 5. Fibrosis score (from 0 to 25) was calculated by semi-quantitative density of fibrosis (score from 0 to 5) multiplied by semi-quantitative fibroblast score (from 0 to 5). A histological scoring was used for evaluation inflammation.

Statistics

The results are presented as means ± standard deviation for continuous variables, and as percentages for categorical variables. Fischer's exact test was carried out for comparisons between categorical variables and the nonparametric Mann-Whitney test was used for non-paired continuous variables. Comparisons between more than two groups were performed with the nonparametric Kruskal-Wallis test. An estimation of the p value by the Chi-square test was carried out for the comparison concerning the number of cases per group. A P-value of <0.05 was considered indicative of significance. Statistical analysis was conducted using SPSS software version 24.0 (IBM Corp., Armonk, NY). GraphPadPrism (Graphpad Software, La Jolla, CA, USA) software was used for statistical analysis information displayed in figures.

Results

High-yield production of EVs in bioreactors triggered by turbulent flow

Murine SCs were cultured in 3D on the surface of micro-support beads in spinner flasks. Stirring speed in these bioreactors was tuned in order to generate a turbulent flow featuring a Kolmogorov length of 35 μm. Murine SCs were stimulated during 4 hours. Typically, this turbulence set-up may enable the production of about 500 000 EVs per cell in 4 hours while about 10 times less is produced in 48 h of starvation.⁵² Herein, a total of 10¹³ murine SC EVs were obtained in 1 L bioreactors and were then isolated by ultracentrifugation and characterized by cryo-TEM, NTA and Exoview. Cryo-microscopy analysis evidenced membrane-enclosed round-shaped structures featuring high size polydispersity between 70 and 500 nm (Fig. 2A). Size distribution characteristic to EVs mainly spanning from 100 to 250 nm was observed. The values for the mean size and mode obtained by NTA were 136.2 nm and 94.8 nm, respectively, with a size distribution standard deviation

of 70.4 nm (Fig. 2B and C). In more details, 90% of the particles were smaller than 228.6 nm, 50% of the particles were smaller than 112.7 nm, while 10% of the particles were smaller than 81.6 nm.

EV markers were characterized for a murine SC EV batch produced by the turbulence method *via* Exoview analysis. The obtained data indicated that the particles could be immune-captured by anti CD63 and CD81 antibodies. Minimal capture was observed by anti CD9 antibody and the same applied to negative control mouse IgG antibody (Fig. 2D). Fluorescence images enabled to visualize of single immune-captured EVs further labelled by fluorescence antibodies, especially anti CD81 (Fig. 2E). Minimal fluorescence was detected for calnexin negative cytosolic marker when using an anti-calnexin fluorescent antibody in permeabilized immune-captured EVs.

As a final formulation, EVs were combined to PF-127 hydrogel at 20% just before use. The rheological properties of PF-127 hydrogel were assessed by the evolution of dynamic storage modulus (G') and loss modulus (G'') as a function of the temperature, confirming the thermoresponsive properties of the obtained hydrogels (Fig. 2F). A transition temperature near 20 °C was determined at G' and G'' cross over.

Cecostomy enabled the induction of a post-surgical col-cutaneous fistula model in rats

A surgical colo-cutaneous fistula model was performed *via* a cecostomy at day 0 (Fig. 3A and B). After the surgical procedure, all fistulas featured an external orifice diameter ≥4.5 mm. Besides, all fistulas were permeable, featuring faeces secretion output, in 100% of the cases. The model allowed the formation of a communication resembling a digestive fistula-like disease with skin and intestinal lesions. The presence of a fistula tract was observed by MRI (Fig. 3C). There was no complication related to fistula induction surgery. The mean of weight before to surgery was 265.2 ± 8.3 g. Therapy follow-up after surgery was 60 days. At the end of the follow-up mean weight was 274.4 g ± 20.8 g.

Percutaneous EV delivery in the thermoresponsive gel allowed EV retention in the fistula tract

We investigated the interest of the percutaneous EV administration embedded in the thermoresponsive gel (Fig. 3D) compared to other administration strategies. A thorough biodistribution study was out of the scope of the present paper. Our aim was to appraise the potential relevance of the proposed administration strategy providing a general picture of biodistribution patterns obtained by other delivery approaches. For that, an EV radiolabeling procedure with [⁸⁹Zr]zirconium was conceived. The direct labelling of DFO-EVs with [⁸⁹Zr][Zr(ox)₄]⁴⁻ was unsuccessful. Therefore, a [⁸⁹Zr][Zr(8-HQ)₄] labelling procedure was used and this complex was obtained in 52% yield (67.6 MBq), with radiochemical purity of 97.5%. [⁸⁹Zr]Zr-EVs were obtained in a 32.5% yield (22 MBq) with a radiochemical purity of 89.5%. Animals were treated with [⁸⁹Zr]Zr-EVs *via* percutaneous intra-fistula administration in saline, percutaneous intra-fistula administration embedded in



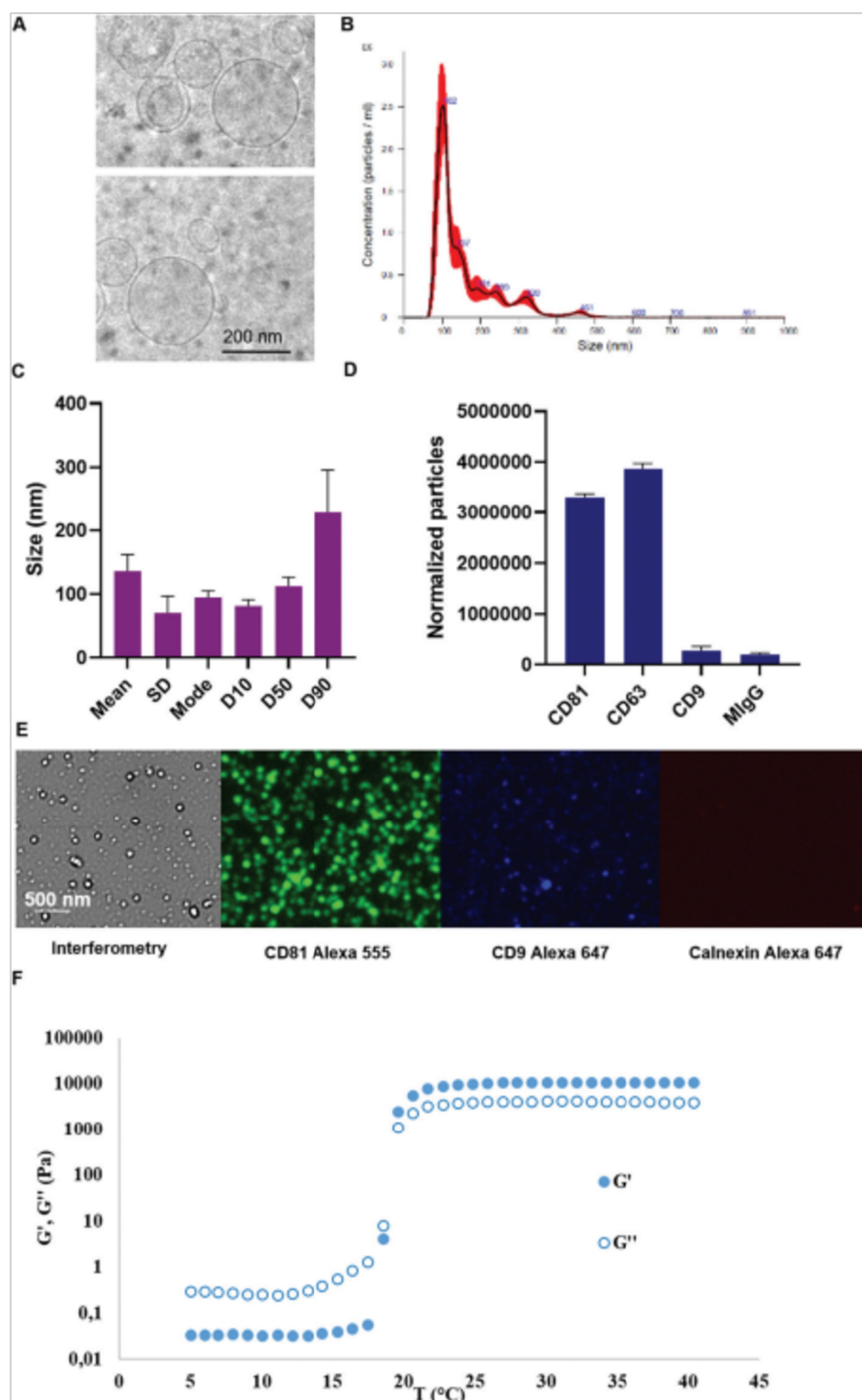


Fig. 2 Characterization of murine stem cell (SC) EVs and Pluronic F-127 hydrogel. Cryogenic transmission electron microscopy images of SC EVs (A). Size distribution of SC EVs, averaged on 5 recorded videos (B) and the mean, mode, standard deviation and percentiles of the size of EV samples, represented as the mean \pm SEM of three independent batches by nanoparticle tracking analysis (C). EV marker analysis by ExoView for SC EVs using CD9, CD63 and CD81 capture antibodies with a mouse IgG2a (MlgG2a) used as negative control capture antibody (D). Representative fluorescence images of the multiplexed EV marker analysis by ExoView for SC EVs using CD81 capture antibodies. Refractometry images are shown as well as fluorescence images using CD81 and CD9 detection antibodies (positive markers) and calnexin fluorescence detection antibody (negative marker) (E). Evolution of dynamic storage modulus (G') and loss modulus (G'') of a Pluronic F-127 at 20% at a heating rate of $5\text{ }^{\circ}\text{C min}^{-1}$ from $5\text{ }^{\circ}\text{C}$ to $40\text{ }^{\circ}\text{C}$ for the determination of the sol-gel transition temperature (G' and G'' cross over) (F).



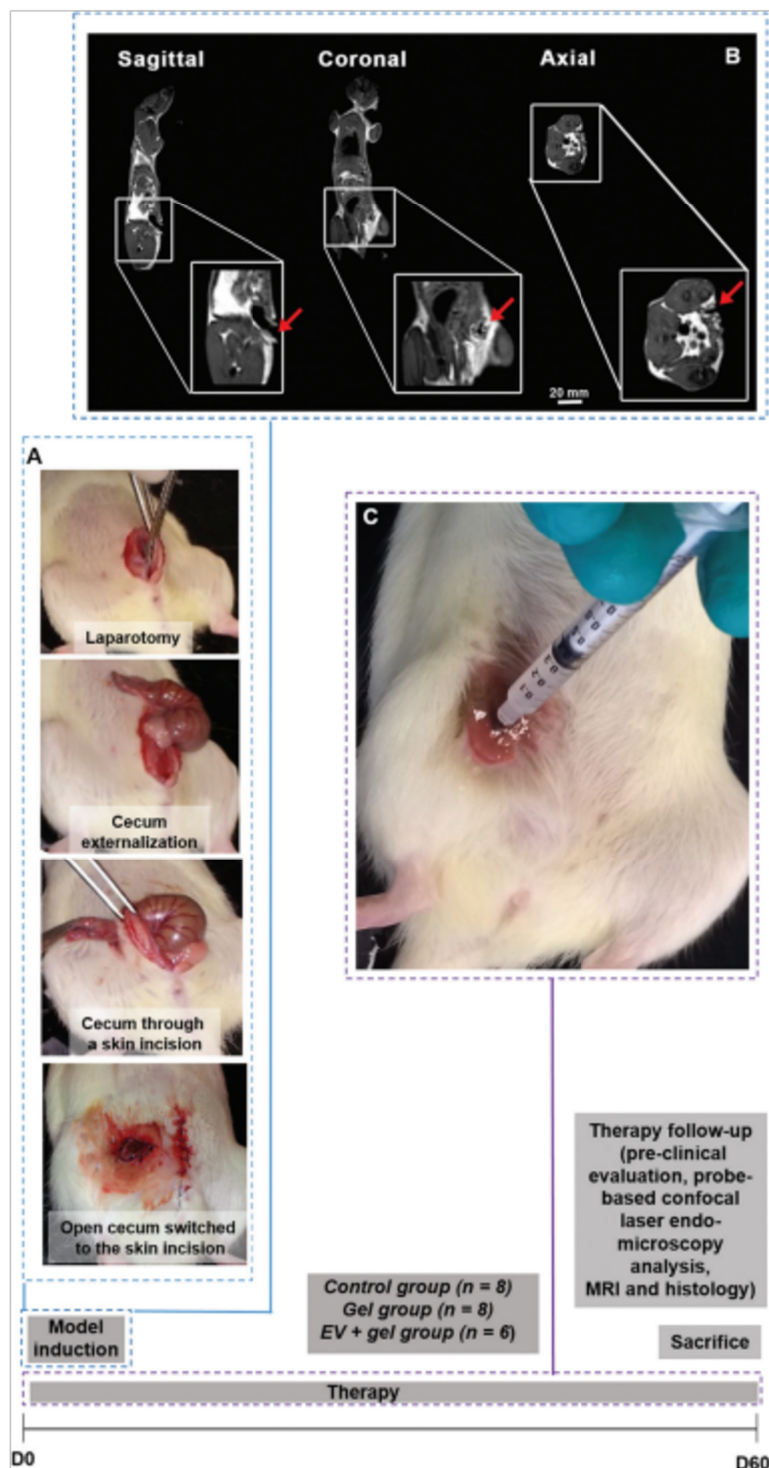


Fig. 3 Design of the *in vivo* experiments and their main steps. Study timeline and sample size per group are displayed. Surgical procedure for the colo-cutaneous fistula model induction from laparotomy to cecum communication to the skin in rats (A). T1 weighted MRI illustrating the colo-cutaneous fistula model. The red arrows indicate the fistula in different anatomical planes: sagittal, coronal, and axial (B). Percutaneous administration of the EVs into the Pluronic F-127 gel via the fistula external orifice (C).

the thermoresponsive gel or *via* intravenous injection in saline (Fig. 4). We observed that the percutaneous $[^{89}\text{Zr}]\text{Zr-EVs}$ treatment in saline induced a disseminated tracer uptake in the digestive tract at 1 h. A fast decrease in the SUV of 75% in the

fistula site was evidenced between 1 h and 24 h (56 and 14, respectively). Concerning the $[^{89}\text{Zr}]\text{Zr-EVs}$ delivery percutaneously in the thermoresponsive gel, PET signal mainly remained limited to the site of interest and the SUV decrease



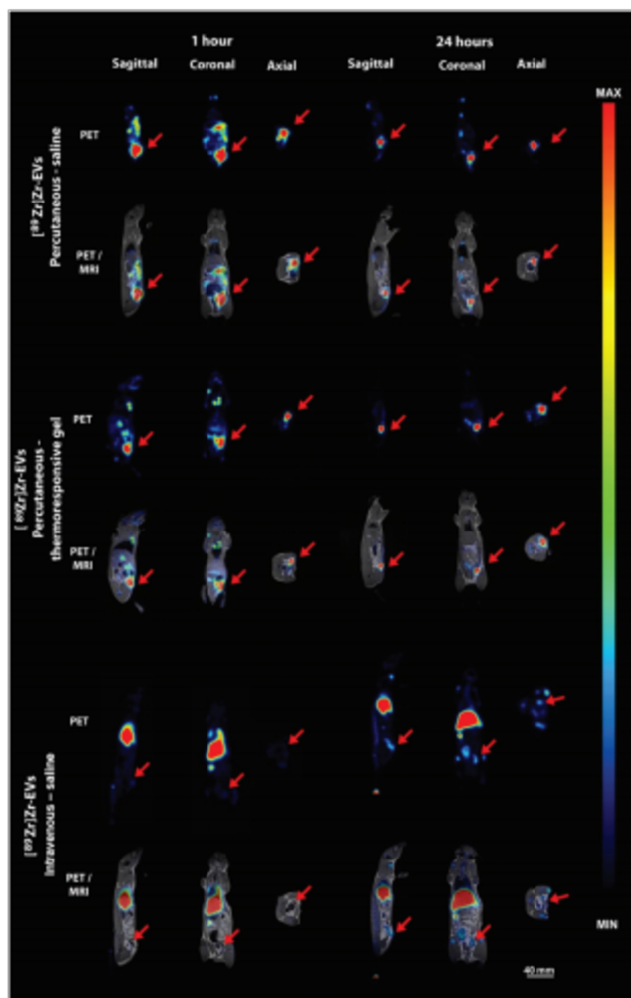


Fig. 4 PET and PET/MRI images illustrate the biodistribution and local uptake of radiotracer labeled EVs (^{89}Zr -EVs), 1 hour and 24 hours after local percutaneous administration, in suspension or embedded in the thermoresponsive gel, or intravenous injection (3 anatomical planes are illustrated). Red arrows indicate the radiotracer uptake in the colostomy fistula. Note that the percutaneous treatment without gel (first line) revealed faster standardized uptake value (SUV) decrease in the fistula site, SUV mean between 1 hour and 24 hours decreased around 75%. For the percutaneous treatment with gel (middle line), the SUV at the fistula site decreased around 30% between 1 hour and 24 hours. For the intravenous injection (bottom line), there was a ^{89}Zr Zr-EVs uptake increase of 400% between 1 hour and 24 hours. However, SUV levels were 3 times lower when compared to local percutaneous administration.

was of 30% between 1 hour and 24 hours (30 and 21, respectively). After intravenous injection, ^{89}Zr -EVs in saline mainly displayed a liver accumulation pattern along with minor uptake in the fistula site and joints. An increase of 400% was observed for the SUV in the fistula site between 1 hour and 24 hours (1.5 and 7.5, respectively) indicating EV homing. However, this SUV level were 3 times inferior when compared to local percutaneous administration. At day 7th post-administration, imaging was performed for animals treated with EVs embedded in the gel administered percutaneously or following

EV intravenous injection in saline. The tracer signal was still mainly limited to the fistula site featuring a SUV decrease of just 15% compared to SUV at 24 hours for the percutaneous EV administration into the thermoresponsive gel condition (Fig. S1†). Comparatively, an almost 4-fold decrease in the SUV at the fistula site was observed for the intravenous injection condition when comparing time points 7 days and 24 hours. As control conditions, the PET biodistribution of the of the ^{89}Zr [Zr(ox) $_4$] $^{4-}$ tracer alone or linked to the conjugation ^{89}Zr [Zr(DFO)] molecule was recorded at 24 hours and 7 days after intravenous administration in saline or percutaneous administration in the thermoresponsive gel. Data showed a biodistribution pattern different from the ones observed for the ^{89}Zr -EVs (Fig. S2†). For instance, the intravenous administration of ^{89}Zr [Zr(ox) $_4$] $^{4-}$ indicated a bone biodistribution (spine) quite different for the liver biodistribution observed for the intravenous ^{89}Zr -EVs injection. This seems to indicate the effective tracer conjugation to EVs.

EV therapy in the thermoresponsive gel decreased fistula output and external orifice diameter

Repeated weekly treatment during 60 days with the gel or the gel with EVs was well tolerated by animals. No evident signs of local irritation, allergy or toxicity were observed. No statistically significant difference in mean body weight was evidenced for animals in the control, gel and EVs + gel group at day 60th after the begin of the therapy (Fig. S3†). Fistula output was evaluated at day 30th. One case of no fistula output (absence of faeces secretion) from 8 animals was observed for the control and also for the gel group, corresponding to 12.5%, for both. Two cases of no fistula output from 6 animals were observed for the group treated with EVs and gel, corresponding to 33% of the animals (Fig. S4†). Fistula closure, output and external orifice diameter were evaluated at day 60th. One case of fistula closure per group was observed for each group, corresponding to 12.5%; 12.5% and 16.7% of the cases for the control, gel and EVs + gel groups. The frequency of no fistula output was significantly different when comparing the control group (1 case from 8: 12.5% of the animals) to the EVs + gel group (4 cases from 6: 66.7% of the animals) (Fig. 5A). No significant difference was observed when comparing the frequency of no output for the control group to the gel one (2 from 8 cases: 25% of the animals) nor the gel group to the EVs + gel one. The external orifice diameter distribution showed a clear shift towards smaller size ranges. The size of external orifice diameter ranges increased in the following sequence EVs + gel < gel < control animals (Fig. S5†). The frequency of fistulas featuring an external orifice diameter <3 mm was significantly different when comparing the control group (1 case from 8: 12.5% of the animals) to the EVs + gel one (4 cases from 6: 66.7% of the animals). No significant difference in the frequency of fistulas featuring an external orifice diameter <3 mm was observed when comparing the control group and the gel one (3 cases from 8: 37.5% of the animals) nor the gel group to the EVs + gel one (Fig. 5B). The macroscopic view of the fistulas revealed a higher and



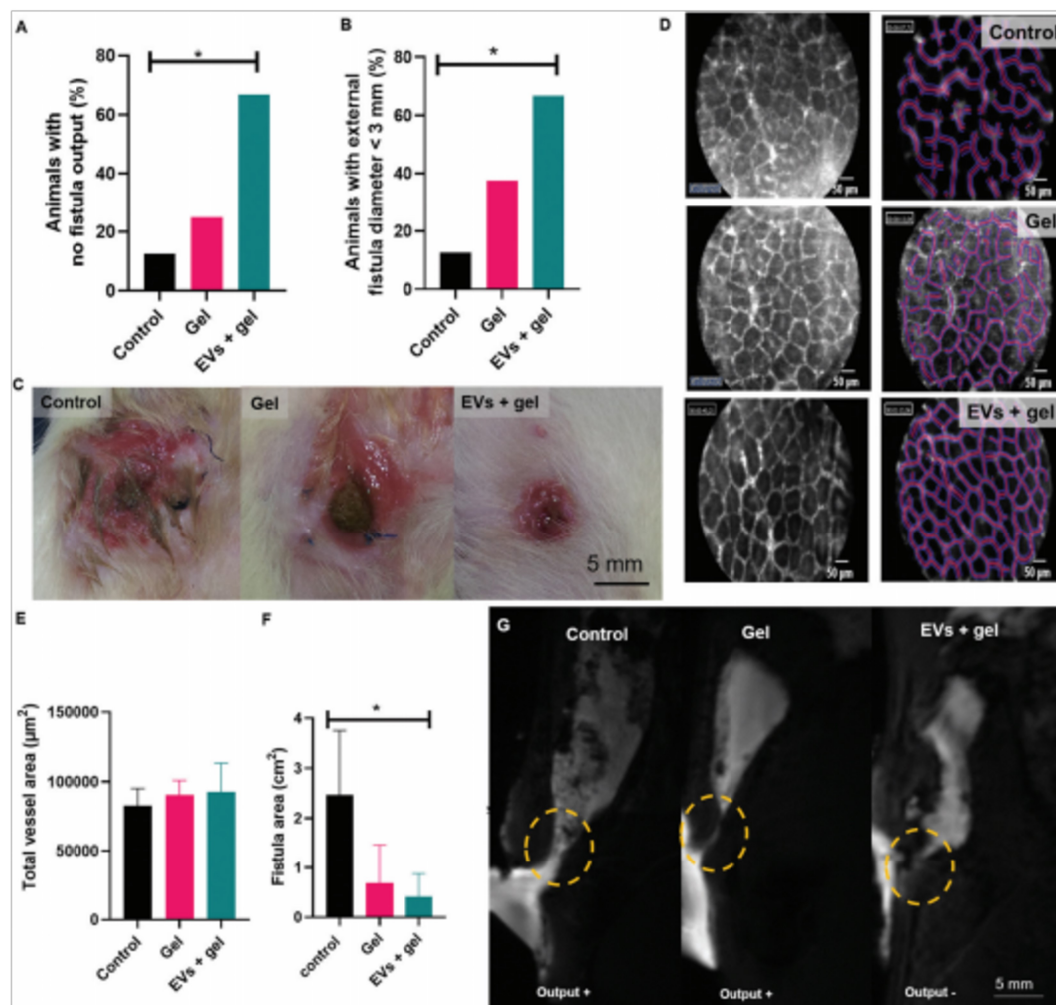


Fig. 5 Potency evaluation *in vivo* by a multi-modal approach in rats treated with the gel or EVs + gel in comparison to an untreated negative control. Preclinical evaluation of fistulas at day 60 indicating the percentage of animals per group featuring the absence of faeces (no output) at the external fistula orifice (A) as well as percentage of animals per group featuring an external orifice diameter inferior to 3 mm (B). For both A and B, *p* values were estimated by Chi-square test based on the number of cases per group featuring the presence (output+) or absence of feces (output-) at the external fistula orifice (A) and number of cases featuring an external orifice diameter inferior or superior to 3 mm (B). Macroscopic aspect of external fistula orifices at day 60 for the three groups (C). Therapy follow-up by probe-based confocal laser *endo*-microscopy analysis at day 60 showing vessel network (D) and the quantification of total vessel area (E) for each group. Therapy follow-up by MRI analysis at day 60 showing fistula area quantification (F) and representative related images (G) for each group (fistula tracts were indicated by yellow dotted circles). For both E and F, *p* values were computed using Mann Whitney test.

extended zone featuring an inflammatory aspect surrounding the external orifice for control and gel groups in comparison to fistulas from the EVs + gel group (Fig. 5C).

EV therapy in the thermoresponsive gel seemed to improve vascularization while reducing fistula area: an image-based therapy follow-up

Vascularization parameters were analysed by probe-based confocal laser endomicroscopy. No significant difference was observed for group comparison in terms of vessel diameter, total vessel length, total vessel area, mean vessel diameter nor functional capillary density length, for instance (Table S1†). There was a trend towards a higher functional capillary density

area and total vessel area for animals from the group gel or EVs + gel one in comparison to control (Fig. 5D, E and Table S1†). This trend did not reach statistical significance probably due to the reduced sample size.

Fistula metrics was assessed by MRI analysis right after the percutaneous injection of a gadolinium contrast agent *via* the external fistula orifice at day D60. There was a trend towards a reduced fistula length when comparing EVs + gel group to control (Table S2†). MRI data evidenced a significant reduction of the fistula area when comparing EVs + gel group to control (Fig. 5F and G). No significant reduction of the fistula area was observed when comparing the gel group to control nor the gel group to the EVs + gel one.



Histological analysis indicated a decrease in inflammation and fibrosis and an increase in neo-vessel density induced by EV therapy in the thermo-responsive gel

Histological analysis was carried out to assess inflammation, fibrosis and vascularization. A histology score (integrating epithelial damage, mucosal inflammation and submucosal inflammation parameters as indicated in the Table S3†) evidenced a significant difference when comparing the control group to the EVs + gel one (Fig. 6A). A significant difference was also observed when comparing the gel group and EVs + gel one. Accordingly, the density of polynuclear neutrophils (PNN) was significantly lower for the EVs + gel group than observed for the control one (Fig. 6B). The histological evaluation validated the trend observed by probe-based confocal laser endomicroscopy as the density of neo-vessel score was significantly higher for the EVs + gel group in comparison to control (Fig. 5C). This is also in agreement with *in vitro* pro-angiogenic (pro-migratory) properties of the EVs (Fig. S6†). A significant reduction in the fibrosis score was also evidenced when comparing the EVs + gel group to the control one (Fig. 6D). Besides, a significant difference in the fibrosis score was also observed when comparing the gel group and the EVs + gel one. Extensive fibrosis and mucosa damage was observed for the control group (Fig. 6E). Fibrosis and mucosa damage seemed more limited in the gel group (Fig. 6F). Fibrosis was limited in the gel and EVs + gel group, which featured minimal mucosa damage (Fig. 6G). Table S4† provides additional histology data.

Discussion

Herein we investigated a biomaterial-based cell-free local therapy for post-surgical fistulas. This combined therapy relied on turbulence SC EVs embedded in a PF-127 thermo-responsive hydrogel administered percutaneously in colo-cutaneous fistulas.

As far as we know, this is the first time that SC-EVs incorporated in a thermo-responsive hydrogel are investigated for the therapy of post-surgical colo-cutaneous fistulas as an alternative to cell therapy. In a previous report from our team, SC organized in double cell sheets showed a therapeutic effect in an equivalent fistula model in mice.⁵⁹ In the attempt to shed light on the mechanisms of cell therapy for fistula treatment, our group performed *in vivo* experiments with doubled labelled SCs for fluorescence and MRI tracking. SC detection at fistula site was showed just following transplantation, but it could not be clearly evidenced 7 and 14 days post-treatment. We showed that SC cell sheet therapeutic benefits on fistula healing were accompanied by a paracrine effect with an increase in the expression of anti-inflammatory cytokines and intestinal growth factors involved in tissue repair.⁵⁹

As EVs are known to participate in SC paracrine role,^{1,2} we decided to test herein an EV-based cell free therapy. SC EVs were produced *via* a high-yield scalable strategy in bioreactors. The turbulence method in 4 h may enable up to a 10-fold

increase in EV production yield compared the state-of-the-art starvation approach in 48 h.⁵² Our turbulence strategy is fully integrated to the cell culture in stirred tank in cGMP bioreactors. We evidenced that the turbulence approach was scalable based on turbulence physical laws *via* the Kolmogorov equation.⁵² The choice of the turbulence approach enabling high-yield, scalability and cGMP compliance is one of the translational strengths of our investigation. The comparison between turbulence SC EVs and state-of-the-art ones in terms of properties and potency *in vitro* is provided elsewhere.⁵²

Turbulence SC EVs were combined to PF-127 thermo-responsive hydrogel for local colo-cutaneous fistula therapy. The aim of the hydrogel was two-fold: (i) to act as a sealant promoting fistula mechanical occlusion and (ii) to constitute a delivery system for EVs modulating their release and biodistribution. Concerning fistula sealants, other biomaterials such as porcine plug or fibrin glue⁶⁰ have been investigated in a clinical setting for post-surgical fistula therapy. However, the use of these biomaterials may be limited by abscess formation⁶¹ and spontaneous expulsion.⁶² We have recently evidenced in a case report that PF-127 gel may be a thermo-actuated fistula sealant of interest. It was well-tolerated by the patient while inducing a reduction in the external fistula orifice diameter and secretion output volume in combination to a stent placement.⁶³ Due to its thermo-responsive properties, this biomaterial can be administered as a liquid *via* a catheter. Its gelation *in situ* at body temperature is expected to enable its retention at the fistula tract, limiting the circulation of secretions and thus favoring the healing process.

As we have mentioned, the use of the PF-127 gel herein goes beyond a sealant agent also providing a delivery system for EV administration locally in the rat model of colo-cutaneous post-surgical fistula. In this regard, we developed an EV labelling strategy with a radiotracer in order to identify the biodistribution pattern of EVs following their administration either percutaneously in the gel or in saline in comparison to an intravenous administration in saline. We selected [⁸⁹Zr]zirconium as the radionuclide as its half-life of 3.3 days enables the *in vivo* tracking from hours to days post administration. EVs were tagged with [⁸⁹Zr]zirconium *via* a double labelling strategy. In a first attempt, we investigated EV labelling by the direct reaction between DFO-EVs⁵⁴ with [⁸⁹Zr][Zr(ox)₄]⁴⁻. This strategy was not successful. Therefore, we decided to use [⁸⁹Zr][Zr(8-HQ)₄] to react with DFO-EVs which could concurrently (i) transfer part of the ⁸⁹Zr⁴⁺ to the DFO bounded to EVs or (ii) cross the lipid membrane delivering ⁸⁹Zr⁴⁺ into the EV inner compartment. Control experiments showed that the biodistribution of [⁸⁹Zr][Zr(ox)₄]⁴⁻ or [⁸⁹Zr][Zr(DFO)] was quite distinct from the one observed for [⁸⁹Zr]Zr-EVs. This supports the assumption of a successful EV radiolabelling by our proposed double strategy. As far as we know, this is the first time that EVs are tagged with [⁸⁹Zr]zirconium *via* this double labelling strategy, which allowed tracking up to 7 days post-administration. For instance, different EV radiolabelling strategies with [^{99m}Tc]Tc⁶⁴ or [¹²⁴I]I⁶⁵ were reported in the literature with a tracking time up to and 5 h and 72 h, respectively.



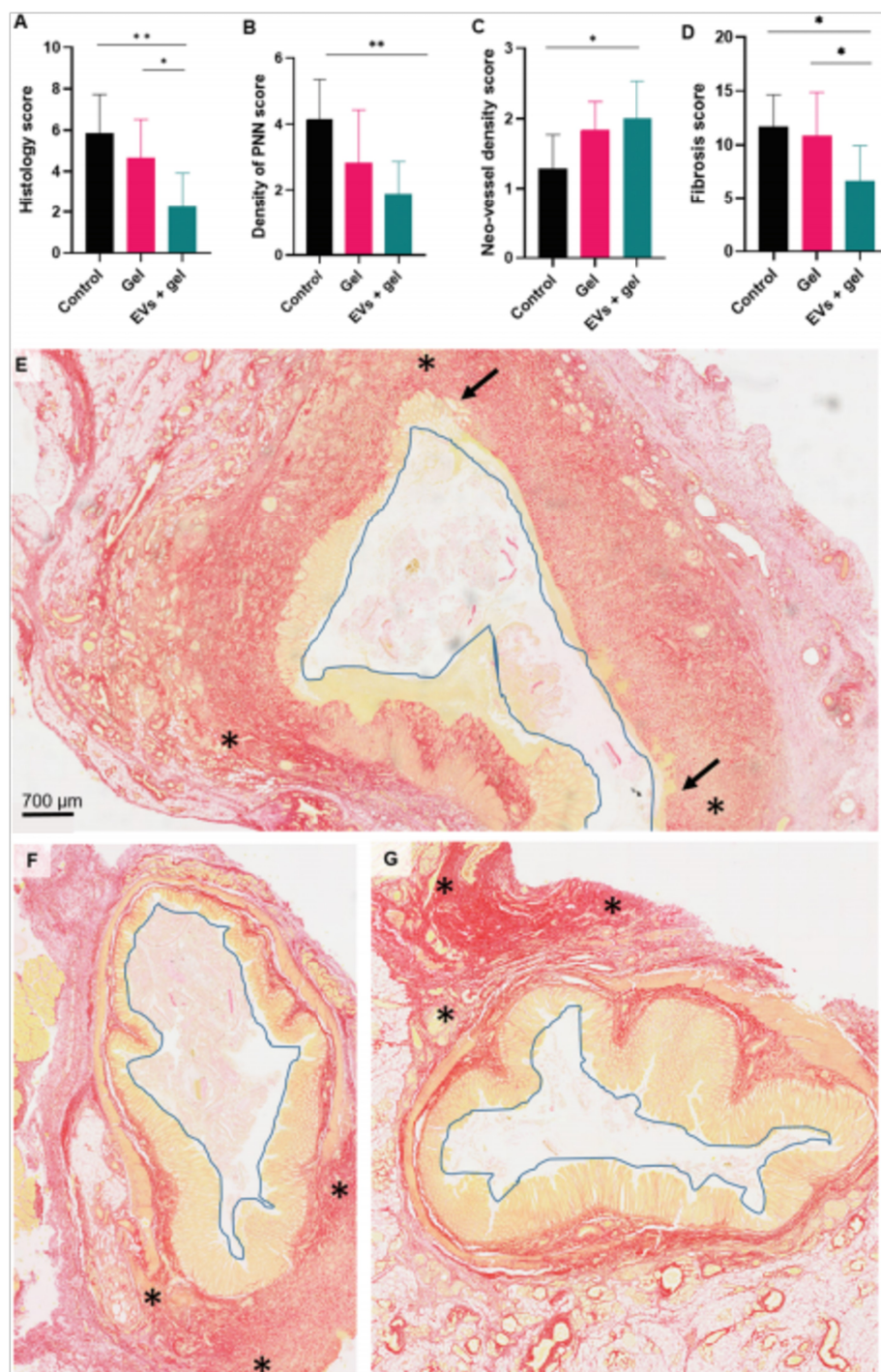


Fig. 6 Potency evaluation *in vivo* by histology in rats treated with the gel or EVs + gel in comparison to an untreated negative control. Therapy follow-up for the three groups by histological analysis at day 60 assessing a histology score based on epithelial damage, mucosal inflammation and submucosal inflammation parameters (A). Therapy follow-up for the three groups by histological analysis at day 60 assessing the polynuclear neutrophils (PNN) density (B), neo-vessel density (C) and fibrosis (D) scores. Mann Whitney test was used for computing p values in group comparison for all scores. Histology analysis (Sirius red staining) of the colo-cutaneous fistulas from control (E), gel (F) and EVs + gel (G) groups. Note that the fistula orifice was delineated in blue color and main fibrosis regions, in red staining, were delimited by *. The epithelium was identified by an orange staining. Important epithelial damage was observed for the control group and its extent was indicated by black arrows.

By using the [⁸⁹Zr]Zr-EVs, we observed that the highest SUV at the fistula region were obtained following percutaneous administration. Besides, signal detection was mainly

restrained to the site of interest when administration was performed percutaneously *via* the thermoresponsive hydrogel. Signal persisted with a 30% reduction in 7 days. In a related



literature approach, a pH-responsive hydrogel featuring immediate gelation upon administration into physiological systems was investigated for local EV delivery. A sustained EV delivery until day 4 post-administration was observed with EV localization in the tissue surrounding the injection site.⁶⁶ In a myocardial infarction model disease, it was reported that SC EV local delivery in the heart of rats *via* an alginate hydrogel improved EV cardiac retention.⁶⁷ *Ex vivo* 2D optical microscopy coupled to 3D optical tomography revealed a stronger fluorescent signal from DiR-labeled EVs for animals treated with in EVs incorporated in the gel compared to those treated with EVs alone.

Considering the potential interest of local percutaneous EV administration in the PF-127 hydrogel indicated by our biodistribution data, we next evaluated the therapeutic outcome of this strategy in colo-cutaneous fistulas.

The combination of EVs and PF-127 gel biomaterial was previously tested in the therapy of post-surgical oesophageal fistulas in pigs by our team.⁶⁸ Of note, a different producer cell type (primary AdSCs) was used in the precedent study. In this preceding investigation, we showed by rheological analysis that the dispersion of EVs into PF-127 gel did not impact its thermoresponsive properties. EV integration into the gel also seemed to have minor impact on EV integrity. We evidenced that EV release was expected to be driven by the saliva flow exerting a dilution effect on the gel. Indeed, we showed that the aqueous dilution of the gel to a concentration inferior to 13% notably induced a viscosity decrease and the release of EVs.⁶⁸ Based on these data, we chose the PF-127 concentration of 20%. In principle, we could choose any concentration $\geq 14\%$. However, at 14% there would be a transition to a liquid state releasing the EVs even at a minimal dilution, resulting in a burst EV release. We chose a concentration superior to this in order to enable the gel to withstand to a dilution factor of about 1.5 before transition to a liquid state to allow EV release in a more sustained way.

In our previous study, an anti-inflammatory, anti-fibrotic and pro-angiogenic effect was observed for animals treated with EVs embedded in the PF-127 hydrogel with total fistula healing being observed in this pig esophagocutaneous disease model.⁶⁸ Considering the differences in fistula tract size and secretion constitution/volume, the EV release and fate as well as the therapeutic outcome following the administration of EVs and PF-127 gel biomaterial in a colo-cutaneous fistula in rats may be indeed distinct from the one observed in the esophagocutaneous fistulas in pigs. Importantly, gel dilution factor is expected to be reduced in the colo-cutaneous model as the faeces secretion features inferior aqueous constitution in comparison to the saliva secretion in the oesophagus. The smaller fistula tract size in the colo-cutaneous rat model in comparison to the esophagocutaneous fistula model in pigs is expected to influence the gel residence time as the “reservoir” volume of these tracts are different. In brief, the combined EV/biomaterial fate and the obtained therapeutic outcome may be influenced by fistula characteristics, which depend on the

involved organs. Despite these differences, we observed a therapeutic effect following the percutaneous EV administration in the gel for both the colo-cutaneous model and the esophagocutaneous one. The investigation of our approach in different fistula settings is of interest in order to extend the clinical relevance of the local delivery of EVs in the PF-127 thermoresponsive hydrogel.

Conclusions

Intestinal fistulas secondary to surgery are a major health problem. Current unmet needs require innovative strategies. We evidenced here the therapeutic potential of a biomaterial-based cell-free local EV therapy for the management of colo-cutaneous post-surgical fistulas. Thanks to a turbulence flow stimulation, EVs were produced in high-yield in an approach that combines scalability and cGMP capabilities, which is a translational asset of our study. EVs were tagged with [⁸⁹Zr]zirconium *via* a double labelling strategy, which allowed tracking up to 7 days post-administration. This radiolabeling approach enabled us to identify the pattern of EV biodistribution following intravenous injection in saline in comparison to percutaneous administration in saline or PF-127 gel in a colo-cutaneous fistula model in rats. We observed that the highest EV retention at the fistula site was observed following percutaneous administration. Besides, fistula retention of EVs was more prominent when their percutaneous administration was performed embedded in the thermoresponsive hydrogel. We assessed therapy outcome following the latter administration strategy by evaluating fistula output and external orifice diameter by pre-clinical macroscopic analysis; vascularization by probe-based confocal laser endomicroscopy and fistula metrics by MRI. Histology was equally assessed. Based on this multi-modal approach, we evidenced that the local administration of SC EVs in the thermoresponsive hydrogel reduced colo-cutaneous fistula external diameter, area, output, fibrosis and inflammation while increasing the density of neo-vessels in comparison to the negative PBS control. Altogether, our study brings prospects for a local minimally-invasive EV delivery relying on a thermo-actuated administration strategy in PF-127 gel in colo-cutaneous fistulas. Importantly, PF-127 gel has been previously approved for clinical use (Legoo, Genzyme), which may facilitate the clinical translation of our approach. This study extends insights from a previous investigation of our team that showed successful post-surgical esophagocutaneous fistula therapy by the equivalent strategy. As a future perspective, additional Crohn's disease and post-surgical fistula models involving different digestive organs should be also investigated to appraise post-surgical fistula disease heterogeneity. Hopefully, these further studies may confirm the versatile potential of the proposed approach as a next-generation therapeutic option for digestive fistula management in a “one-fits-all” basis. The translational assets of the proposed strategy may accelerate and render economically feasible pharmaceutical development steps toward clinical investi-



gation in the perspective to tackle unmet needs and benefit patient care.

Author contributions

Conception, design, and study supervision: Amanda Karine Andriola Silva, Gabriel Rahmi, Florence Gazeau. Acquisition of data: Max Piffoux, Alba Nicolás-Boluda, Arthur Berger, Alice Grangier, Imane Boucenna, Caroline Cristiano Real, Fabio Luiz Navarro Marques, Daniele de Paula Faria, Amália Cinthia Meneses do Rego, Chloe Broudin. Analysis and interpretation of data: Arthur Berger, Olivier Clément, Christophe Cellier, Carlos Alberto Buchpiguel; Writing, review, and/or revision of the manuscript: Amanda Karine Andriola Silva, Gabriel Rahmi, Claire Wilhelm, Florence Gazeau, Arthur Berger

Conflicts 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, Claire Wilhelm are co-founders of the spin-off EverZom. The other authors are no conflicts to declare.

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Discussion :

Les résultats de cette première étude sur un modèle de fistules colo-cutanées chez le rat, montrent une meilleure bio-distribution des VEs appliquées localement. En effet, l'analyse en TEP-IRM à 7 jours retrouve un signal toujours présent en regard du trajet de fistule. Ces résultats plaident pour une application locale de la thérapie vésiculaire.

Les résultats de cette première étude sur un modèle de fistules colo-cutanées chez le rat, confirment l'effet cicatrisation des VEs. En effet, le taux de fermeture des fistules était significativement plus important dans le groupe gel + VE. La fermeture des fistules était observée dans 57% (n=4) dans le groupe gel + VE, dans 25% (n=2) dans le groupe gel seul et 12% (n=1) dans le groupe témoin (p=0,0363). Par ailleurs, seul le groupe traité par le gel + VE a montré une réduction statistiquement significative de la fréquence des fistules d'un diamètre supérieur à 3 mm (p=0,003). Sur le plan histologique, le score d'inflammation et de fibrose histologique étaient significativement moins importants dans le groupe Gel+VE (p=0,01). On observe une réduction du nombre de macrophage (p=0,047), de polynucléaire neutrophile (p=0,002), et de la néo-vascularisation (p=0,019) dans le groupe Gel+VE vs groupe témoin. Ces résultats sont confirmés en micro-endoscopie confocale, avec une architecture des glandes irrégulières dans 71,4% des cas dans le groupe témoin vs 14,3% dans le groupe Gel et le groupe GEL+VE (p=0,067). On observe une disparition des cellules caliciformes dans 46,7% des cas dans le groupe témoin vs 16,7% dans le groupe Gel et 36,7% dans le groupe GEL+VE (p=0,002).

Ces résultats confirment, les données retrouvées dans l'étude de A. Silva et al, sur les fistules œsophagiennes chroniques étaient traitées par $1,3 \cdot 10^{11}$ vésicules dérivées d'AdSC incluses dans du gel de poloxamer. Cette étude préliminaire confirme que les VEs produites à fort rendement présentent des propriétés régénératives. En effet, l'application de gel avec VE

induisait une fermeture significative des fistules associée à une diminution de l'inflammation et de la fibrose.

Dans cette étude, nous avons utilisé un modèle de fistule colo-cutané chez le rat. Il s'agit d'un modèle de fistule animal connu dans la littérature. Ce modèle a notamment été validé pour étudier les phénomènes inflammatoires et cicatriciels mis en jeu au cours du développement d'une fistule digestive dans les maladies inflammatoires [91]. Cependant, ce modèle de fistule chirurgical décrit par Bültmann *et al.* en 1998 est éloigné des fistules digestives post-opératoire que l'on retrouve chez l'homme [191]. En effet, l'une des principales causes de fistule post-opératoire est la chirurgie oeso-gastrique avec des taux de fistules d'environ 10% [27,28]. Ces premiers résultats encourageants, nous invitent à évaluer l'effet paracrine des VEs dans des modèles animaux plus proches des fistules post-opératoire retrouvées chez l'homme.

Article 2: Creation of a gastro-cutaneous fistula model after sleeve gastrectomy in small and pig animal

Introduction

Après ces premiers résultats encourageants, nous nous sommes intéressés à des modèles de fistules digestives post-opératoires proches des fistules que l'on retrouve chez l'homme. La chirurgie de l'œso-gastrique et son risque de fistules post-opératoires représentent un défi important pour la prise en charge des malades. Plusieurs modèles animaux ont été décrits pour étudier les fistules oeso-gastriques chez le rat, le lapin ou le porc (tableau 5). A notre connaissance aucun modèle de fistules post-sleeve gastrectomie n'a été étudié. Pourtant, chaque année, environ 60.000 patients bénéficient d'une prise en charge chirurgicale de l'obésité en France. Plus de 50% des patients sont maintenant traités par SG, avec un risque de fistules d'environ 2 à 3% [32,41,43–47].

Tableau 5 : Modèle animal de fistule oeso-gastrique

| Référence | Type fistule | Modèle | Technique chirurgicale |
|--|----------------|--------|--|
| Kodama et al ; Endoscopy 2000 [192] | Gastro-cutanée | Rat | Anastomose gastro-cutané avec drain laissé en place |
| Paré et al ; Physiol. Behav. 1973 [193] | Gastro-cutanée | Rat | Tunnelisation sous cutané d'un drain |
| Skorjanec et al ; Dig. Dis. Sci 2009 [194] | Gastro-cutanée | Rat | Ouverture de la poche gastrique sur 3 mm et création gastrostomie ouverte sur 2 mm |
| Smith et al ; Aliment. Pharmacol. Ther 2001[195] | Gastro-cutanée | Rat | Anastomose gastro-cutané avec drain laissé en place |
| Yurtçu et al ; Int. J. | Oeso-cutanée | Lapin | Création chirurgicale d'une oesophagostomie |

Pediatr

Otorhinolaryngol.

2009 [196]

Aldaqa et al Int. Gastro-cutanée Lapin Anastomose gastro-cutané avec drain laissé en place

Surg. 2015 [153]

Rahmi et al ; Surg Oeso-cutanée Porc Mise en place de deux drains en région cervicale

Innov. 2016 [197]

Silva et al ; ACS Oeso-cutanée Porc Mise en place de deux drains en région cervicale

Nano 2018 [175]

Dans cette deuxième partie du travail de thèse, nous avons cherché à développer de nouveaux modèles animaux de fistules digestives post-SG. Dans un premier temps, nous nous sommes intéressés au petit animal. Nous avons créé des fistules gastro-cutanées chez le rat adulte après réalisation d'une SG longitudinale par agrafage mécanique par laparoscopie. La fistule gastro-cutané a été créée par tunnélisation de la poche gastrique fundique appelé rumen chez le rat. Le rumen a été ouvert sur quelques millimètres au sommet de la ligne d'agrafes, puis a été suturé à la peau. Les premiers résultats obtenus chez 16 rats avec 100% de fistules perméables à six semaines, nous ont encouragé à évaluer la technique chez le grand animal. Nous avons cherché à transposer le modèle chez le porc. Après avoir réalisé une SG par voie laparoscopique chez le porc, nous avons fixé deux drains à l'extrémité de la ligne d'agrafes que nous avons ensuite tunnélisé jusqu'à la peau. Plusieurs modèles de confection de la SG ont été évalués chez huit porcs, puis validés dans une nouvelle population de six porcs retrouvant une fistule perméable à quatre semaines.

Title: Creation of a gastro-cutaneous fistula model after sleeve gastrectomy in small and pig animal

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Abstract

Gastric fistula after sleeve gastrectomy (SG) is a rare but severe and life threatening complication, involving in most cases a long and complex management. The aim of this study is to create an experimental model of gastric fistula after sleeve gastrectomy in rats and pigs.

Methods: A surgical gastro-cutaneous fistula after SG was performed in 16 female 12-week old Wistar rats, and in 8 female 3-months old Large White pigs. The primary outcome was the fistula patency rate assessed clinically at the end of the follow-up for rat and pig model.

Results: A surgical gastro-cutaneous fistula after SG, was performed in 8 rats for the technical development of the sleeve gastric fistula model, and 8 for the validation of model. Fistula were patent in 100% cases at this end of the follow up. Two rats featured total body weight loss superior to 30% at the end of the follow-up. A surgical gastro-cutaneous fistula after SG was performed in 8 pigs for the technical development and 6 for the validation of the model. Fistula were patent in 100% of the cases at the end of the follow-up in the model validation step. The endoscopic and radiological assessment indicated that the internal orifice of fistulas was open in 100% of the cases. No severe adverse event was observed in validation group.

Conclusion: In this study we present a first animal model of gastro-cutaneous leak after SG in both rats and pigs.

Introduction

Gastric fistula after sleeve gastrectomy is a rare (2%) but severe complication, involving in most cases a long and complex management [1–7]. The endoscopic management has clearly redesigned and improved the management of these fistulas, but it requires many procedures during a long period with also major morbidity [8]. Moreover, when endoscopic conservative treatment fails, fistula results in a chronic fistula (>12 weeks) and a surgical treatment is mostly required with mutilating techniques as total gastrectomy [9]. In order to decrease the induced morbidity, the number of procedures, the delay to recovery, it is of outmost importance to develop relevant models in order to investigate innovative therapies. The aim of this study is to create an experimental model of gastric fistula after sleeve gastrectomy in rats and pigs. The rat model has the advantage to be cost-effective and implementable in most animal experimentation facilities. It may be of high interest to screen several therapies. The pig one is more clinically relevant, being a valuable option to confirm the therapeutic effect of treatments selected based on the rat model.

Methods

Population

This study was conducted at the Biosurgical Research Laboratory of the Alain Carpentier Foundation in Paris. It was approved by the local ethics committee of Descartes University (N°27476-2019081016097890 for the rat model and N°2018040219589382 for the pig one). A surgical gastro-cutaneous fistula after sleeve gastrectomy was performed in 16 female 12-week old Wistar rats, with a mean weight (range) of 484.4 g (274.0 – 810.0 g), and in 8 female 3-months old Large White pigs, with mean weight (range) of 46.1 Kg (42.0 – 54.0 Kg). The animals were housed in a dedicated animal facility, 2 per cages for rat and in individual cages but in same room for pigs, with regulated temperature, ventilation, and respecting light-dark cycles. Rats and pigs received a liquid diet on the day prior to surgery and were fasted overnight

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before operation.

For the rat model, anaesthesia was performed under 2% of isoflurane anaesthesia (Baxter, Maurepas, France). Rats received an analgesic therapy at a dose of 0.05mg/kg (buprenorphine) subcutaneously before surgery and postoperatively at D1.

For the pig model, animals received before surgery a pre-anaesthesia by intra muscular injection of ketamine (20mg/kg) and azaperone (2mg/Kg). Induction of general anaesthesia was performed by intra-veinous injection of propofol (4mg/kg) together with 2% of isoflurane anaesthesia (Forene, Abbott, France) after oro-tracheal intubation. Pigs received an analgesic therapy at a dose of 0.05mg/kg (buprenorphine) subcutaneously before surgery and postoperatively at D1.

Rats and pigs were placed in supine position with feet spread apart with the abdomenshaved before surgery. Standard aseptic procedures were used throughoutly.

In rat model: After a 20 mm median laparotomy and exposure of the liver, the entire greater curvature of the stomach was identified. Pylorus and Esogastric junction were individualized and separated from the left crus of the diaphragm. The dissection and sectioning of the gastrocolic ligament and gastrosplenic ligament were performed. Particular attention was given to short vessels, which needed a ligation. The sleeve gastrectomy was performed by the use of a vertical stapler (EndoGIA – 45mm-purple cartridge-Covidien) from 5 mm of the pylorus to the upper part of the rumen (equivalent of the fundus for rats), avoiding stenosis of the remnant gastric tube. The distal part of the residual rumen was passed with a staggered passage through the abdominal muscle (posterior and anterior fascia of the rectus abdominals) and the subcutaneous space. It resulted in a 5-mm-long tract that was then attached to the skin. For that, four stitches (Vicryl 4/0) were done at the site of a 3x3 mm biconcave incision on the left flank

1 of the rat creating the gastro-cutaneous fistulas. These steps are illustrated in Fig. 1a, fig. 1b, and
2 Fig. 1c. The final aspect is illustrated in Fig. 1d.
3

4 The first step to induce the surgical gastro-cutaneous fistula after sleeve gastrectomy was to
5 complete gastric dissection, place a 36-French bougie tube and advanced it to the level of the
6 pylorus. The induction of the sleeve began 3 to 4 cm from pylorus. Three to four vertical
7 staplers (GIA 80mm, green cartridge, Medtronic) were fired along the calibration tube. At the
8 top of the staple line, the stomach was opened to introduce 2 catheters (one 24 Fr Pezzer catheter
9 in parallel with a T-tube catheter); then, catheters were guided and fixed to the skin to create
10 gastro-cutaneous fistulas. After surgery, upper endoscopy was performed systematically with
11 opacification to control the appropriate positioning of catheters and permeability of the surgical
12 montage.
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26 In order to find the best model with relevant similarity to the human disease conditions and
27 acceptable tolerance with low morbidity rate, three experimental set-ups were tested over time:
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- 31 1) The fundic pouch was preserved (Figure 2a): Three pigs were operated with the stapling
32 achieving the greater curvature just below the fundic pouch in order to let a little reserve
33 for alimentation and to easily guide the stomach and the catheters to the parietal wound.
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- 39 2) The fundic pouch was resected (Figure 2b): Two pigs were operated with the stapling
40 achieving the cardia near the eso-gastric (EG) junction in order to reproduce the real
41 surgical model performed in human.
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- 47 3) The fundic pouch was partially resected (Figure 2c): Three pigs were operated. with a
48 light stenosis performed at the lesser curvature and the stapling achieving the fundus,
49 four to six centimeters from the EG junction (Figure 3).
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In all pig models, stapling were removed 2 weeks after by general anesthesia. Clinical, endoscopic, and radiology outcomes were evaluated at base line, just after the splapling were removed by general anesthesia.

Post-operative care

After surgery, all animals were kept in separate boxes. After one week, rats were kept at two per boxes. At post-operative day (POD) 1, rats received day one after surgery a subcutaneous injection of 5mL of an isotonic glucose solution and an injection of 0.5µl/g body weight of penicillin G (20000IU) in intra-muscular. At POD-1 and 2, they had access to 50ml of liquid diet per day (Pre and Postoperative diet-C0200, Genestil) and water. From POD-3 on, animals had access to standard solid food (Genestil, Altromin- Breeding diet Rats/Mice Vacuum C1314). Each rat received Buprenorphine (0.05mg/kg) subcutaneously from POD-1 to POD-3 if necessary.

Pigs received an antibiotherapy during 5 days by (Cefazoline). Liquid diet was administred during all the follow-up (Genestil).

Follow-up

Animals were followed for 6 weeks after surgery in rat group, and 4 weeks after surgery in pig group (Figure 4). Animals were weighed on a weekly basis until the end of the follow-up. Clinical and radiological data were collected (figure 5). The primary outcome was the patency of fistula at the end of the follow-up. The external fistula orifice was evaluated by a skilled gastroenterologist to assess its opening and inflammatory aspect. External fistula orifice were considered open respectively in rat and pig model if a 20G (0.8 mm) plastic catheter and plastic catheter 6 Fr Huibregtse® guiding catheter (Cook Medical, North Carolina, USA) could be introduced. Size of external orifice were assess used slide rule. At this end of the follow-up, animals were killed by an intracardiac injection of thiopental in rat model and by intra-venous

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injection of Dolethal® in pig model.

Endoscopic and radiological assessment

In pig model internal orifice of fistula was evaluated by upper gastro-intestinal endoscopy (Gastroscope EG-590WR Fujifilm, Tokyo, Japan). At D14 and D28, an endoscopic evaluation under general anesthesia was also performed by a skilled gastroenterologist to investigate internal fistula orifice opening and inflammatory aspect. Similarly, catheterization of the fistula tract was systematically attempted, and inflammation was defined by the presence of an ulcerated orifice. External fistulography was performed by injection of 5 ml of a contrast agent (Xenetix 250[®], Guerbet, France), under fluoroscopy monitoring.

Results

Both models required two steps in order to be robust and representative of the human disease. The first step was dedicated to the conception and second one to test the reproducibility. Results are detailed for each animal model as follows.

Rat model

A surgical gastro-cutaneous fistula after sleeve gastrectomy was performed in 8 rats for the technical development of the sleeve gastric fistula model, and in 8 rats for the validation of the model. The median follow-up after surgery was 45 (+/- 2.4) days. At the end of the follow-up, the mean weight was 101.6 g +/- 106.7. Percentage (range) of total body weight loss was 16.4% (0.65% - 45.15%). Fistula was patent in 100% of the cases at the end of the follow-up. For two rats, total body weight loss was superior to 30% at the end of follow-up.

Pig Model

A total of 8 adult (45–55 kg) pigs were used for the technical development of the sleeve gastric fistula model, and 6 pigs for the validation of the model.

1 Concerning the first step, three pigs were operated with the fundic pouch preserved. In this case,
2 none had patent fistula at the end of the follow-up. For another group of 2 pigs, the fundic pouch
3 was completely resected. All pigs were dead in first week, secondary to dysphagia and
4 dehydration. In last group of 3 pigs, the fundic pouch was partially resected and gastric fistula
5 was created 6 cm under the cardia. All pigs survived at the end of the follow-up. Fistulas were
6 patent in 100% of the cases, and the percentage of total body weight loss was 1.4% +/- 1.1.
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9 For the validation of the pig model, a surgical gastro-cutaneous fistula after sleeve gastrectomy
10 was performed in 6 pigs with the stapling achieving the fundus 5.1 (4.5-6.0) cm from the EG
11 junction. The mean time of surgery was 71 min. The mean weight before the surgery was 46.1
12 kg +/- 4.1. The follow-up after surgery was 14 days. At the end of the follow-up, the mean
13 weight was 1.2 kg +/- 0.8. Percentage of total body weight loss was 2.8 % +/- 1.8. Fistulas
14 were patent in 100% cases at this end of the follow up.
15

16 *Endoscopic and radiological assessment*

17 Endoscopic and radiological assessment were performed only for the pig model. The internal
18 fistula orifice was open in 100% of the cases. In the radiological evaluation, 100% of
19 fistulographies displayed patent gastro-cutaneous fistulas (Figure 5).
20

21 *Several adverse events*

22 For the pig model, dehydration was a severe adverse event in two pigs with completely resected
23 fundic pouch. These two pigs were sacrificed before the end of the follow-up. No severe adverse
24 event was observed for the validation group.
25

26 **Discussion**

27 In this study, to the best of our knowledge, we present the first animal model of gastro-
28 cutaneous leak after sleeve gastrectomy in both rats and pigs. After the conception and the
29 validation of surgical procedure in rat model, we performed gastro-cutaneous fistula after sleeve
30 gastrectomy in the pig model. In the rat model, gastro-cutaneous fistula was permeable at the
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end of the follow-up in 100% of rats. This result was confirmed in the validation group. The rate of adverse events was observed to be 12.5 %. The most important risk was weight loss superior to 30%. The development of the pig model was more difficult. In fact, in development group, when the fundic pouch was preserved, the fistula was totally closed at the end of the follow-up. When the fundic pouch was completely resected, the problem was the mortality due to dehydration and dysphagia. Dehydration was probably due to direct communication of fluid between the cardia and gastric fistula. In fact, when the fundic pouch was completely resected, the fistula started at the top of the staple line around 2 cm under the EG junction. Finally, the fundic pouch was partially resected and the fistula was created at distance from the EG junction. In this group, all pigs survived and fistulas were patent. Gastro-cutaneous leak after sleeve gastrectomy with partially fundic pouch resected in the pig model was confirmed in validation groups. Animal model of sleeve gastrectomy was already reported [10–13]. It is the first study to report animal model of gastro-cutaneous leak after sleeve gastrectomy. Digestive fistulas are challenging conditions associated to low remission rates, high morbidity and refractoriness [14,15]. Pharmacological and endoscopic approaches result in poor therapeutic outcome [16,17]. Rescue surgery is required when minimally invasive strategies fail or in case of life-threatening complications [15]. This complex surgery will attempt to close the fistula and reconnect the gastrointestinal tract with a risk of mutilating surgery [18]. Post-surgical fistulas therapy challenges recently motivated the investigation of cell therapy as alternative [15,19]. Besides, other emergent biotherapies based on extracellular vesicles would be of interest, as already demonstrated for esophago-cutaneous and colo-cutaneous fistulas [20]. The present models provide valuable opportunities for that.

53 **Conclusion**

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In this study, to the best of our knowledge, we present the first animal model of gastro-cutaneous leak after sleeve gastrectomy in both rats and pigs. This new model of gastro-

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2 cutaneous leak after sleeve gastrectomy will allow the development of new strategies for
3 minimally invasive regenerative therapy.
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10 Informed Consent: Informed consent does not apply.
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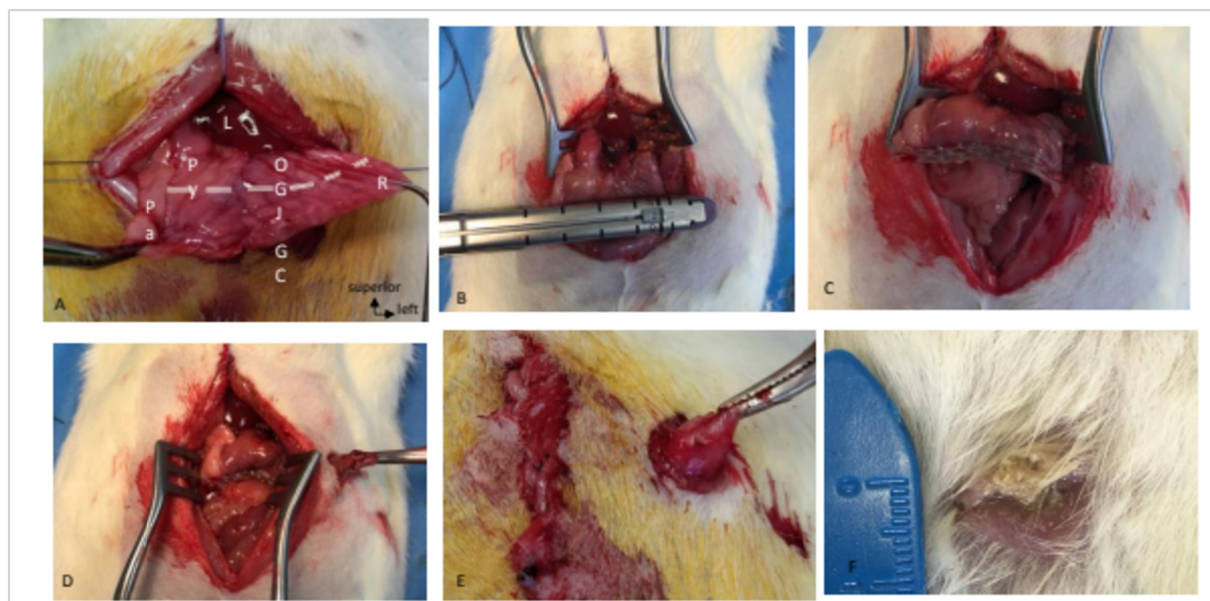
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14 Conflict of Interest: The authors declare that they have no conflict of interest.
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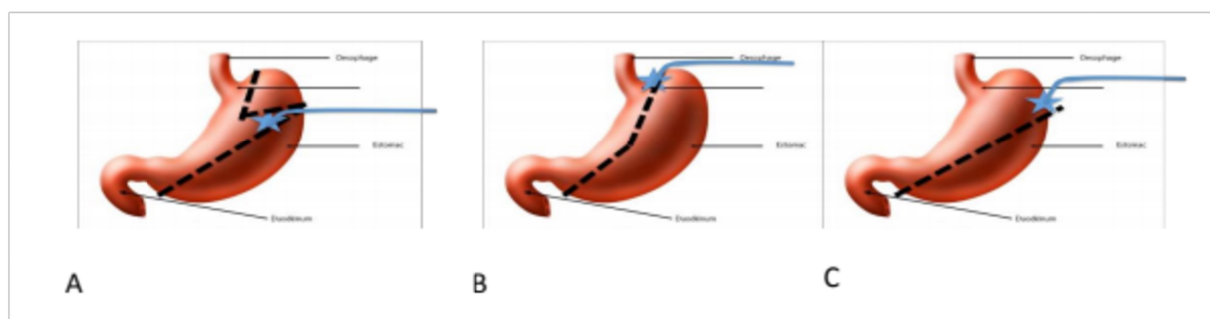
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Figure 1: General disposition, and landmark for sleeve gastrectomy in a rat model. A, Pa : pancreas, L : liver, Py : pylorus, OGJ : esogastric junction, GC : great curvature of the stomach, R : rumen. The discontinuous line refers to the sectional plan for sleeve gastrectomy, beginning from 5 mm to the pylorus. B, section of the stomach, and application of a vertical stapler (EndoGIA – 45mm-purple cartridge-Covidien); C, definitive aspect after vertical sleeve gastrectomy; D, creation of the fistula by a staggered passage through the abdominal muscle and subcutaneous tissues; E, creation of the external fistula; F, definitive aspect of the fistula, 45 days after surgery.



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Figure 2: Three experimental model of surgical gastro-cutaneous fistula after sleeve gastrectomy in pig model. A, The fundic pouch was preserved, the stapling achieved at the greater curvature just below the fundic pouch. B, The stapling achieved at the cardia near the

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eso-gastric junction. C, The fundic pouch was partially resected and a lightly stenosis was performed at the lesser curvature. The stapling achieved at the fundus four to six centimeters from the EG junction.

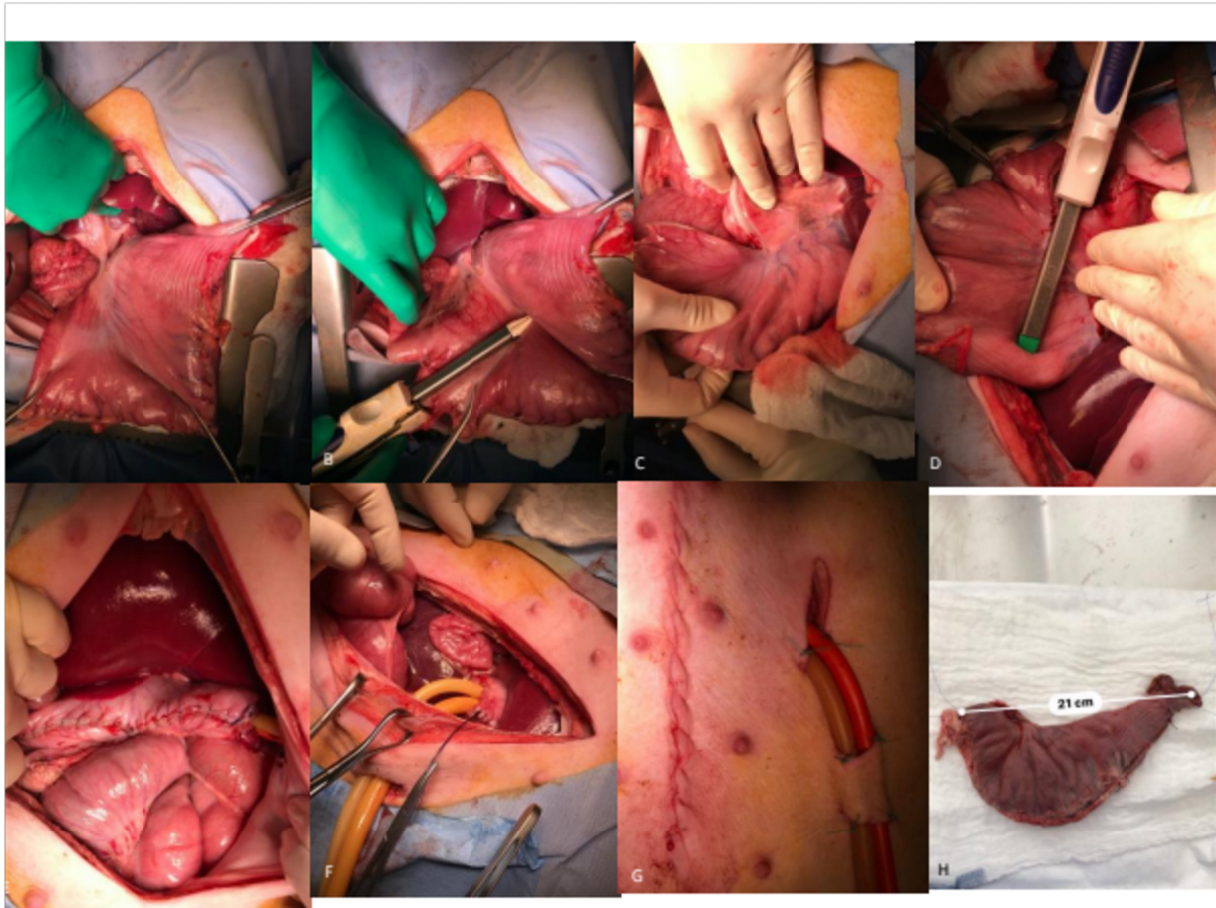


Fig. 3: Procedure of gastric leak after sleeve gastrectomy in pig model. A, gastric dissection; B, Gastric calibration by 36-French bougie tube was placed and advanced to the level of the pylorus. Creation of the sleeve begins 3 to 4 cm from pylorus. C and D, three to four vertical staplers (GIA 80mm, green cartbridge, Medtronic) were fired along the calibration tube. E, A manual suture was performed along the staple line. F and G, At the top of the staple line, the stomach was opened to introduce 2 catheters (one 24 Fr Pezzer catheter in parallel with a T-tube catheter); then, catheters were guided and fixed to the skin to create gastro-cutaneous fistulas. H, excluded stomach removed after surgery

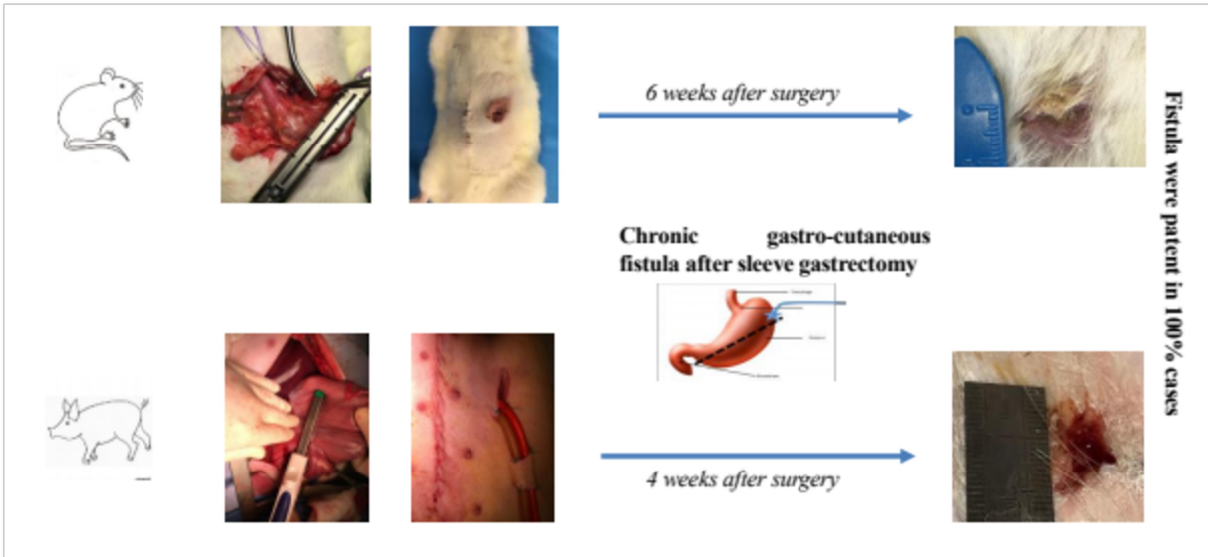


Fig. 4: Summary of animal's follow-up after surgery in rat group, and pig group.

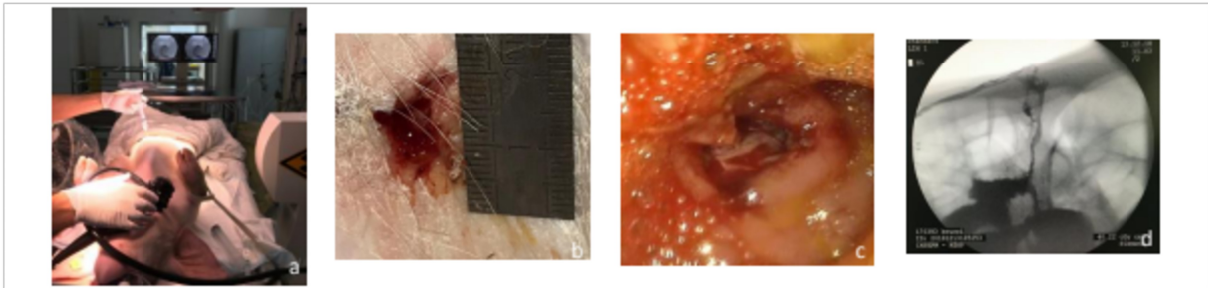


Fig. 5: Clinical, endoscopic, and radiological assessment of gastric leak after sleeve gastrectomy in pig model after 28 days. a; position of the pig, during clinical, endoscopic and radiological assessment, performed under general anesthesia; b, external fistula; c, endoscopic view of internal fistula orifice; d, radiological opacification of the fistula.

Discussion

Nous avons pu créer deux nouveaux modèles de fistules digestives hautes après SG laparoscopique. Il s'agit de deux modèles proches des fistules digestives après SG chez l'homme. Dans ces deux modèles nous avons pu créer des fistules gastro-cutanées chroniques qui restent ouverte après six semaines chez le rat et quatre semaines chez le porc.

Grace à sa simplicité, et sa reproductibilité le modèle de fistules gastro-cutanées sur SG chez le rat, permettra l'évaluation de nouveau traitement régénératif. L'utilisation d'un modèle de petit animal, permet la simplification des expérimentations et une évaluation multimodale des effets thérapeutiques. Cependant avant toute transposition chez l'homme il est nécessaire d'évaluer la sécurité et l'efficacité de la thérapie vésiculaire chez le gros animal.

Article 3: Treatment of chronic gastrocutaneous fistula after sleeve gastrectomy in a rat and PIG models by Extracellular Vesicles embedded with Pluronic gel

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Introduction :

La morbidité globale après chirurgie bariatrique varie de 0 à 17,5 % et la mortalité globale de 0 à 1,2 % [37–41]. De plus, le taux de reprises chirurgicales peut aller jusqu'à 13% [42]. La fistule digestive est l'une des principales causes de reprise chirurgicale. Après SG le taux de fistules est d'environ 2 à 3% [32,41,43–47]. Il s'agit d'une complication grave, lourde de conséquences chez des patients jeunes opérés pour une pathologie ne menaçant pas le pronostic vital à court terme. La prise en charge de ces fistules nécessite des soins médicaux lourds avec des délais de cicatrisation entre 4 et 6 mois. En cas de péritonite post-opératoire, le taux de mortalité de cette fistule atteint 20%. La thérapie cellulaire et sous cellulaire apparaît comme une voie prometteuse dans la prise en charge mini-invasive des fistules post-SG.

À la suite de nos premiers résultats sur un modèle de fistules colo-cutanées chez le rat, nous avons cherché à transposer ces résultats dans deux nouveaux modèles de fistules post-opératoires proches des contraintes humaines. De plus, avant de pouvoir envisager une transposition chez l'homme de la thérapie vésiculaire, il est nécessaire d'évaluer l'efficacité et la sécurité d'utilisation des VEs issues d'AdSC humaines dans la prise en charge des fistules post-opératoires.

Dans cette étude nous avons évalué dans un modèle de rats de fistules gastro-cutanées plusieurs types de thérapie régénérative (AdSC humaines, VEs issues d'AdSC humaine seules ou associées au gel de pluronic F127). Les VEs ont été produites à haut rendement avec une méthodologie implémentable dans des bioréacteurs compatibles avec une production future de grade GMP (Good Manufactory Practice) et toutes les thérapies ont été administrées par voie percutanée.

Dans le modèle de rat, les meilleurs résultats thérapeutiques ont été observés pour les groupes AdSC, VEs et gel + VEs en considérant le taux et la cinétique de fermeture de la fistule ainsi que les scores d'inflammation par rapport au contrôle. Bien que des résultats plus favorables aient été obtenus dans le groupe VEs, aucune différence statistiquement significative n'a été observée. L'approche thérapeutique avec le résultat le plus faible a été observé pour le groupe gel. Ensuite, nous avons validé les thérapies sélectionnées dans un modèle de porc. Des groupes de traitement ont été sélectionnés pour être testés dans le modèle porcin. Les résultats obtenus dans le modèle porcin ont validé ceux du modèle de rat. L'approche proposée permet de cribler plusieurs thérapies candidates dans le modèle de fistules de rats tandis que la validation est effectuée dans un modèle cliniquement pertinent. Sur la base de cette approche, nous avons démontré que l'administration locale de VEs issues d'AdSC humaines réduit le débit de la fistule gastro-cutanée, la fibrose et l'inflammation par rapport aux groupes de contrôle. Cette étude a mis en évidence le potentiel thérapeutique des VEs administrées localement et dans un hydrogel thermorésistant pour la gestion des fistules gastro-cutanées post-chirurgicales difficiles à traiter dans le cadre d'une stratégie dite « sub-cellulaire » peu invasive.

TREATMENT OF CHRONIC GASTROCUTANEOUS FISTULA AFTER SLEEVE GASTRECTOMY IN A RAT AND PIG MODELS BY EXTRACELLULAR VESICLES EMBEDDED WITH PLURONIC GEL

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Key-words: extracellular vesicles, intestinal post-surgical fistulas, thermoresponsive hydrogel, regenerative medicine.

Abstract :

Introduction : Gastric fistulas after sleeve gastrectomy are a rare but severe complication, involving in most cases a long and complex management. Innovative therapies are imperative to fill this unmet medical need. We propose herein a two-sided approach. First, we screened therapies of distinct complexity based on biomaterial, adipose tissue-derived stromal cells (ADSC) or their extracellular vesicles (EVs) in a rat model of gastro-cutaneous fistulas. ADSC EVs were produced by a high-yield scalable approach in bioreactors and all therapies were administered percutaneously. Next, we validated selected therapies in a clinically-relevant pig model.

Method : A surgical gastro-cutaneous chronic fistula model after sleeve gastrectomy was performed in Wistar rats and White Pigs. In the rat model, animals were randomized in 5 groups being treated with PBS (control group), thermo-responsive Pluronic F-127 hydrogel at 20% (gel group), 10^7 ADSCs (cell group), 2×10^{11} ADSC EVs with or without thermoresponsive Pluronic F-127 hydrogel at 20% (EV and gel + EV groups, respectively). Control, gel and gel + EV (10^{12} ADSC EVs) groups were selected for validation via the gastro-cutaneous fistula model in pigs. Treatment was performed two times: 2 and 3 weeks after surgery under general anesthesia. Rats and Pigs were respectively weekly followed for 45 days and 14 days after surgery. Clinical, radiological and histological data were collected. The primary outcome was the rate of fistula external orifice closure at this end of follow-up.

Results : In rat model, the best therapeutic outcomes were observed for the cell, EV and gel + EV groups considering fistula closure rate and kinetics as well as inflammation scores when compared to control. Although more favorable results were obtained were obtained in the EV group, no statistically significant difference was observed. The therapeutic approach with the lowest outcome was observed for the gel group. As a second step, groups were selected to be tested in the pig model. The gel + EV group was chosen from the best performers due to the facilitated administration strategy in large animals featuring an extensive fistula defect. The gel group was chosen to represent the worst performant therapy and both groups were compared to control. Results in the pig model validated the rat model ones.

Conclusion: The proposed approach enables to screen several candidate therapies in the rat fistula model while validation is performed in a clinically relevant model. Based on this approach, we demonstrated that the local administration of ADSC EVs in the hydrogel reduced gastro-cutaneous fistula output, fibrosis and inflammation when compared to the control groups. This study pointed-out the therapeutic potential of SC EVs administered locally and in a thermoresponsive hydrogel for the management of challenging post-surgical gastro-cutaneous fistulas in a minimally-invasive cell-free strategy.

Introduction:

Obesity is a complex chronic disease with a considerable socio-economic impact. Prevalence of obesity is estimated at 15% of the adult population in Europe [198]. Surgical treatment of obesity is more effective in the long term than medical treatment alone. However, it is associated with a higher morbi-mortality. Indeed, the rate of serious adverse events can be as high 37%, and the rate of surgical resumptions up to 13% [42]. Gastric fistula after sleeve gastrectomy is a rare (2%) but severe complication, involving in most cases a long and complex management. The endoscopic management has clearly redesigned and improved the management of these fistulas, but it requires many procedures during a long period with also major morbidity. Moreover, when endoscopic conservative treatment fails, fistula results in a chronic fistula (>12 weeks) and a surgical treatment is mostly required with mutilant techniques as total gastrectomy. Stem/stromal cell (SC) therapy currently provides hope for the treatment of fistula, which fail to respond to conventional treatments. Regenerative treatment based on SC therapy has generated a lot of enthusiasm in the scientific community. SC therapy may be used in many complex pathologies. Due to ethic and safety consideration, SC were preferred to pluripotent embryonic stem cells for regenerative medicine [199]. Most of animal and human study was conducted with SC.

These SC can be harvested from liposuction or lipoaspiration of human adipose tissue (ADSC: adipose-tissue derived stromal cells). Initially, the scientific community thought that action of these SC from their ability to differentiate, replacing the target damaged tissue. However, the interest in SCs for regenerative medicine has rapidly moved beyond their ability to differentiate into multiple cell types, with preclinical demonstration of their propensity to promote wound healing and angiogenesis, as well as their immunomodulatory properties and anti-fibrotic effects [142–145]. In addition, the results of several preclinical studies seemed to question the direct effect of SCs on tissue regeneration. It has been shown that very few SCs

graft durably to the wound site [15,146]. In the presence of an excessively inflammatory environment, SCs have a limited life span and die prematurely [146]. The therapeutic effect of SCs seems to occur in a time interval too short to be attributed to their cellular differentiation [15]. These results have prompted a paradigm shift in cell therapy towards mainly paracrine mechanisms of action [147].

The paracrine effect has been reported as the main mechanism of action of EVs secreted by SCs [147]. EVs or "cell dust" represent an innovative and promising tool in regenerative medicine. EVs derived from SCs reproduce the paracrine effect of the parent cells without proliferation risk. They are more resistant and easily preserved. EVs are nano-sized particles (40-5000 nm) released by the cells. They are composed of lipids, proteins, mRNA and miRNA, and their content depends on the cells that secrete them [6–8]. EVs play a direct role in the restoration of tissue function and homeostasis by modulating cell recruitment, proliferation, and differentiation [9–11]. Physiologically, EVs mediate intercellular communication. The regenerative medicine effect of EVs has been demonstrated in several preclinical study models, such as: myocardial ischaemia/reperfusion injury, acute kidney injury, type 2 diabetes mellitus, liver fibrosis, intervertebral disc degeneration, etc [21,161,173]. There is currently a shift in regenerative medicine from cell therapy to subcellular therapy based on EVs. The immune status of SC EVs (potentially lacking the MHC41 complex), allows them to be used as a "standard" allogeneic treatment [14,16]. These EVs have already demonstrated a real healing effect of regenerative treatment of digestive fistula in rat and pig model [175,176]. In those studies, EVs were from murin and pig SCs and investigated for the therapy of esophago-cutaneous or colo-cutaneous fistulas. Human SC-derived extracellular vesicles were never been evaluated for their pro-healing effect in gastro-cutaneous fistulas. This aims of this study was to evaluated safety and efficacy of human ADSC EVs in comparison to biomaterial or cell therapy for the management of

gastro-cutaneous fistulas after sleeve gastrectomy. ADSC EVs were produced by a high-yield scalable approach based on EV release via a turbulent flow enabling the production of clinically relevant doses in 1L bioreactor [176]. In this two-sided study, a rat model of gastro-cutaneous fistula after sleeve gastrectomy was used to screen therapies of distinct complexity based on biomaterial, ADSC or their extracellular vesicles alone or in combination with the biomaterial. Selected therapies were validated in a clinically relevant pig model of gastro-cutaneous fistula after sleeve gastrectomy.

Material & Method:

EVs and gel production

Human ADSCs were obtained from Cella-easy (France). Alpha MEM supplemented with 5% human platelet lysate, 100 U/mL heparin was employed for cell culture in 150 cm² flasks until confluence. Then, cells were used for EV production by the turbulence approach [176].

Surgical procedure

Surgical procedure in rat and pig model were detail in previous publication. This study was conducted at the Biosurgical Research Laboratory of the Alain Carpentier Foundation in Paris. It was approved by the local ethics committee of Descartes University (N°27476-2019081016097890 for the rat model and N°2018040219589382 for the pig one).

Rat model

Wistar female rats were operated by sleeve gastrectomy at day-0 of the study. A 2-cm laparotomy was performed. Stomach was identified and the great curvature dissected with elective ligation of short vessels from splenic artery. One centimetre from the pylorus, a tri staple purple EndoGIA[®] 60mm was applied for create a sleeve gastrectomy.

The upper part of the remnant stomach (rumen) on the staple line was sported for the fistula creation. A winding pathway was created throw the aponeurosis and abdominal muscles in order to create an indirect gastrocutaneous fistula-like pathway. Staple line was opened on 3 mm, and gastric mucosa was fixed to the rat skin by 4 stiches (PDS-5.0).

Pig model

To induce the surgical gastro-cutaneous fistula after sleeve gastrostomy, the first steps were to complete gastric dissection, place a 36-French bougie tube and advanced it to the level of the pylorus. The induction of the sleeve began 3 to 4 cm from pylorus. Three to four vertical staplers (GIA 80mm, green cartbridge, Medtronic) were fired along the calibration tube. At the top of the staple line, the stomach was opened to introduce 2 catheters (one 24 Fr Pezzer

catheter in parallel with a T-tube catheter); then, catheters were guided and fixed to the skin to create gastro-cutaneous fistulas. After surgery, upper endoscopy was performed systematically with opacification to control the appropriate positioning of catheters and permeability of the surgical montage. The fundic pouch was partially resected with a light stenosis performed at the lesser curvature and the stapling achieving the fundus, four to six centimeters from the EG junction. Splapling were removed 2 weeks after by general anesthesia.

Experimental procedure

Randomization

After surgery animals were randomized in different groups of fistula treatment.

In the rat model, Female Wistar rats (Janvier, 250–300 g) were randomized in: control group treated by 0.6 ml of PBS alone (control group, n =8); thermo- responsive Pluronic F-127 (Sigma-Aldrich) hydrogel at 20% (gel group, n = 8); 0.6 mL of thermoresponsive Pluronic F-127 hydrogel at 20% containing 1×10^{12} EVs turbulence-triggered EVs injected into the fistula lumen and the same dose in 0.6 ml PBS injected around the external orifice (gel + EV group, n = 8); 2×10^{12} turbulence-triggered EVs alone in 0.6 ml PBS , half of the dose being injected around the external orifice and the other half in the fistula tract wall (EV group, n = 8), and 10^7 human ADSCs in 0.6 ml of PBS half of the dose being injected around the external orifice and the other half in the fistula tract wall (cell group, n= 7).

For the pig study, female 3-months old Large White pigs were randomized in three groups treated with 5 mL of PBS injected into the fistula lumen (control group, n = 8), 5 mL of thermo- responsive Pluronic F-127 (Sigma-Aldrich) hydrogel at 20% injected into the fistula lumen (gel group, n = 8) or 5 mL of thermoresponsive Pluronic F-127 hydrogel at 20% containing turbulence-triggered EVs at 1×10^{12} EVs ml⁻¹ concentration, injected into the

fistula lumen and the same dose in in 0.6 ml PBS injected around the external orifice (gel + EV group, n = 8).

Follow-up

Animals were followed for 6 weeks after surgery in rat group, and 4 weeks after surgery in pig group. Treatment was started two weeks after surgery in both animal model. For the rat model, treatment was repeated once every week during 60 days under 2% of isoflurane anesthesia. For the pig model, treatment was repeated under general anesthesia one time three weeks after surgery. At this end of the follow-up, animals were killed by an intracardiac injection of thiopental in rat model and by intra-venous injection of Dolethal® in pig model. Randomization groups and follow-up were summarized in Figure 1.

Clinical assessment

The primary outcome was the patency of fistula at the end of the follow-up. The external fistula orifice was evaluated by a skilled gastroenterologist to assess its opening and inflammatory aspect. External fistula orifice were considered open respectively in rat and pig model if a 20G (0.8 mm) plastic catheter and plastic catheter 6 Fr Huibregtse® guiding catheter (Cook Medical, North Carolina, USA) could be introduced. Size of external orifice were assess used slide rule. Time to occlusur fistula was evaluated. Rate of closure fistula was assess in each groups. Inflammation was defined by the presence of an erythema around orifice (cutaneous inspection). Clinical score of inflammation between 0 to 5 was calculated, this score associated erythema (0: normal skin, 1: inflammatory skin over less than 90° circumference, 2: inflammatory skin over 90, 3: inflammatory skin over the whole circumference) and ulceration (0: no present, 1: superficial, 2: necrosis).

Radiological assessment

In the rat model, radiological assess was performed to evaluated activity of inflammation by PET-CT scanner. Rats were fasted overnight with free access to water. Rats were anesthetized (2 ± 0.5 % isoflurane in dioxygen), weighed and glycemia was measured in blood drawn from the caudal ventral artery using an Accu-Chek® Aviva Nano A (Accu-Chek, France). A 26G needle catheter (Fischer Scientific, France) connected to a 5cm polyethylene tubing (Tygon Microbore Tubing, 0.010" x 0.030"OD; Fisher Scientific, France) was inserted in the caudal vein for radiotracer injection. $25,0 \pm 1.5$ MBq of 2'-deoxy-2'-[18F]fluoro-D-glucose (FDG; Advanced Applied Applications, France) in 0.3mL saline was injected via the catheter. Rat were left on a warming pad for 30 min and then installed into the PET-CT dedicated bed. Respiration and body temperature were registered. Body temperature was maintained at 34 ± 2 °C and anesthesia was controlled on the breathing rate throughout the entire PET-CT examination. CT was acquired in a PET-CT scanner (nanoScan PET-CT; Mediso Medical Imaging Systems, Hungary) using the following acquisition parameters: semi-circular mode, 50kV tension, 720 projections full scan, 300ms per projection, binning 1:4. CT projections were reconstructed by filtered retro-projection (filter: Cosine; Cutoff: 100%) using the software Nucline 3.00.010.0000 (Mediso Medical Imaging Systems, Hungary). 70 min post tracer injection, PET data were collected for 10 min in list mode and binned using a 5ns time window, with a 400-600keV energy window and a 1:5 coincidence mode. Data were reconstructed using the Tera-Tomo reconstruction engine (3D-OSEM based manufactured customized algorithm) with expectation maximization iterations, scatter and attenuation correction. Volumes-of-interest (VOI) were delineated on the fistula tract (pathway) between the stomach and the skin using the PMOD software package (PMOD Technologies Ltd, Zürich, Switzerland). FDG accumulation was quantified as Standard Uptake Value (SUV, ratio of the radioactivity concentration in VOI on the whole-body concentration of the injected radioactivity). The standardized uptake value (SUV) was

analysed. SUV-max is determined as the highest value of FDG uptake of the pixel in the volume of interest (VOI) or fistula pathway. The hot average is defined as the mean of the 5 highest values in the VOI. The total lesion glycolysis (TLG) is the product of the SUV mean by the volume of the VOI.

In pig model fistula was evaluated at D14 and D28, under general anesthesia by external fistulography was performed by injection of 5 ml of a contrast agent (Xenetix 250[®], Guerbet, France), under fluoroscopy monitoring. This radiological assess was performed to evaluated track fisula and estimated size and volume of fistula.

Endoscopic assessment

In the pig model, the internal orifice of the fistula was evaluated by upper gastro-intestinal endoscopy (Gastroscope EG-590WR Fujifilm, Tokyo, Japan). At D14 and D28, an endoscopic evaluation under general anesthesia was performed by a skilled gastroenterologist to investigate internal fistula orifice opening and inflammatory aspect. Similarly, catheterization of the fistula tract was systematically attempted, and inflammation was defined by the presence of an ulcerated orifice. Endoscopic score of inflammation between 0 to 5 was calculated, this score associated erythema (0: normal mucosa around fistula, 1: inflammatory mucosa over less than 180° circumference, 2: inflammatory mucosa over 180°; ulceration (0: no present, 1: superficial, 2: necrosis); and bleeding (0: no present, 1: spontaneous bleeding).

Histological assessment

After sacrifice, a gastro-cutaneous fistula site and its periphery were collected and embedded in Formol 4%. Specimens were first sectioned perpendicular to the center of the fistula and then the two fragments obtained were fully sectioned to obtain thin tissue sections of 7 µm, which were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) for mucins,

glycogen, etc, and Sirius Red for measurement of fibrosis. Morphological analysis and specific measurements were performed by the same pathologist: presence of a fistula tract; presence, location and maximum thickness of fibrosis around the fistula; presence and semi-quantitative analysis of inflammatory infiltrate around the fistula tract. Fibrosis rate was determined by Fiber^o software developed from Matlab[®]. Pixel stained by Red Sirius was identified by the software (quantitative method) and a ratio was calculated between fibrosis pixel and every other pixel of the section. The mean of the results of three red sirius slices was used as a reference for the mean fibrosis rate for each animal.

All procedures and assessment since surgery to sacrifice were summarized in figure 1.

Statistics

The results were presented as means \pm standard deviation for continuous variables, and as percentages for categorical variables. Fischer's exact test was carried out for comparisons between categorical variables and the nonparametric Mann-Whitney test was used for non-paired continuous variables. Comparisons between more than two groups were performed with the nonparametric Kruskal-Wallis test. An estimation of the p value by the Chi-square test was carried out for the comparison concerning the number of cases per group. Time to occlusion fistula in each group of treatment were compared using the Kaplan–Meier method with the log-rank test. A P-value of <0.05 was considered indicative of significance. Statistical analysis was conducted using SPSS software version 24.0 (IBM Corp., Armonk, NY). GraphPadPrism (Graphpad Software, La Jolla, CA, USA) software was used for statistical analysis information displayed in figures.

Results:

Results were presented in two step, first in small animal (rat model), and results were validated in big animal (pig model).

First step, 40 rats, old of 12-months were operated by sleeve gastrectomy with the induction of a gastro-cutaneous fistula. Thirty-nine rats had been included in the study. One rat died on post-operative day 1 due to a haemorrhage on a splenic short vessel injury. One rat in control group has been euthanized 20 days after surgery because of an uncontrolled extracellular dehydration responsible for a weight loss above 30% of the initial weight (according to the ethical project). Flow-chart was represented on Figure 2a. The best therapeutic outcomes were observed for the cell, EV and gel + EV groups considering fistula closure rate. Number of gastrocutaneous closed fistula in EV group is 7 from 8 (87.5%), higher than in gel group with 2 from 8 (25%) ($p=0.041$) and control group with 1 from 8 (12.5%) ($p = 0.007$). Gel + EV group featured a number of fistula closure of 5 from 8 (62.5%) and the cell group 4 from 7 (57.1%), without statistical differences. Details were showed on Figure 3. Treatment by EVs increased the rate of closure fistula, with 95.4% +/- 12.9% with EV injected alone, and 67.1 +/- 46.5% with gel + EV. This result was statistically significant compared to control group with only 13.8 +/- 52.3% of closure rate ($p=0.001$), but no statistical difference was found respect to the Gel alone (49.2 +/- 37.6%) and the ADSC group (59.1 +/- 56.5%). Data were represented on Figure 4. Extracellular vesicles alone and embedded with gel + EV were related to an anti-inflammatory activity with a mean score of 0.25 +/- 0.46 and 0.38 +/- 0.52 in comparison to control with 2.0 +/- 1.07 ($p<0.005$). ADSC decrease the inflammatory score with a score about 0.57 +/- 0.53 ($p<0.005$). The score of the gel group was 0.88 +/- 0.83 and highlights an anti-inflammatory property of the gel alone ($p=0.031$). No difference was found between the anti-inflammatory property between EV, gel + EV and ADSC using this score ($p=0.912$). Details were illustrated in figure 5.

The Kinetics of gastrocutaneous fistula healing was displayed with Kaplan Meier graph and log rank test. It was evidenced that EVs allows a faster fistula closure in comparison to the control group ($p=0.019$) and to the gel group ($p=0.006$). EV did not increase the fistula closure speed respect to the gel + EV ($p= 0.174$) and to the ADSC ($p=0.063$) but seems to be close to the significant statistical level. Gel + EV was statistically different from the control group ($p=0.030$). The cell group did not increase the fistula closure kinetics in comparison to the control group ($p=0.056$) (Figure 6).

Radiological assessment

Nineteen rats had been randomized and underwent PET-TDM with ^{18}F FDG on post-operative day (POD) 44. Five rats had been allocated to the EV group, 5 to the extracellular vesicles and gel group, 4 to the ADSC group, 3 to the gel group, and 2 in the control group. Despite significant statistical results, EV and gel + EV allows a reduction of inflammation by reducing SUV max, Hot average and Total lesion Glycolysis (TLG) comparing with ADSC group, gel group and control group. The Figure 7 compares a control group rat against an EV group rat, and highlights the reduction of ^{18}F FDG captation and inflammation in the fistula tract and the end of the study on POD-44. Distribution of SUVmax in the different groups was represented on figure 8. Results were detailed in supplementary Table 1.

Histological assessment

Fibrosis rate in fistula tract was significantly decreased in EV group with 39.55 +/- 9.14% and GEL + EV group with 34.56 +/- 12.64% respect to the control group with 50.89 +/- 10.27% ($p < 0.001$) (Figure 9). No statistical difference has been found with ADSC group with a fibrosis rate of 34.10 +/- 11.30% compared to the EV and gel + EV group ($p = 0.230$) (Supplementary table 2).

In this first part, we showed that human EVs had a clinical, radiological and histological pro-healing effect, to closure gastro-cutaneous fistula in rat model. Based to this first result on small animal, we evaluated in next part safety and efficacy of human EVs in pig model of gastro-cutaneous fistula after sleeve gastrectomy.

Second step: validation in Pig Model

Surgical gastro-cutaneous fistula after sleeve gastrectomy were performed in 27 white pigs, 3 months old. Three pigs, deaths due to dysphagia and dehydration before randomization, during to first 2 weeks follow-up (Figure 2b). The mean weight before the surgery was 47.2 kg +/- 3.2. At the end of the follow-up, the mean weight was 44.1 kg +/- 4.7, and percentage of total body weight loss (TBWL) was 6.6%. TBWL was similar in three groups at this end of follow-up ($p = 0.637$). Fistulas were patent in 100% cases at POD-14. After randomization and started fistula treatment we did not observed any severe adverse even and/or allergic effect due to treatment. The frequency of fistula output was significantly increasing in gel + EVs group, compared to control group ($p = 0.010$) and gel group ($p = 0.041$) (figure 10 and supplementary table 3). No significant difference was observed when comparing the frequency of output for the control group to the gel one ($p = 1$). Clinical inflammation score was decreasing significantly between each group ($p = 0.002$) (Figure 11). Into endoscopic assessment, frequency of internal fistula orifice closed was significant increasing in gel + EVs

group comparing to control group ($p=0.026$). Endoscopic inflammation score was decreasing significantly between each group (Figure 11) ($p= 0.008$). Fistula tract was closed in 12.5% (control group) versus 75.0% (gel + VE group) ($p=0.041$). Volume of fistula tract was significant decreasing in gel + VE group (vs control group; $p= 0.003$) (vs gel group; $p= 0.018$) (Figure 11). Velocity of closure fistula was increasing in gel + VE group ($p=0.007$) (Figure 12).

Histological analysis were in progress and not available now, due to delayed caused by COVID epidemic.

Discussion :

This study showed that human EVs had a clinical, radiological and histological pro-healing effect, to closure gastro-cutaneous fistula in rat and pig model. Originality of our study is to evaluated safety and efficacy of human EVs in regenerative treatment in small and big animal. Based to this first result on small animal, we were evaluated in next part safety and efficacy of human EVs in pig model of gastro-cutaneous fistula after sleeve gastrectomy. It is first time that rat and pig model of gastro-cutaneous fistula after sleeve gastrectomy were used to compare different regenerative treatment. The proposed approach enables to screen several candidate therapies in the rat fistula model. In rat model, the best therapeutic outcomes were observed for the cell, EV and gel + EV groups considering fistula closure rate and kinetics as well as inflammation scores when compared to control. Although more favorable results were obtained in the EV group, no statistically significant difference was observed. The therapeutic approach with the lowest outcome was observed for the gel group. Metabolic radiological assessment by PET-TDM with ^{18}F FDG on POD-44, despite significant statistical results, EV and gel + EV allows a reduction of inflammation by reducing SUV max, comparing with ADSC group, gel group and control group.

As a second step, groups were selected to be tested in the pig model. The gel + EV group was chosen from the best performers due to the facilitated administration strategy in large animals featuring an extensive fistula defect. The gel group was chosen to represent the worst performant therapy and both groups were compared to control. Results in the pig model validated the rat model ones.

Regenerative medicine are challenging of post-surgery fistula. Gastric fistula after sleeve gastrectomy is a rare (2%) but severe complication, involving in most cases a long and complex management. When endoscopic conservative treatment fails, fistula results in a chronic fistula (>12 weeks) and a surgical treatment is mostly required with mutilant

techniques as total gastrectomy. SC therapy currently provides hope for the treatment of fistula, which fail to respond to conventional treatments. Regenerative treatment based on SC therapy has generated a lot of enthusiasm in the scientific community. Few data were published on SC therapy hope for the treatment of eso-gastric fistula. In one study, a model of chronic gastrocutaneous fistula in rabbits treated with autologous stromal vascular fraction (SVF, containing ADSC) was injected into the fistula. Three weeks after treatment, examination of the external orifices and histological analysis of the fistula tract showed almost complete healing in the SVF group [153]. Most of study used enterocutaneous or colcutaneous fistula in rat model for assess benefic effect of SC therapy [152–155]. One question, is it possible to used SC therapy from another species would find the same results in other species (for example human SC used in rat model) ? A first study, using a rat model of enterocutaneous fistula, after performing a caecostomy-type enterocutaneous fistula, showed an improvement in the closure of the external orifice with local injection of human SC [155]. Another study evaluated the potential of human bone marrow-derived SC sheets in a mouse model of colocutaneous fistulae. Two weeks after transplantation, clinical assessment, MRI, histological analysis and confocal probe endomicroscopy results indicated improved healing in the human SC groups compared to the control group. SC therapy resulted in less inflammation, less fibrosis, better microvascularisation, smaller fistula area and a decrease in the maximum size of the fistula [152]. As far as we know, this is first study where human EVs are used for treatment of post-surgery fistula. Futhermore, efficacy of human EVs are like than human ADSC. As EVs are known to participate in SC paracrine role [18,200]. Human EVs are produced via a high-yield scalable strategy in bioreactors. This production of EVs is fully integrated with cell culture in stirred tank in cGMP bioreactors [176]. EVs production are a challenge to launch wide scale clinical trials. Good manufacturing practices is a necessary rule to optimized production with high yields of EVs. The main advantages of EVs

are their ease of storage, preservation, distribution and administration compared to ADSCs. The legislation makes them a simpler drug to develop than SC, with a lower neoplastic risk than SC, autonomous and able to multiply. They may represent a new therapeutic tool for the future, with advantages comparable to SC therapy, with greater ease of use and a better safety profile.

Conclusion: The proposed approach enables to screen several candidate therapies in the rat fistula model while validation is performed in a clinically relevant model. Based on this approach, we demonstrated that the local administration of ADSC EVs in the hydrogel reduced gastro-cutaneous fistula output, fibrosis and inflammation when compared to the control groups. This study pointed-out the therapeutic potential of SC EVs administered locally and in a thermoresponsive hydrogel for the management of challenging post-surgical gastro-cutaneous fistulas in a minimally-invasive cell-free strategy. These results are encouraging and invited to started clinical translation to patients with postoperative fistula.

Informed Consent: Informed consent does not apply.

Conflict of Interest: The authors declare that they have no conflict of interest.

Figures

Figure 1; Summary of all procedures and clinical, radiological, endoscopic and histological assessments since surgery to sacrifice in rat model (A) and pig model (B).

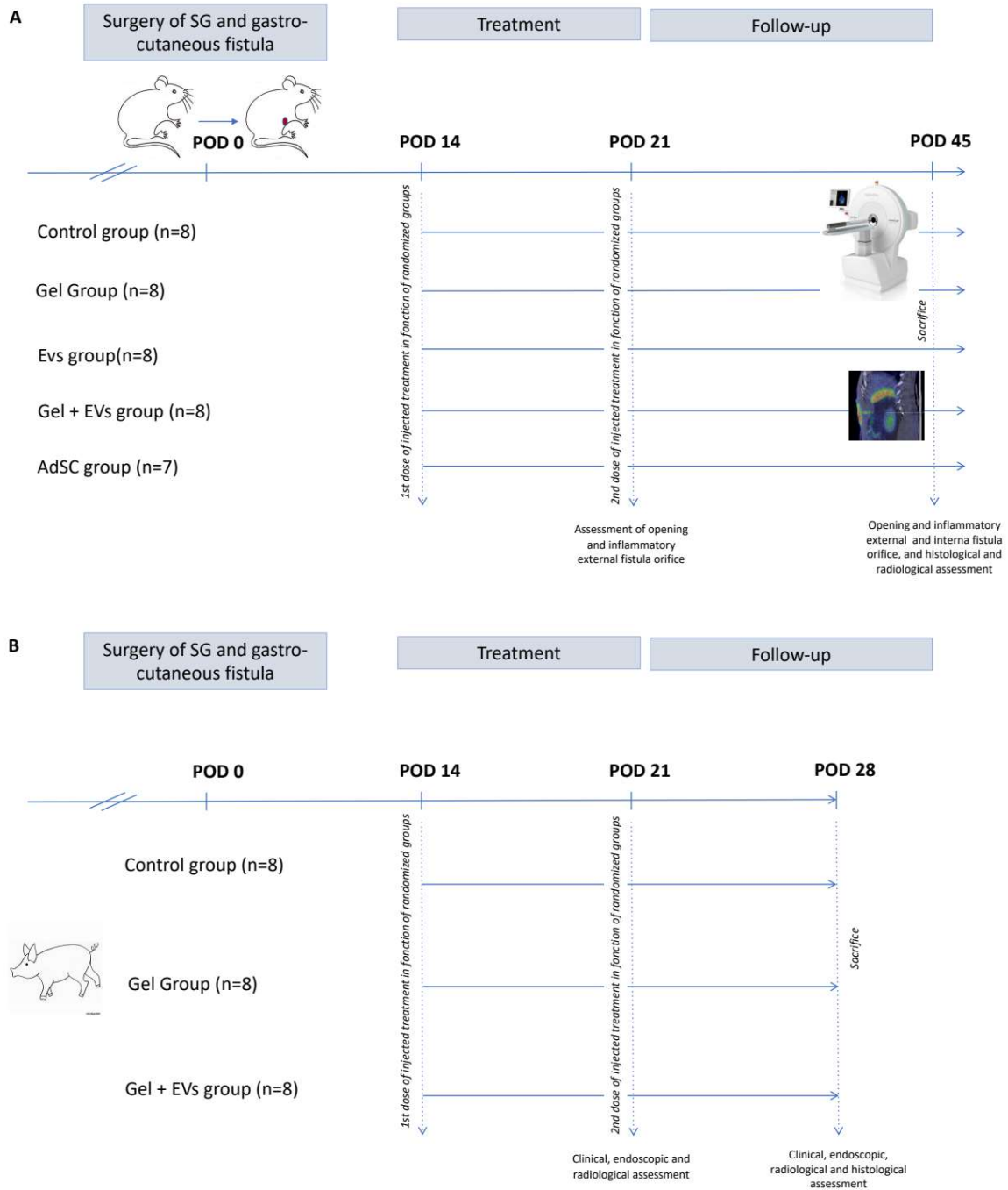


Figure 2 ; Flow chart

Figure 2a ; Flow chart for rat model

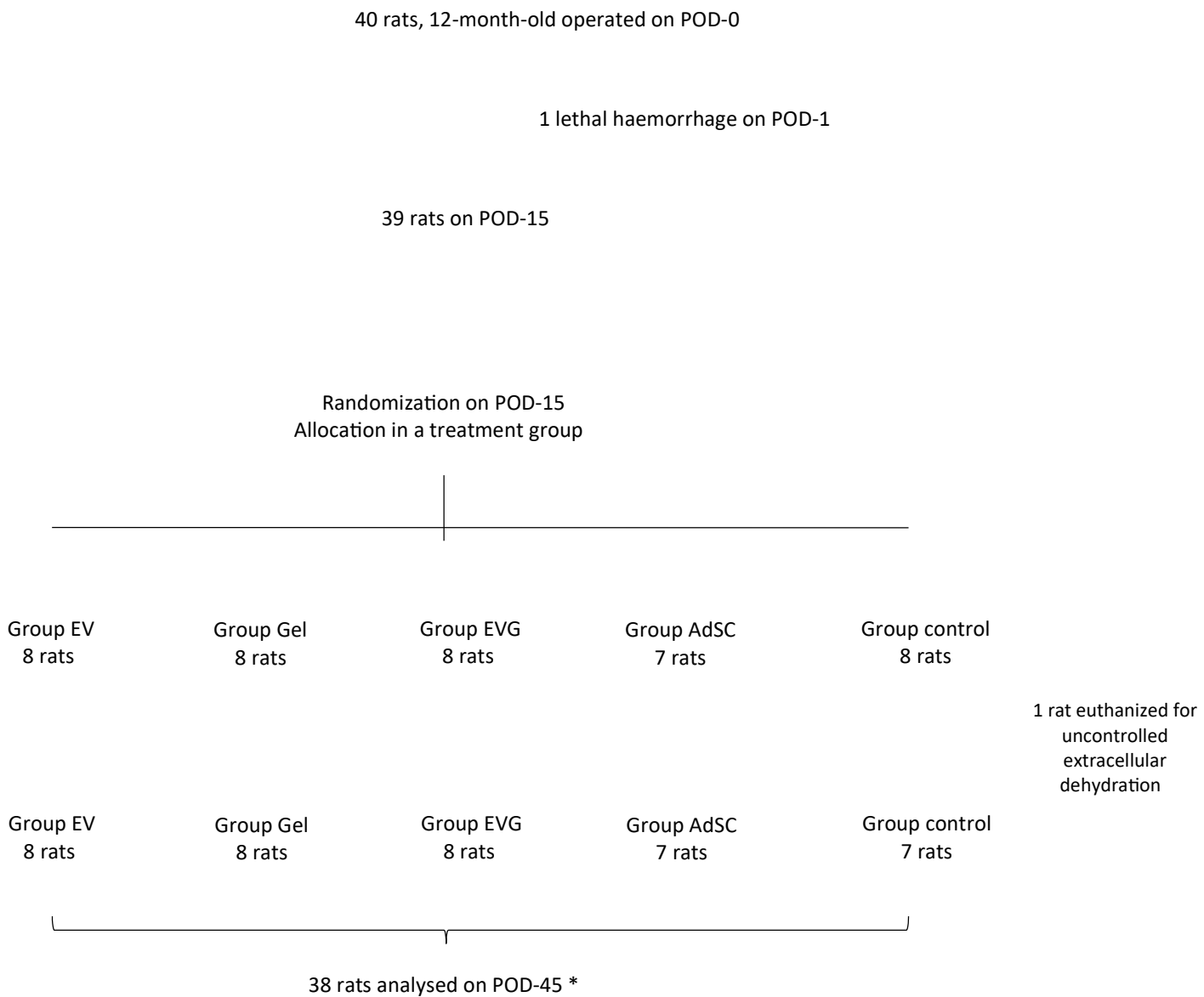


Figure 2b ; Flow chart for pig model

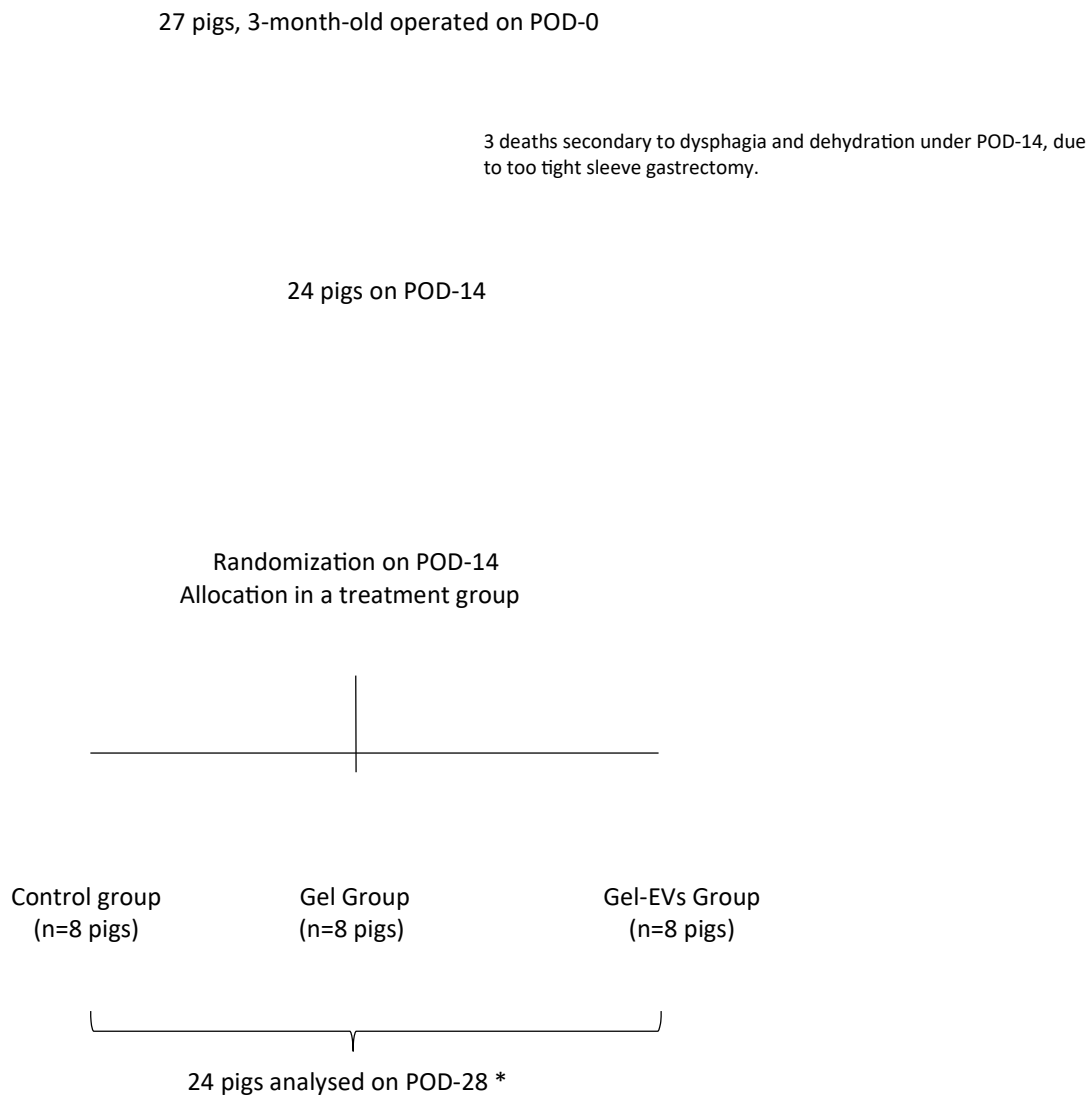
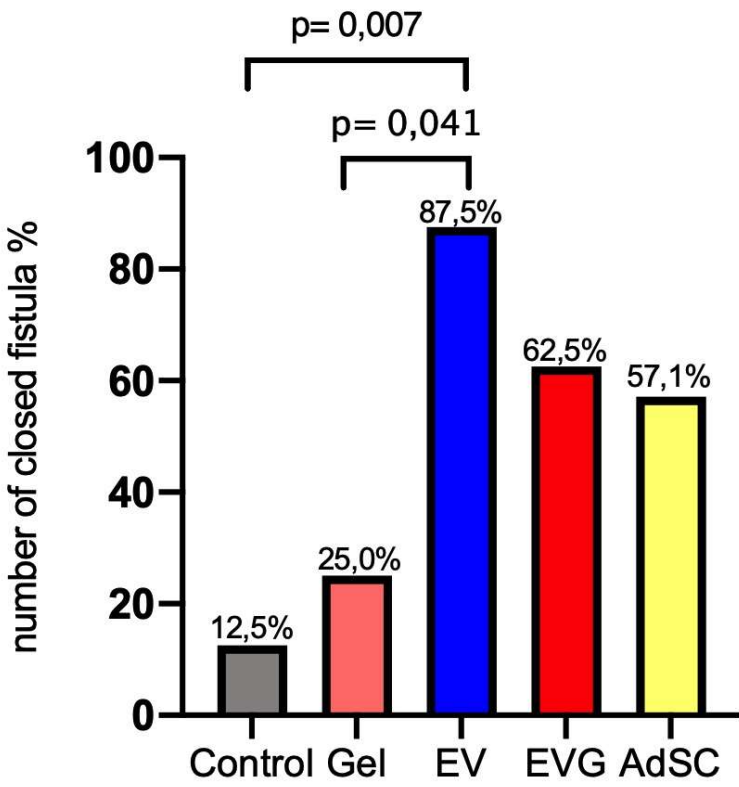
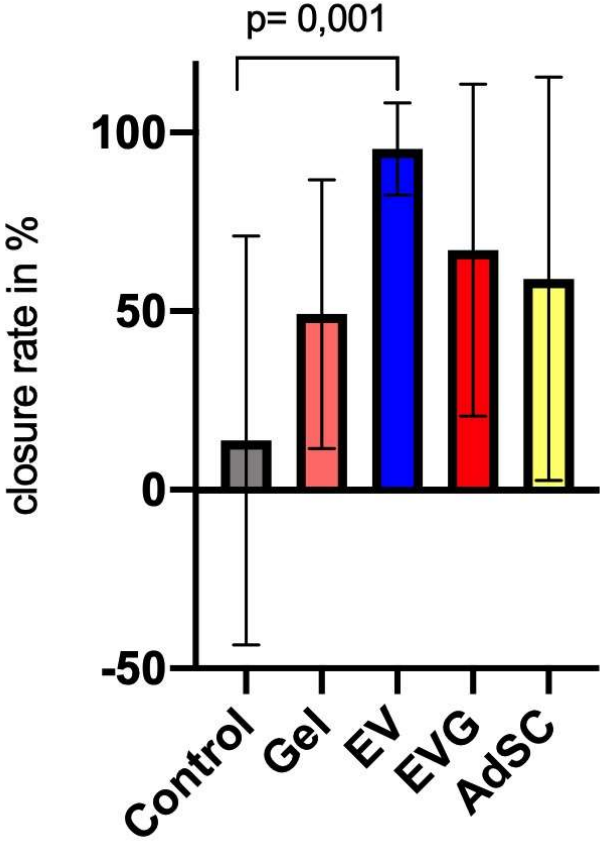


Figure 3: Number of closed fistula on POD-45



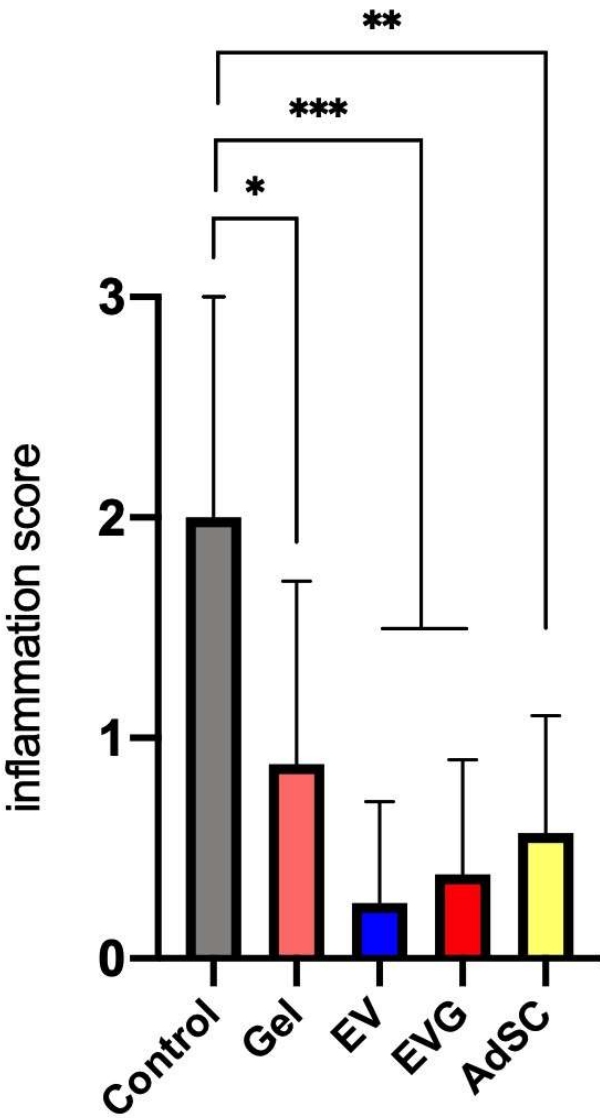
Number of fistula closure is expressed in percentage and p-value was estimated by Chi-square test, EV = extracellular vesicles; EVG = extracellular vesicles and gel ; AdSC = adipose stem cells.

Figure 4: Percentage of external gastrocutaneous fistula closure on POD-45



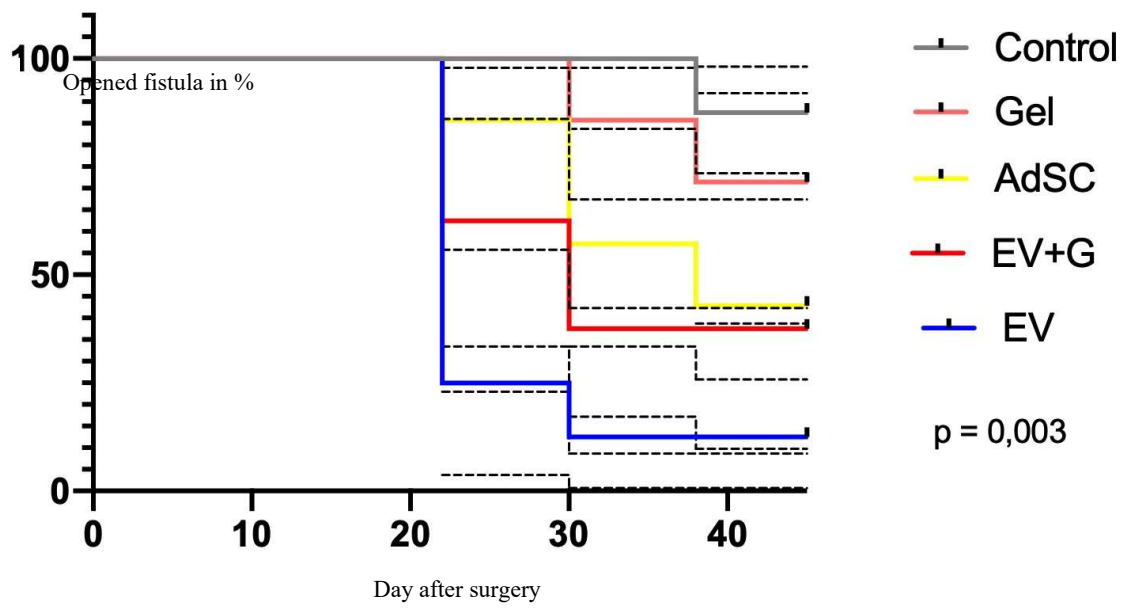
p-value was estimated by Mann-Whitney test; EV = extracellular vesicles; EVG = extracellular vesicles and gel ; AdSC = adipose stem cells.

Figure 5: Clinical inflammation score of external fistula orifice on POD-45



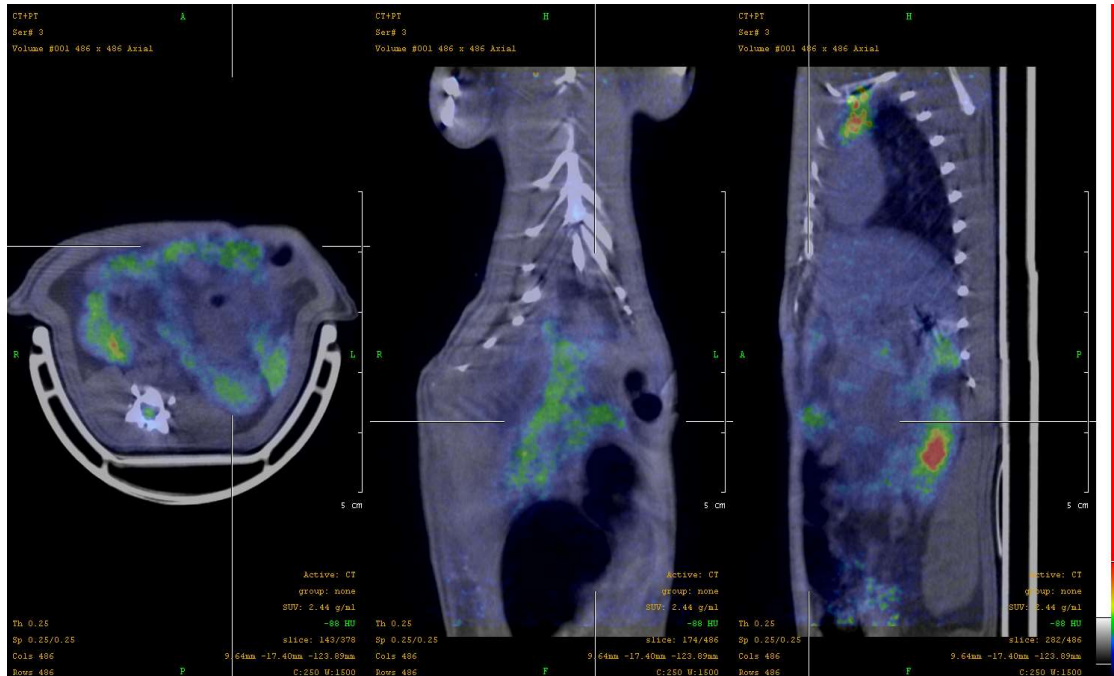
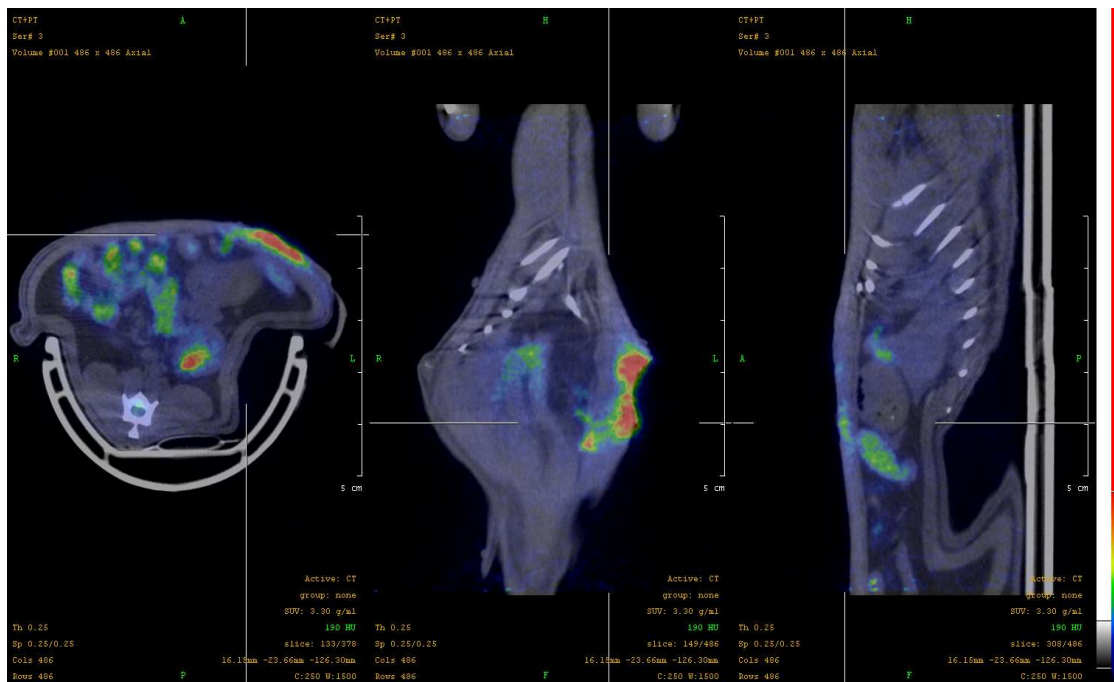
Inflammation score on POD-45 is represented by groups, *: $p < 0,05$; **: $p < 0,005$; *** : $p < 0,0005$. EV = extracellular vesicles ; EVG = extracellular vesicles and gel ; AdSC = adipose stem cells.

Figure 6: Kaplan Meier of cinetic closure rate



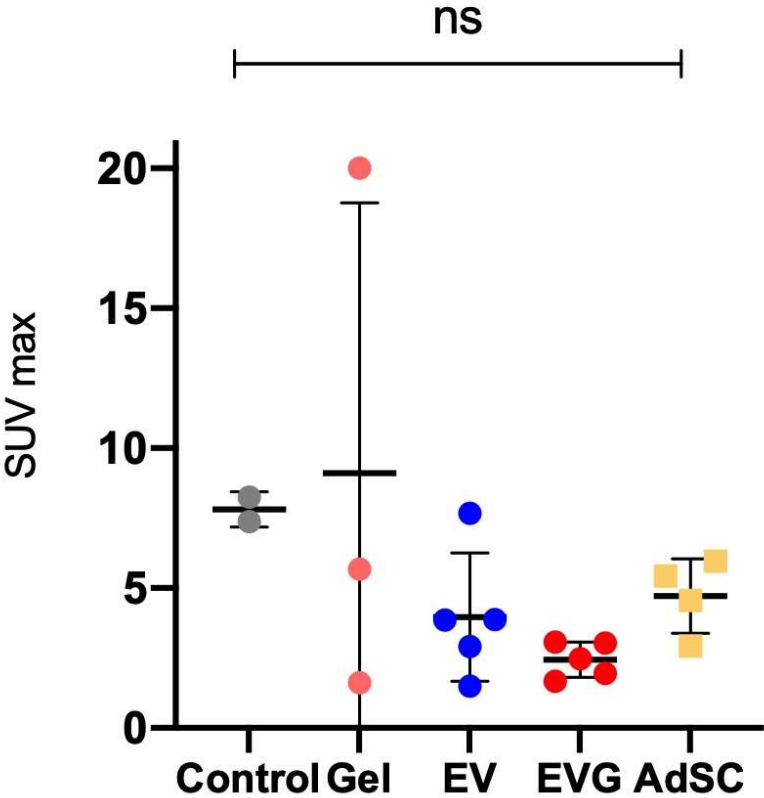
Kaplan Meier curves illustrates the number of fistula closure across the time until POD-45. The logrank test is significant with $p=0,003$; EV = extracellular vesicles; EV+G = extracellular vesicles and gel; AdSC = adipose stem cells.

Figure 7: PET TDM in a control group rat and EV's group rat on POD-44



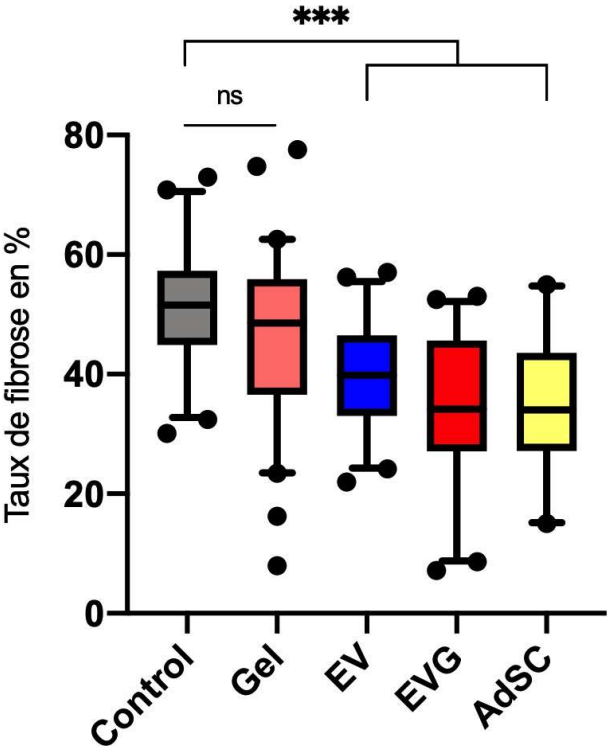
PET-TDM in a control rat; with axial, coronal and sagittal view of the fistula tract. The white arrow indicates the pathway of the fistula. PET-TDM in a EV's rat; with axial, coronal and sagittal view of the fistula tract. The white arrow indicates the pathway of the fistula.

Figure 8: SUV_{max} according different group of treatment on POD-44



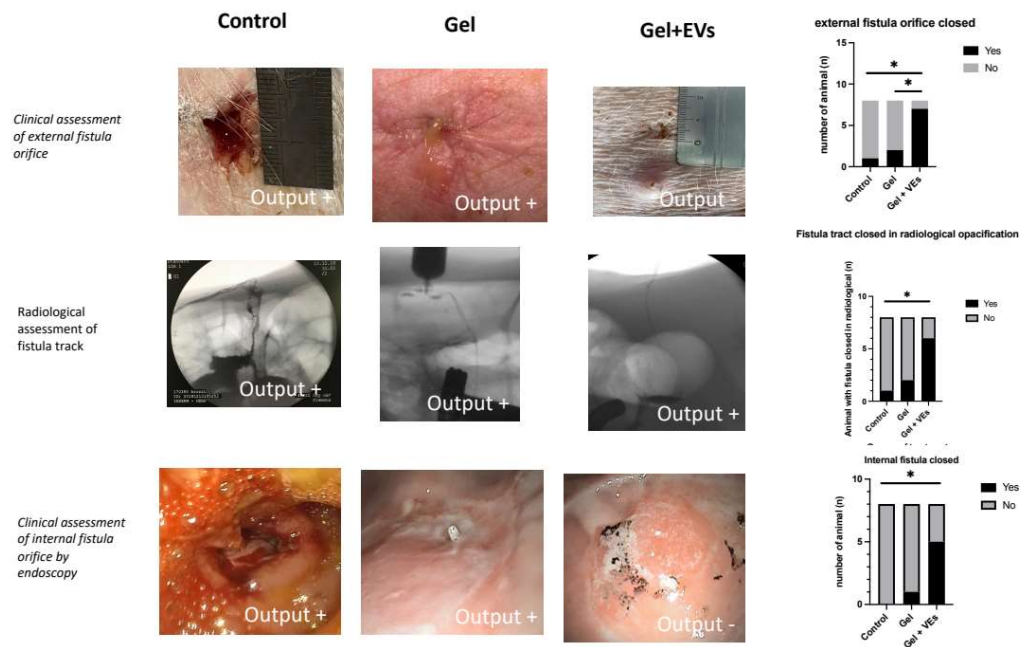
SUV_{max} are represented through the different groups. *Ns* = non-significant.

Figure 9: illustration of fibrosis among different groups of treatment on histological analysis with *Fiber*° software.



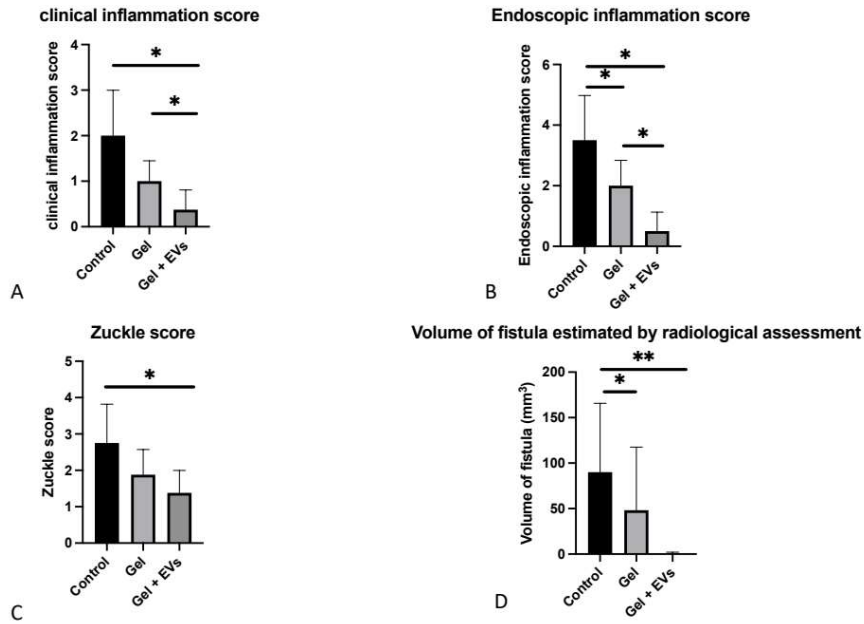
Fibrosis is expressed in percentage among the different group of treatment, ns : no-significant; *** : $p < 0,0005$.

Figure 10: Clinical, radiological and endoscopic assessment of gastro-cutaneous fistula in pig model.



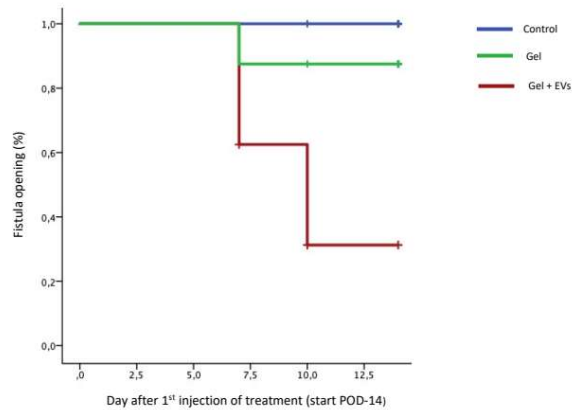
*: $p < 0,05$

Figure 11; Clinical (A), surgical (B), and endoscopic (C) assessment of local inflammation around fistula track. (D) Volume of fistula estimated by radiological assessment in pig model.



*: $p < 0,05$; **: $p < 0,005$

Figure 12; Kaplan Meier curves illustrates the number of fistula closure across the time until POD-28 in pig model.



The logrank test was significant in each groups ($p=0,007$), comparison between control vs gel group ($p= 0.31$), Gel vs Gel + EVs ($p= 0.04$) and control vs Gel + Evs ($p= 0.006$). EVs = extracellular vesicles; Gel + EVs = extracellular vesicles and gel.

Supplementary tables

Supplementary table 1: results of PET according groups of treatment and TEP parameters measured.

| Parameters of PET | Groups of treatment | | | | | p-value |
|-------------------------------------|---------------------|-------------------|--------------------|--------------------|-------------------|---------|
| | Control | Gel | EV | EVG | AdSC | |
| SUV max | 7.815 +/- 0.44 | 9.103 +/- 7.89 | 3.966 +/- 2.05 | 2.440 +/- 0.56 | 4.718 +/- 1.15 | 0.153 |
| Hot average | 7.591 +/- 0.45 | 8.526 +/- 7.45 | 3.782 +/- 1.98 | 2.368 +/- 0.52 | 4.426 +/- 1.15 | 0.152 |
| Total Lesion Glycolysis (TLG) | 0.202 +/- 0.08 | 0.136 +/- 0.09 | 0.068 +/- 0.052 | 0.099 +/- 0.038 | 0.122 +/- 0.10 | 0.478 |

Standardized Uptake Value (SUV) is expressed by groups of treatment. Results are expressed as mean +/- SEM (standard error of the mean); EV = extracellular vesicles; EVG = extracellular vesicles and gel ; AdSC = adipose stem cells.

Supplementary table 2: distribution of fibrosis determined by Fiber^o software on gastric fistula histological analysis.

| | Control | Gel | EV | EVG | AdSC | p-value |
|----------------------|----------------|------------|-----------|------------|-------------|----------------|
| Rate of fibrosis (%) | 50.89 | 46.12 | 39.55 | 34.56 | 34.10 | < 0,0001 |
| SEM | 10.27 | 13.65 | 9.14 | 12.64 | 11.30 | |

Fibrosis is expressed as the mean rate +/- standard error of the mean (SEM).

Supplementary table 3: characteristics as a function of treatment.

| | All (n=24) | Control (n=8) | Gel (n=8) | EVs (n=8) | <i>p</i> ^a | | | |
|---|-----------------------|--------------------------|----------------------|----------------------|-----------------------|---------------|---------------|---------------|
| | | | | | All | C vs G | C vs E | G vs E |
| Fistula output; n(%) | 14(58.3) | 7(87.5) | 6(75.0) | 1(12.5) | 0.005 | 1 | 0.010 | 0.041 |
| External fistula orifice closed; n(%) | 10(41.6) | 1(12.5) | 2(25.0) | 7(87.5) | 0.005 | 1 | 0.010 | 0.041 |
| Clinical inflammation score; mean(SD) | 1.13(1.03) | 2.0(1.19) | 1.0(0.53) | 0.38(0.51) | 0.002 | 0.039 | 0.006 | 0.036 |
| Internal fistula orifice closed; n(%) | 6(25.0) | 0(0.0) | 1(12.5) | 5(62.5) | 0.009 | 1 | 0.026 | 0.119 |
| Endoscopic inflammation score; mean(SD) | 2.0(1.75) | 3.5(1.77) | 2(1.0) | 0.50(0.75) | 0.008 | 0.048 | 0.005 | 0.012 |
| Fistula tract closed in radiological opacification; n(%) | 9(37.5) | 1(12.5) | 2(25.0) | 6(75.0) | 0.024 | 1 | 0.041 | 0.132 |
| Volume of fistula estimated by radiological assessment (mm ³); mean(SD) | 46.32(77.66) | 90.01(90.56) | 48.29(82.84) | 0.66(1.62) | 0.002 | 0.171 | 0.003 | 0.018 |
| Zuckle score; mean(SD) | 2.0(1.10) | 2.75(1.28) | 1.88(0.83) | 1.38(0.74) | 0.087 | 0.143 | 0.028 | 0.178 |

^a *Mann Whitney test / Fisher test.*

Discussion

L'originalité de cette étude est d'évaluer des traitements régénératifs, issus d'AdSC humaines. Il s'agit de la première étude évaluant l'efficacité et la sécurité d'utilisation des VEs issu d'ADSC humaines dans la prise en charge de fistules gastro-cutanées post-opératoires. La force de cette étude est son évaluation des thérapies dans un modèle de fistules gastro-cutanées sur sleeve gastrectomie chez le rat. Les résultats obtenus chez le petit animal ont ensuite été validés sur un modèle de fistules gastro-cutanées sur sleeve gastrectomie chez le porc.

Dans le modèle de rat, les meilleurs résultats thérapeutiques ont été observés pour les groupes AdSC, EVs et gel + EVs en considérant le taux et la cinétique de fermeture de la fistule ainsi que les scores d'inflammation par rapport au contrôle. Le taux de fistules gastro-cutanées fermées était plus faible dans le groupe contrôle, 12,5% (n= 1), que dans le groupe Gel + VEs, 62,5% (n= 5). La cinétique de fermeture des fistules gastro-cutanées guéries été plus rapide dans le groupe gel + EV par rapport au groupe témoin (p=0,030). Le groupe gel + EV est lié à une activité anti-inflammatoire avec un score moyen de 0,38 +/- 0,52 par rapport au groupe contrôle avec 2,0 +/- 1,07 (p<0,005).

Dans le modèle porcin : Le taux de fermeture des fistules tend à augmenter de manière significative dans le groupe VEs+Gel, comparé au groupe contrôle (p= 0,010) et au groupe Gel (p=0.041). Le score d'inflammation clinique a diminué de manière significative entre chaque groupe (p= 0,002). Dans l'évaluation endoscopique, le taux de fermeture de l'orifice interne à augmenter de façon significative dans le groupe VEs+Gel par rapport au groupe témoin (p=0,026). Le score d'inflammation endoscopique a diminué de manière significative entre chaque groupe (p= 0,008). Le tractus de la fistule était fermé dans 12,5% (groupe témoin) contre 75,0% (groupe VEs+Gel) (p=0,041).

Cette étude confirme l'efficacité et la sécurité d'utilisation des VEs associées ou non à un gel de Pluronic dans la prise en charge des fistules digestives post-opératoires. Les VEs utilisées sont issues d'AdSC humaines et produites par une technique à haut rendement implémentable en grade GMP. Ces résultats sur un modèle de rat est validé dans un modèle porcine permettront d'envisager une transposition vers l'utilisation des VEs chez l'homme dans le cadre d'une étude phase I/II.

Article 4: Enhancing digestive fistula healing by the off-label use of a thermoresponsive vessel occluder polymer associated with esophageal stent placement: A case report.

Arthur Berger, Eric Caudron, Guillaume Perrod, Imane Boucenna et al. Clin Res Hepatol Gastroenterol. 2020

Introduction :

Les fistules digestives sont souvent complexes, avec des trajets non rectilignes et de tailles variables. L'administration de la thérapie cellulaire ou sub-cellulaire constitue un défi ; en effet, la voie systémique ont une demi-vie courte et une internalisation rapide par le foie et la rate [185–187]. Cela souligne l'importance de l'administration **locale** plutôt que systémique pour améliorer la rétention de la thérapie cellulaire sur le site d'intérêt [188].

Les travaux de recherche précédent ont montré l'intérêt de l'utilisation d'un gel de poloxamer 407 comme transporteur des VEs afin de permettre l'application des VEs sur l'ensemble du trajet de fistules. Le gel de poloxamer 407 (Pluronic F127) est déjà commercialisée et bénéficie d'un marquage CE et d'une approbation par la Food Drug Administration (FDA) comme agent vaso-occlusif en radiologie interventionnelle. Ce gel pourrait également avoir de propriétés cicatrisantes, chez l'animal comme chez l'homme [189]. L'utilisation du gel de Pluronic augmente le temps de contact entre les molécules thérapeutiques et le tissu cible. Ce gel thermosensible est facilement injectable à travers un cathéter sous la forme liquide (permettant le remplissage de l'intégralité du trajet de la fistule indépendamment de sa taille et de sa morphologie irrégulière). Dans les travaux chez l'animal, nous avons utilisé un Gel de poloxamer 407 20%, dont la transition sol/gel se produit aux environs de 20°C, permet une

injection sous forme liquide pour remplir de manière homogène le trajet de fistules et une gélification rapide lors de l'administration pour retenir les VEs dans le trajet de fistules. Par ailleurs, l'utilisation du gel seul avait un effet sur le taux de fermeture des fistules oeso-cutanées chez le porc par rapport au groupe contrôle [175].

Le cas clinique ci-dessous illustre la première utilisation pour fistules digestives chez l'homme du gel de Pluronic F127. Le patient présentait une fistule chronique colo-cutanée au regard de l'anastomose colo-gastrique (contexte d'atrésie de l'œsophage opéré). Devant l'impossibilité de proposer une prise en charge chirurgicale de cette fistule colo-cutanée. L'utilisation du gel de pluronic F127 a été proposée au patient après avis du comité d'éthique local. Le traitement a réduit le diamètre de l'orifice de la fistule de $4,0 \pm 0,5$ à 1 mm et le débit journalier de la fistule a diminué de 425 ± 65 à 23 ± 4 ml, en comparant les mois avant et après le traitement.



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CASE REPORT

Enhancing digestive fistula healing by the off-label use of a thermoresponsive vessel occluder polymer associated with esophageal stent placement: A case report

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KEYWORDS

Fistula;
 Endoscopic
 treatment;
 Thermoresponsive
 vessel occluder
 polymer

Summary This case report relates to the first-in-man use of a vessel occluder gel medical device as a fistula occluder in a repurposing strategy. A patient with chronic colcutaneous fistula received an off-label treatment with a thermoresponsive Poloxamer 407 gel (20%) via percutaneous administration and injected under endoscopic control. Treatment consisted in the association of esophageal stent placement and gel injection. The product was administered just after the stent placement at $< 20^{\circ}\text{C}$ in its liquid form, gelling at body temperature to form a fistula plug. However, the stent was removed at day 26 because of major pain and the fistula was still present. Treatment was continued a total of 14 administrations of thermoresponsive Poloxamer 407 gel during 7 weeks via the external fistula orifice. The treatment reduced fistula orifice diameter from 4.0 ± 0.5 to 1 mm and fistula daily output decreased from 425 ± 65 to 23 ± 4 mL, when comparing the months before and after treatment. Gel administration was not

Abbreviations: SEMS, Self-expandable metal stent; VEGF, Vascular endothelial growth factor; TGF- β 1, Transforming growth factor-beta 1.

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associated with any toxic effects. The therapeutic outcome remained stable 1 year after treatment. The external fistula diameter and the fistula output were similar to what was observed after the last Poloxamer 407 gel administration.

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Introduction

Fistulas are a challenging public health burden related to Crohn's disease or secondary to surgery affecting millions of people worldwide with a high morbidity [1–4]. We present evidence of the potential therapeutic benefit of an intra-fistula treatment with a thermoresponsive Poloxamer 407 gel (20%) as a fistula occluder. It consisted in the first-in-man off-label use of this vessel occluder polymer gel that received market authorisation in the US and Europe (LeGoo®, Sanofi) as a IIb medical device.

Case

A 21-year-old male patient presented a cologastric anastomotic stenosis complicated by a supra-anastomotic colocutaneous fistula in the median supra-umbilical region. A colon interposition procedure was indicated following a post-natal diagnosis of esophageal atresia. This patient had a complex medical history with prenatal diagnosis of diaphragmatic cupola hernia, chronic multi-factorial respiratory failure and neurocognitive disorders. Three attempts to dilate endoscopically the cologastric anastomotic stenosis were unsuccessful. The last dilation was complicated by a perforation, which was closed by endoscopic through-the-scope clips (Boston Scientific, USA). The use of a standard clip approach for fistula management failed, due to the fistula location and the difficulty to position the clips appropriately. Then, a combined technique was proposed: a covered self-expandable metal stent (SEMS) 8 cm in length (MI TECH, South Korea), placed into the stenosis under radiological control (Fig. 1A–C), in association with an application of Poloxamer 407 gel as a fistula occluder. This strategy was accepted by the patient after ethical consideration in accordance with the results provided by our preclinical study [5]. Informed patient consent and a written patient's mother consent were obtained before therapy. Poloxamer 407 gel 20% in saline magistral formula was prepared in the Pharmacy Department of HEGP (Supplementary Fig. 1). Rheological analysis was performed indicating a sol-gel transition temperature at about 20 °C (Supplementary Fig. 2). The procedure was carried out using a standard adult gastroscope (GIF-H190, Olympus, Japan) under general anesthesia with orotracheal intubation. The endoscopy evidenced a normal esocolic anastomosis, but also a severe stenosis at the cologastric anastomosis. The internal fistula orifice was situated 2 cm above the cologastric anastomosis. A macroscopic view and an endoscopic one of external and internal fistula orifice, respectively, are shown in Fig. 1D and E. The radiological analysis evidenced a short fistula track of about 10 mm long and 3 mm wide with an important fibrosis in the internal orifice. In a first step, the internal

orifice was intensively abraded and after stents placement, the Poloxamer 407 gel at 20% was injected percutaneously. A total of 1 mL of gel was injected under endoscopic control before and after stent placement, as indicated in Fig. 1F and G. However, due to a severe abdominal pain, the stent was removed 26 days after its placement. Even if the fistula output decreased significantly, the external fistula diameter was still present with daily digestive secretions. Therefore, the patient received 1 mL of Poloxamer 407 gel twice a week during 7 weeks via percutaneous administration without anesthesia.

Fistula external orifice diameter progressively reduced from 4.0 ± 0.5 mm to 1.0 ± 0.5 mm during the 7 weeks of therapy (Fig. 2A). Fistula output decreased drastically from 425 ± 65 mL to 23 ± 6 mL in the following days post-treatment. The output continued to decrease slightly and remained stable at 10 ± 1 mL from the 4th to 7th week of gel application (Fig. 2B). The observed therapeutic benefit (Fig. 2A–C) was probably due to the combined effect of the stent, by alleviating stenosis pressure, and the gel, by promoting fistula occlusion. At 1 year, the external orifice had a dot shape without significant digestive secretions. The patient did not heal from the stenosis, or the fistula. After one year, the external fistula diameter and the fistula output were similar to what was observed after the last Poloxamer 407 gel administration.

Discussion

We showed for the first time that digestive fistula therapy may be enhanced by the percutaneous administration of thermoresponsive polymer gel in association with an esophageal stent placement. The thermoresponsive Poloxamer 407 hydrogels offer the advantage of filling irregular defects and reaching low-accessible areas while bypassing surgical interventions and the related discomfort as well as complications. Due to its thermoresponsive properties, the product could be administered as a liquid injected through a catheter, whose gelation in situ at body temperature enabled it to be retained at the fistula site. Poloxamer 407 hydrogel is expected to act mechanically limiting the circulation of secretions through the fistula orifice, which has a beneficial effect in the healing process.

The main limitations of esophageal stent placement are stent migration and abdominal pain. In our case, the stent was removed because of major pain. Poloxamer 407 hydrogel could be regarded as an additional treatment associated with an endoscopic technique or alone after endoscopic failure. In addition to the mechanical effect, an ancillary effect may be related to the intrinsic properties of Poloxamer 407 hydrogel, which is known to favour healing by increasing microvessel density while enhancing the

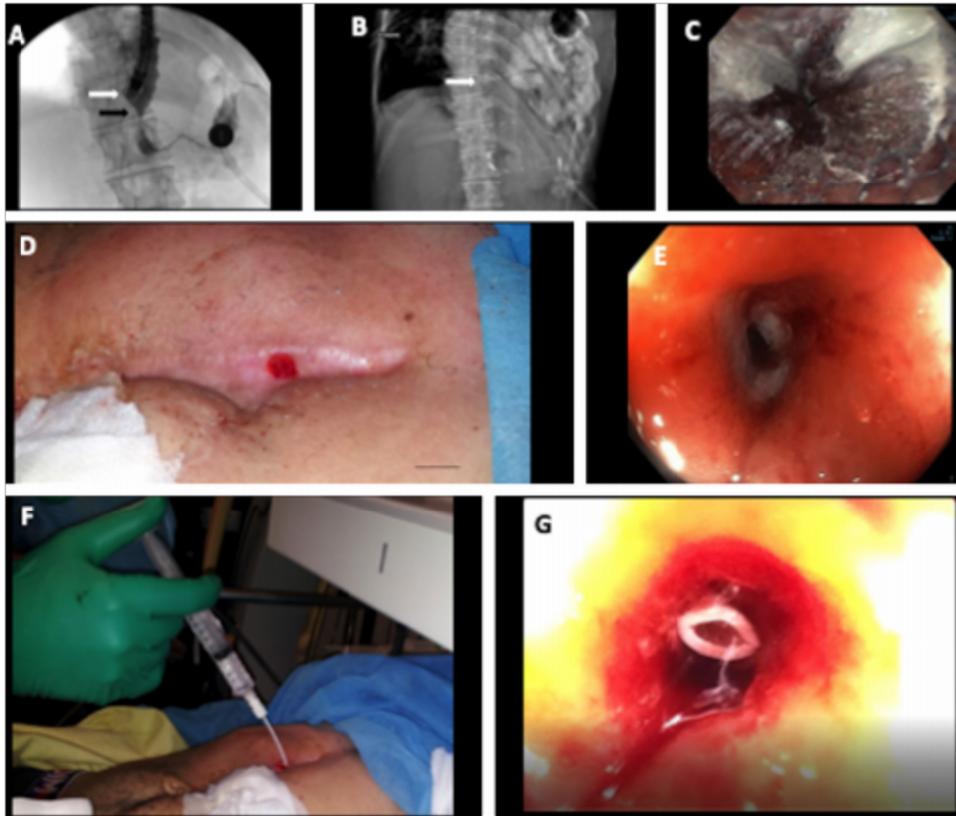


Figure 1 Treatment of a patient presenting a chronic colcutaneous fistula with a self-expansible metallic stent (SEMS) and poloxamer 407 gel as a fistula occluder. A. Fluoroscopy image showing the occlusion of the fistula after gel injection (white arrow) and the anastomotic stenosis (black arrow) just before stent placement. B. Radiological monitoring after stent placement into the stenosis (white arrow). C. Proximal part of the fully covered SEMS (endoscopic view). D. Macroscopic view of fistula external orifice. E. Endoscopic view of fistula internal orifice. F. Poloxamer 407 gel administration via a catheter positioned into the external fistula orifice G. fistula tract reaching the internal fistula orifice.

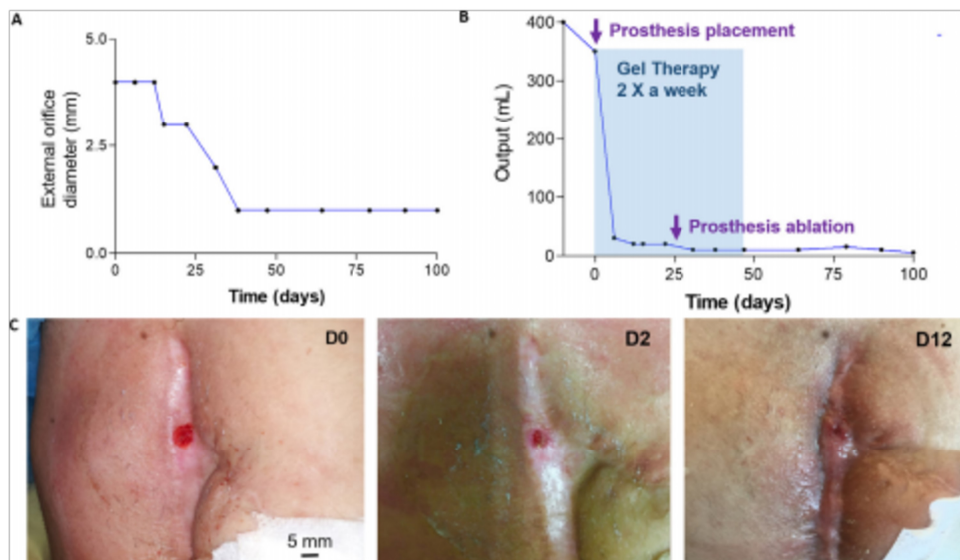


Figure 2 Partial fistula healing via stent placement and poloxamer 407 gel injection. A. Fistula external orifice diameter and B. output as a function of the time as well as its aspect at days 0 (just before therapy), 2 and 12.

expression of vascular endothelial growth factor (VEGF) and transforming growth factor-beta 1 (TGF- β 1) [6–9]. Poloxamer 407 hydrogel has been investigated for burn [10] and wound healing [7]. The Poloxamer 407 gel is expected to be eliminated via dilution with fistula secretions. Renal excretion is reported for Poloxamer 407 gel vessel occluder product (PMA P110003: FDA Summary of Safety and Effectiveness Data for Legoo®).

In conclusion, we report herein the first-in-man clinical report on the use of Poloxamer 407 gel as fistula occluder product combined with an esophageal stent placement. The treatment reduced fistula orifice diameter and fistula daily output decreased. The therapeutic outcome persisted 1 year after treatment. Importantly, Poloxamer 407 gel may benefit from a quite straightforward regulatory pathway considering that the gel has received FDA and EMA approval as a vessel occluder.

Ethics

Informed patient consent was obtained for publication of patient information.

Disclosure of interest

C.W., F.G., A.K.A.S and G.R. are co-founders and shareholder of Evora Biosciences. The other authors declare that they have no competing interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.clinre.2020.06.001>.

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Discussion

Ce cas clinique montre l'intérêt potentiel de l'utilisation d'un gel thermosensible de type Pluronic F127, comme traitement régénératif des fistules digestives. Ce gel a initialement été développé comme traitement vaso-occlusif, mais il semble également présenter des propriétés pro-cicatrisantes et mécanique occlusif [189]. L'utilisation de ce traitement chez l'homme hors indication thérapeutique peut être envisagé devant l'impasse thérapeutique en considérant les données pré-cliniques encourageantes sur l'innocuité et l'effet potentiel du gel. Ce premier résultat chez l'homme est donc prometteur, pour envisager son utilisation comme véhicule des VEs afin de potentialiser l'effet régénératif mais il est nécessaire de tester cette stratégie sur un nombre plus important de patients.

Discussion et perspectives

La gestion des fistules digestives post-opératoires est complexe et nécessite une prise en charge pluridisciplinaire, longue, associant la nutrition, la radiologie, l'endoscopie et la chirurgie. Cependant, cette approche mini-invasive visant à aider à une cicatrisation dirigée est imparfaite, avec un risque important de ré-intervention. Une chirurgie de sauvetage est parfois nécessaire lorsque les stratégies mini-invasives échouent et/ou en cas de complications mettant en jeu le pronostic vital [30]. Cette chirurgie est complexe, elle tentera de fermer la fistule et de reconnecter le tractus gastro-intestinal avec un risque de stomie définitive, de syndrome de grêle court, et de récurrence des fistules [26,30,31,124]. Les défis posés par la prise en charge des fistules post-chirurgicales ont récemment motivé l'étude de la thérapie cellulaire comme alternative mini-invasive [30,125]. Le traitement à base de CSM donne des résultats encourageants grâce à leur effet régénératif mais surtout à leur effet paracrine. Il existe actuellement un changement d'orientation en **médecine régénérative** en proposant un passage de la thérapie cellulaire à la thérapie « *sub-cellulaire* » basée sur des vésicules extracellulaires. Les VEs apparaissent comme une alternative aux CS.

Ce travail de thèse, a permis d'évaluer les effets des VEs comme traitement régénératif des fistules post-opératoires. Les premiers résultats réalisés sur un modèle connu de fistules colo-cutanées chez le rat ont permis de mieux comprendre la bio-distribution des VEs. Les résultats de bio-distribution des VEs par TEP-IRM avec marquage des VEs par [⁸⁹Zr] ont montré une meilleure distribution des VEs administrées localement. Dans un deuxième temps, nous avons développé un nouveau modèle de fistules gastro-cutanées sur SG chez le rat et le porc. Il s'agit de deux modèles proches des fistules digestives après SG chez

l'homme. Dans ces deux modèles nous avons pu créer des fistules gastro-cutanées chroniques qui restent ouvertes après 6 semaines chez le rat et 4 semaines chez le porc. Puis, nous avons évalué l'efficacité de plusieurs types de traitement par AdSC, VEs, et gel de Pluronic dans la cicatrisation des fistules gastro-cutanées post-SG chez le petit animal (rat). Il s'agit de la première étude évaluant l'efficacité et la sécurité d'utilisation des VEs issues d'AdSC humaines dans la prise en charge de fistules post-opératoires. Les meilleurs résultats thérapeutiques ont été observés pour les groupes AdSC, VEs et gel + VEs en considérant le taux et la cinétique de fermeture de la fistule ainsi que les scores d'inflammation par rapport au contrôle. L'efficacité des VEs apparaît similaire aux AdSC. Ces résultats renforcent la théorie d'un mécanisme d'action principalement paracrines des CS médié par les VEs secrétées par les CS [147]. Les résultats obtenus ont ensuite été validés chez le gros animal (porc). Enfin, nous avons réalisé une première évaluation clinique chez l'homme de l'utilisation d'un gel de pluronic F127 seul comme traitement cicatrisant dans le cadre d'une fistule colo-cutanées sur une anastomose gastro-colique post-opératoire. Ce cas clinique montre l'intérêt potentiel de l'utilisation d'un gel thermosensible de type Pluronic F127 comme traitement régénératif des fistules digestives. L'utilisation de ce traitement chez l'homme hors AMM a été envisagé devant l'impasse thérapeutique et les données précliniques encourageantes sur l'innocuité et l'effet potentiel du gel.

Une nouvelle voie d'application des VEs comme traitement préventive a récemment été évaluée dans notre laboratoire. Nous avons évalué l'intérêt des VEs couplées au Gel de pluronic F127 dans la prévention du risque de sténoses oesophagiennes post-dissections sous muqueuse chez le porc (voir annexe 3) [201]. Dans un deuxième modèle nous avons évalué l'impact des VEs sur la cicatrisation des anastomoses coliques après irradiation chez le rat (article en cours de rédaction). Dans ces deux modèles, l'application de VEs contenues dans

un gel thermosensible avait une action anti-fibrosante et anti-inflammatoire qui améliore la cicatrisation en réduisant le risque de sténoses et de fistules.

Il existe actuellement un changement d'orientation en médecine régénérative en proposant un passage de la thérapie cellulaire à la thérapie par VEs. Au-delà de leur potentiel thérapeutique et des preuves précliniques émergentes sur l'efficacité pour le traitement des fistules, les VEs présentent plusieurs avantages qui encouragent la poursuite des travaux de recherche dans ce domaine. Les traitements utilisant des CS présentent des données plus importantes dans la littérature que les VEs. Mais, les VEs présentent un statut immunitaire dit allogénique, qui permettrait une utilisation avec plus sécurité que les CS [14,16]. L'utilisation d'un traitement cellulaire par CS entraîne des contraintes de production, d'acheminement, de stockage et de délivrance. En effet, les CS doivent être viables au moment de l'administration du traitement. Aujourd'hui, il n'existe qu'un seul traitement commercialisé utilisant des AdSC humaines, il s'agit du Darvadstrocel. La principale limite à l'utilisation de la thérapie cellulaire en pratique clinique courante est le coût du traitement. La dose de Darvadstrocel contenant 120 millions de cellules coûte environ 54 000€. Ce traitement a reçu son AMM en Europe, mais son utilisation reste restreinte et limitée à quelques centres. L'utilisation des VEs pourrait réduire les difficultés de manipulation et de stockage par rapport à l'utilisation de CS [175,202,203]. De plus, les VEs pourraient être utilisées de façons plus large. En effet, elles sont constituées de bicouches phospholipidiques qui offrent la possibilité aux VEs d'être chargées de médicaments, de nanoparticules, de chimiothérapie (...) [5,160]. Les vésicules ainsi chargées peuvent libérer leur contenu dans les cellules de voisinage en fusionnant (ou étant internalisées par d'autres voies) avec la membrane cellulaire. Ces propriétés permettraient d'associer l'effet paracrine des VEs à un agent pharmacologique, comme un immunosuppresseur ou des antibiotiques afin d'améliorer la cicatrisation tissulaire. Les VE dérivées d'AdSC humain présentent un statut immunitaire dit « immunoprivilégié » permettant

théoriquement une utilisation allogénique pour faciliter le développement de ces nouveaux traitements innovants chez l'homme [175]. Cependant, plusieurs défis techniques restent à résoudre avant d'envisager l'utilisation des VEs chez l'homme. Premièrement, la plupart des méthodes de production des VEs sont chronophages et non viables pour imaginer une transposition en pratique clinique courante. Cette contrainte est en partie levée par l'élaboration dans notre laboratoire d'une technique de production des VEs à fort rendement, transposable à grande échelle, applicable à différents types cellulaires et compatible avec une production en bioréacteur de grade GMP [176]. Deuxièmement, il semble nécessaire d'établir des contrôles de la qualité des VEs afin de s'assurer de la sécurité d'utilisation des VEs en pratique clinique et de pouvoir s'assurer de la reproductibilité des caractéristiques des VEs entre les lots. Si des règles de production et d'évaluation de la qualité existent pour les traitements par AdSC, la place de la thérapie par VEs reste mal définie entre médicament biologique et thérapie cellulaire [204]. L'ensemble des résultats présentés ci-dessus permet d'appréhender les effets thérapeutiques, la bio distribution, et l'efficacité des VEs dans la prise en charge des fistules post-opératoires. Il s'agit d'un premier pas vers une transposition d'un traitement par VEs chez l'homme qui se profile en plusieurs étapes qui restent encore à franchir : une production des VEs complètement conforme aux bonnes pratiques de fabrication (GMP), la montée en échelle de ce procédé, l'élaboration d'une stratégie de contrôle qualité, la libération de lots, l'étude des mécanismes d'action, les tests précliniques réglementaires et l'approbation du dossier réglementaire notamment [23].

Conclusions

Les fistules post-opératoires sont des complications graves et complexes qui nécessitent une prise en charge pluridisciplinaire longue. Les traitements actuels sont imparfaits avec en cas d'échec une nécessité de reprise chirurgicale associée à une morbi-mortalité importante. La thérapie cellulaire et par VEs offre une nouvelle voie thérapeutique prometteuse. Les travaux présentés ci-dessus montrent l'intérêt des VEs administrées localement associées à un gel de pluronic F127. Les résultats sur un premier modèle de fistules colo-cutanées chez le rat ont confirmé, par TEP-IRM, que l'administration locale des VEs était la plus adaptée. Nous avons ensuite évalué l'effet de VEs issues d'AdSC humaines pour la prise en charge de fistules gastro-cutanées post-SG chez le rat et le porc. Les résultats ont confirmé une action anti-fibrosante et anti-inflammatoire des VEs qui améliorent la cicatrisation en augmentant le taux de fermeture des orifices de fistules. Les données précliniques encourageantes sur l'innocuité et l'efficacité des VEs issues d'AdSC humaines produites par une technique à haut rendement compatible avec une production de grade GMP, nous encouragent à envisager une utilisation chez l'homme. Ces résultats sur un modèle de rat et validé dans un modèle porcin permettront d'envisager d'avancer vers les prochaines étapes vers l'utilisation des VEs chez l'homme dans le cadre d'une étude phase 1/2.

Publications associées :

Au cours de ces 3 années de thèse, j'ai eu la chance de pouvoir participer à divers travaux de recherche. Le premier est une revue de la littérature sur l'impact de la médecine régénérative dans la prise en charge des fistules digestives (Voir annexe 2). Le deuxième est l'illustration de l'application des VEs comme traitement préventif du risque de sténose oesophagienne post dissection sous muqueuse (Voir annexe 3).

Références des publications citées ci dessus :

[IF: 15.47] Sebbagh, A; Rosenbaum, B; Péré, G; Alric, H; Berger, A; et al. **Regenerative medicine for digestive fistulae therapy: Benefits, challenges and promises of stem/stromal cells and emergent perspectives via their extracellular vesicles.** Advanced Drug Delivery Reviews 2021

[IF: 6,895] Elise Coffin , Alice Grangier , Guillaume Perrod , Max Piffoux , Iris Marangon, Imane Boucenna, Arthur Berger et al. **Extracellular vesicles from adipose stromal cells combined with a thermoresponsive hydrogel prevent esophageal stricture after extensive endoscopic submucosal dissection in a porcine model.** Nanoscale, 2021

Annexes

Annexe 1 : Tableau résumé des différents modèles de fistules digestives publiées chez l'animal.

| Animal model | Fistulous tract | Underlying inflammation | Modeled pathology | Reference(s) |
|---|-----------------------------|-------------------------|---|--------------|
| Pigs | Fistula-in-ano | No | Cryptoglandular/iatrogenic fistula-in-ano | [205–208] |
| Pigs | Fistula-in-ano | No | Cryptoglandular/iatrogenic fistula-in-ano | [209] |
| Pigs | Fistula-in-ano | No | Cryptoglandular/iatrogenic fistula-in-ano | [210] |
| Rats | Fistula-in-ano | No | Cryptoglandular/iatrogenic fistula-in-ano | [211–213] |
| Rabbits | Fistula-in-ano | No | Cryptoglandular/iatrogenic fistula-in-ano | [214] |
| Rabbits | Fistula-in-ano | No | Cryptoglandular/iatrogenic fistula-in-ano | [215] |
| SAMP1/YitFc mice | Fistula-in-ano | Yes | Perianal fistulizing Crohn's disease | [216] |
| Dogs with canine anal furunculosis | Fistula-in-ano | Yes | Perianal fistulizing Crohn's disease | [217] |
| Rats with TNBS-induced chemical colitis | Fistula-in-ano | Yes (chemical colitis) | Perianal fistulizing Crohn's disease | [218] |
| Rabbits | Rectovaginal fistula | No | Obstetric/iatrogenic rectovaginal fistula | [219,220] |
| Dogs | Rectovaginal fistula | No | Obstetric/iatrogenic rectovaginal fistula | [221] |
| Pigs | Rectovaginal fistula | No | Obstetric/iatrogenic rectovaginal fistula | [222] |
| Rats | Enterocutaneous fistula | No | Post-surgical fistula | [223] |
| Mice / Rats | Enterocutaneous fistula | No | Post-surgical fistula | [224–226] |
| Rats | Enterocutaneous fistula | No | Post-surgical fistula | [155] |
| Rats | Enterocutaneous fistula | No | Post-surgical fistula | [227] |
| Rabbits | Enterocutaneous fistula | No | Post-surgical fistula | [153] |
| Rabbits | Enterocutaneous fistula | No | Post-surgical fistula | [228] |
| Pigs | Enterocutaneous fistula | No | Post-surgical fistula | [175,229] |
| Dogs | Colovesical fistula | No | Diverticulitis-related fistula | [230] |
| Rats | Gastrocolic fistula | No | Post-surgical fistula | [231] |
| Rats | Internal intestinal fistula | No | Post-surgical fistula | [232] |

| | | | | |
|----------------------|---------------------------|-----------------------------|---|-------|
| | with peritonitis | | | |
| Rats | Internal duodenal fistula | No | Post-surgical fistula | [233] |
| Immunodeficient mice | Enterocutaneous fistula | Yes (lymphocytic enteritis) | Enterocutaneous Crohn's-related fistula | [234] |

Annexe 2 : Sebbagh, A; Rosenbaum, B; Péré, G; Alric, H; Berger, A; et al. Regenerative medicine for digestive fistulae therapy: Benefits, challenges and promises of stem/stromal cells and emergent perspectives via their extracellular vesicles. *Advanced Drug Delivery Reviews* 2021



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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.

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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 patients will have to live with a permanent diverting stoma [9] with a significant impact on their quality of life. Moreover, especially in Crohn's disease, the recurrence of previously healed fistulae constitutes a common occurrence [10,11]. These poor outcomes associated with gastrointestinal fistulae have prompted continued experimental and clinical research for the development of innovative treatment approaches, notably based on stem cell therapy, so as to add effective new alternatives to current management algorithms.

Stem cell therapy has brought along tremendous hope in the medical community as a potential solution for the treatment of a wide range of complex pathologies. Due to the ethical and safety issues linked with the use of pluripotent stem cells [12] many clinical studies have focused on the use of adult stem cells, such as stromal cells (SCs), a heterogeneous cell population also known as mesenchymal stem cells. SCs are adult, non-hematopoietic, multipotent cells that can differentiate into several cell types – such as osteoblasts, chondroblasts, adipocytes, tenocytes, etc. They are further characterized by an adherent, spindle-shaped morphology, along with the expression of surface markers such as CD73, CD90 and CD105 and the absence of expression of markers of the hematopoietic lineage (CD45, CD19, CD14, CD11b, CD79 α , HLA-DR, etc) [13]. SCs were historically isolated from the bone marrow. Other sources notably include umbilical cord blood, Wharton's jelly, tendons, the synovial membrane, the periosteum, etc. Of particular interest for regenerative medicine, a population of SC-like cells called adipose-derived stromal cells (AdSCs) can easily be harvested at high yields from the adipose tissue through liposuction [14]. Initially, the therapeutic effects of SCs were mostly attributed to their differentiation potential [14] thus providing a replacement for compromised functional cells through homing to the injured tissues. However, the prominent interest of SCs for regenerative medicine has rapidly gone beyond their ability to differentiate into

multiple cell types, with the preclinical and clinical demonstration of their propensity to promote endogenous wound healing and angiogenesis, along with their immunomodulatory properties and anti-fibrotic effects (Fig. 1, [15–34]). Moreover, the results from several animal studies and clinical trials led to challenge the assumption of a direct contribution of SCs to tissue regeneration. On the one hand, it was shown that only very few SCs would durably engraft at the injury site [29,35]. Indeed, after systemic administration, most of the injected cells become entrapped in the pulmonary capillaries [29,35,36], and the presence of an inflammatory environment at the injury site is highly deleterious to stem cell survival [29]. On the other hand, the therapeutic effect of SCs often seemed to occur in an overly short time frame to be solely attributed to their direct differentiation into functional cells [35]. These preclinical results, along with the observation of equivalent therapeutic effects with SC-conditioned medium, prompted a change of paradigm in stem cell therapy towards mostly paracrine mechanisms of action [37].

Amongst all the potential effectors for these paracrine effects, SC-derived extracellular vesicles (EVs) have gained increasing interest for regenerative medicine in recent years. EVs correspond to a heterogeneous group of subcellular entities that are secreted by cells in a constitutive or induced manner [38,39]. They are outlined by a membrane and are usually of a nanometric scale (40–5000 nm). Several subsets of EVs have been described depending on their biogenesis pathway, like exosomes, which stem from the endosomal network and are released upon fusion of late endosomes with the plasma membrane; microvesicles, which are formed by outward budding and rupture of the plasma membrane; or apoptotic bodies, resulting from cell blebbing during programmed cell death [38,39]. However, it is now recommended to favor a classification of EVs based on parameters such as size or biochemical composition, which are technically easier to assess [40]. EVs carry lipids, proteins, mRNAs, miRNAs, and their contents depend both on their parent cells and on their method of production [38,39]. Physiologically, EVs serve as mediators for intercellular communication [38,39]. SC-derived EVs have demonstrated anti-apoptotic properties, along with a propensity to increase cell survival through the activation of the PI3K/Akt pathway, and to promote cell proliferation and wound healing [41–43]. They have also demonstrated anti-inflammatory, immunomodulatory and anti-fibrotic properties (Fig. 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

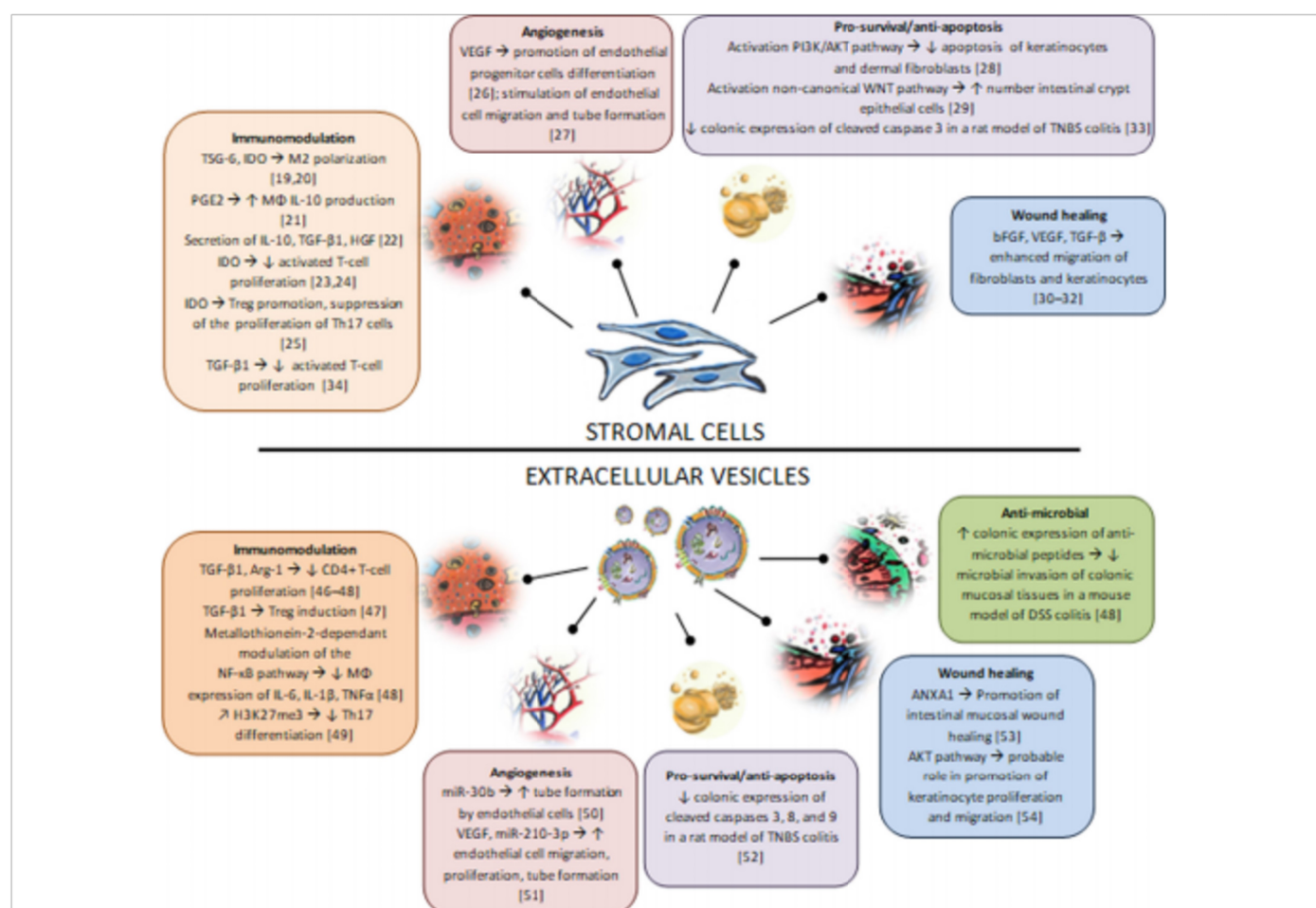


Fig. 1. Some of the putative mechanisms of action of stromal cells and extracellular vesicles of interest for digestive fistula therapy. TSG-6: tumor necrosis factor-stimulated gene 6; IDO: indoleamine 2,3-dioxygenase; PGE2: prostaglandin E2; MΦ: macrophage; IL-10: interleukin 10; TGF-β: transforming 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.

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 Fig. 2.

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]. 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 anasto-

mot 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

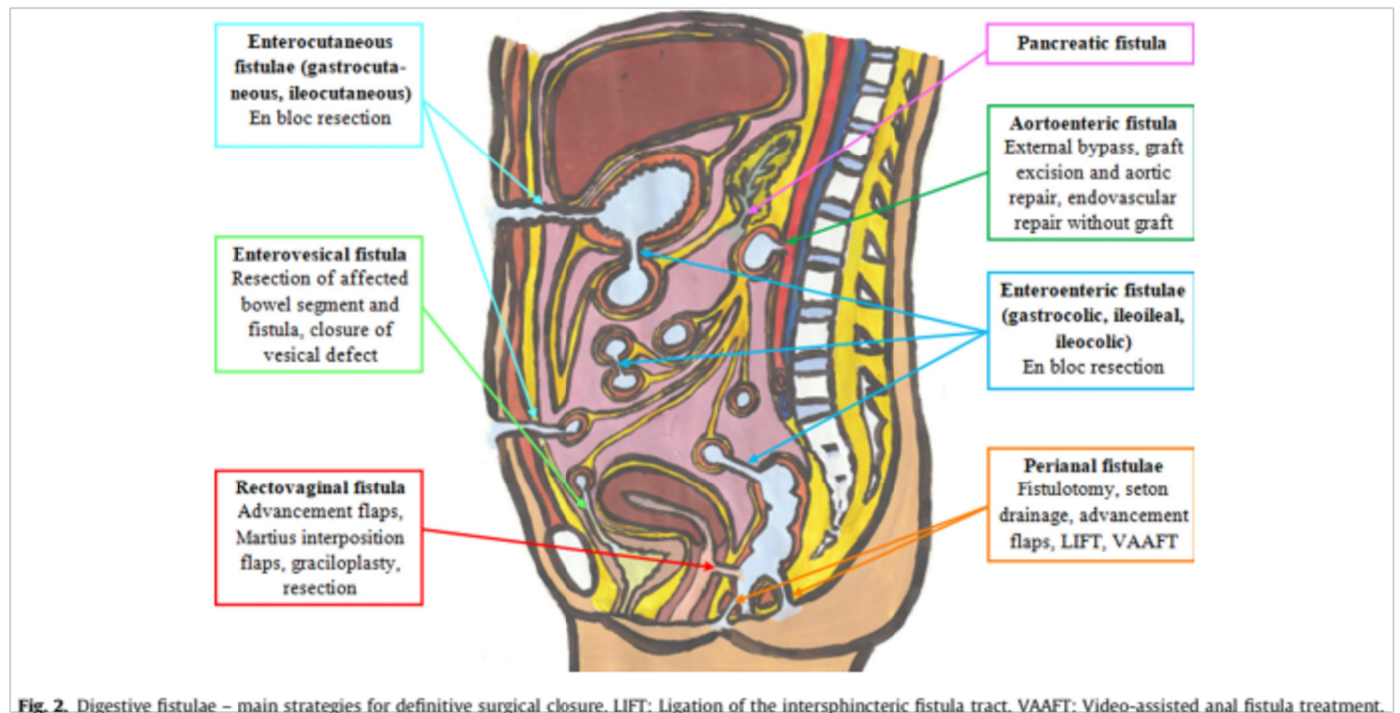


Fig. 2. Digestive fistulae – main strategies for definitive surgical closure. LIFT: Ligation of the intersphincteric fistula tract. VAAFT: Video-assisted anal fistula treatment.

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].

In 15–25% 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 multi-factorial 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 (Fig. 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].

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

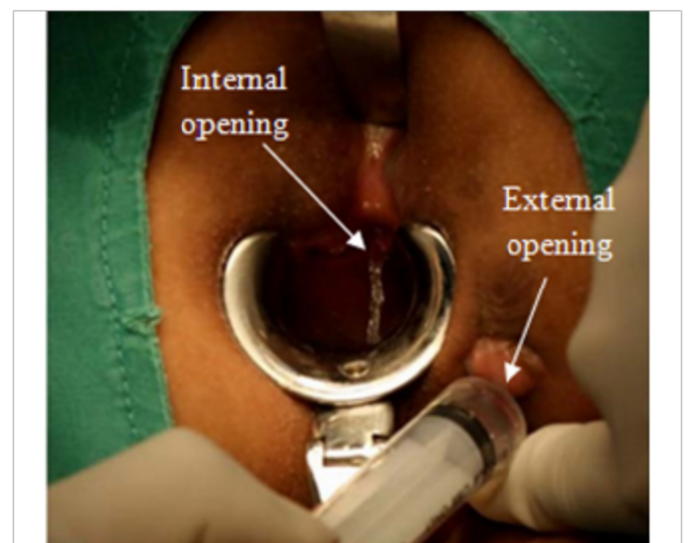


Fig. 3. Perianal fistula. The injection of water, saline or dyes through the external opening facilitates the identification of the internal opening. Adapted with permission from Springer, *Techniques in Coloproctology* "LIFT procedure: a simplified technique for fistula-in-ano". A. Rojanasakul ©2009.

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 4–6 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]. Other biological agents, such as ustekinumab (anti-IL12 and IL23) or vedolizumab (anti-integrin $\alpha_4\beta_7$) are currently under exploration for fistulizing Crohn's disease, however there is still insufficient evidence to recommend their use in this indication [86].

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

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

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

Due to the high morbidity and mortality rates still observed with post-surgical fistulae, preventing their development constitutes a very attractive treatment strategy [5,73].

In this regard, the potential of stem/stromal cells to improve colonic anastomoses healing has been studied in rat models, however a preventive effect of stem cells on the development of anastomotic fistulae could not be found, though the low basal rates of post-surgical fistulae incidence in those models might have biased the results [93].

Some better results were obtained in experimental studies of per-operative local cell sheet placement to prevent post-surgical pancreatic fistulae development [94–96]. Rat models of post-

surgical pancreatic fistulae were established by splenic pancreatic duct transection [94,95] or gastric and splenic pancreatic ducts division with distal pancreatectomy and splenectomy [96]. One study investigated sheets formed with 1 million rat myoblasts on thermoresponsive culture plates [94]. Another compared murine AdSC multilayered sheets in the presence or absence of mannose, formed with 0.5 million magnetically-labeled AdSCs in a collagen/Matrigel solution [95]. A third study compared rat AdSC and bone marrow-derived stromal cell (BM-SC) sheets, formed with 1.1 million cells on thermoresponsive culture plates [96]. With cell sheet treatment, less pancreatic leakage was observed in the early post-operative course, as evidenced by intra-abdominal fluid volume [96] and ascitic pancreatic amylase and lipase concentrations [94,95]. It also led to less associated intra-abdominal damage [94,95], less inflammatory cell infiltration [94–96], an increased tissue regeneration [96] and better survival at 1 month [94]. A different cell source [96] or the adjunction of mannose [95] did not significantly alter these results. Moreover, a control experiment using collagen sheets without cells enabled to exclude a mere physical effect as the mechanism of action of the cell sheets [95].

Overall, these 3 studies tend to show some beneficial effects of cell sheets for the prevention of post-operative pancreatic fistula development. However, as underlined by Kim *et al.* [96] the experimental models used in those studies were not completely representative of the clinical situation. Therefore, further studies with a longer follow-up in more clinically relevant, large animal models are needed before an eventual clinical translation.

The potential of stem/stromal cell therapy has also been studied in experimental models of enterocutaneous fistulae, this time as a curative treatment modality [97–100]. These experimental models, due to the absence of underlying pathology, were more representative of fistulae of post-surgical origin. In spite of extensive differences in terms of protocol, all 4 studies evidenced some promising results.

A first study, in a rat model of enterocutaneous fistula established by performing a 5 mm enterotomy on the caecum and suturing it to the abdominal wall, investigated the local administration of 1 million human AdSCs either through local injection or through their adhesion to the suture material used [97]. At 21 days post-administration, the area of the fistula had decreased considerably in both groups treated with cells, especially in the suture group, compared to the control; however this was only evidenced by image analysis of successive photographs of the external opening, therefore these results, though promising, still remain very preliminary. In another study, gastrocutaneous fistulae were created in rabbits by gastrotomy, and, after two hours for leakage formation, the insertion of a tube in the lesion as a drain. 2 weeks later, the drain was removed and autologous stromal vascular fraction (SVF, containing AdSCs) was injected into the fistula tract [98]. At 3 weeks post treatment, examination of the external openings and histological analysis of the fistula tract evidenced patent tracts with draining, inflamed external openings in the untreated control group and near-complete healing in the SVF group. However, this study presents a limited precision preventing a fully objective comparison between both groups (for instance, the mean percentage of tract closure is given for each group without any indication on intra-group variability). A more thorough study evaluated the potential of human BM-SC sheets in a murine model of enterocutaneous fistulae created by caecostomy [99]. On the third post-operative day, bi-layered sheets, containing 2 million cells either labeled with both magnetic nanoparticles and a fluorescent dye or unlabeled, were positioned into the fistula orifice. 2 weeks after transplantation, clinical evaluation, MRI, histological analysis and probe-based confocal laser endomicroscopy results indicated better healing in both the labeled and unlabeled sheet groups compared to the control with less inflammation and fibrosis,

enhanced microvascularisation, a lower fistula surface and a decreased maximal size of the fistulous orifice. The mechanism of action of the sheets was investigated by following the fate of labeled cells post transplantation and analyzing the expression of diverse signaling molecules, suggesting an indirect up-regulation of host trophic factors rather than a direct contribution of cells to fistula repair. Such experiments unraveling the fate and mechanism of action of the administered stem cells will be crucial in the long run for clinical translation. A fourth study evaluated the benefits of local injection of allogeneic rat AdSCs (4 million cells) in the perifistular tissues in a similar model of caeco-cutaneous fistula in the rat 4 weeks after fistula creation [100]. The control group was injected with a similar amount of saline instead. One month after treatment, 6 fistulae out of 16 had closed in the AdSC group against 1 out of 16 in the control group ($p = 0.033$); and no deaths or treatment-related adverse effects were reported in either group. However, these clinical observations were not completed by either radiological or histological analyses of the fistulous tract.

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

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

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

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

warranting further investigation to confirm them at a larger scale in randomized, placebo-controlled clinical trials.

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

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

Though the potential of EVs as a possible alternative to stem/stromal cell therapy has been extensively investigated for a wide array of diseases, to the best of our knowledge, only two studies so far have described their use as a treatment for post-surgical digestive fistulae [57,58]. In the first of them, esophagocutaneous fistulae were induced in otherwise healthy pigs by inserting plastic stents between the oesophagus and the skin [57]. On the 30th post-operative day, the pigs were treated with either allogeneic AdSC-derived EVs (1.3×10^{11} EVs/mL) embedded in the thermoresponsive hydrogel poloxamer 407 or poloxamer 407 alone. The use of this hydrogel, whose sol/gel transition occurs near 20 °C, enables an injection in liquid form to fill homogeneously the potentially irregular fistula tracts and a swift gelation upon administration to retain the EVs within the fistula tract. Clinical, endoscopic, radiological and histological evaluations on the 45th post-operative day evidenced better healing in the gel and gel + EVs groups compared to the untreated control group; with patent fistula tracts and necrosis in all fistulae of the control group ($n = 6$), 3 completely closed tracts, 1 partially closed tract and 2 thin tracts in the gel group, and 5 fistulae out of 5 demonstrating complete closure in the gel + EVs group. Of note, a decrease in inflammatory cell infiltration and in fibrosis along with increased wound healing and angiogenesis were observed in the treatment groups compared to the control, but this was significant only for the gel + EVs group. Overall, this study provided the first *in vivo* evidence of the interest of local EV administration for the treatment of post-surgical digestive fistulae. A second study in a rat model of post-surgical enterocutaneous fistulae created by caecostomy tended to confirm these results with a decrease in fistula diameter and drainage after administration of 6×10^{11} murine C3H/10T1/2- derived EVs in poloxamer 407 compared to poloxamer 407 alone or saline [58]. A decrease in fibrosis and inflammation along with an increased angiogenesis could also be evidenced histologically. Moreover, a biodistribution study over 7 days after radiolabeling with [^{89}Zr]

zirconium evidenced that local EV administration within poloxamer 407 enabled a better retention of EVs at the fistula site than either local injection in saline or intravenous administration [58]. However, further studies are warranted to replicate these promising results with higher numbers of animals and longer follow-up periods. Notably, a more thorough investigation of the mechanism of action of EVs should be performed before any attempts at a potential clinical translation. Additionally, it would be interesting to vary the amount of EVs administered so as to study a potential dose/response relationship, to compare the therapeutic potential of EVs with that of parent cells in this indication, or to further investigate potential links between mode of delivery, EV retention within the fistula tract and therapeutic effect.

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

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

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

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

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

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

Finally, a mouse model of enterocutaneous fistula was established in 2019 by the subcutaneous xenotransplantation of fetal

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

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

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

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

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

In 2011, a pilot phase I/II clinical trial of multiple BM-SC injections for the treatment of perianal and enterocutaneous

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 (1x) | 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 (1–2x) | 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. | N.A. | 6** | 1 | 1 out of 7 | 0 (1 out of 3**) | | |
| 2 case series | ECF | AdSC (n = 2) | Autologous | Local + FG (1x) | 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 (2–5x) | 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 (1x) | 1–4 × 10 ⁹ /mL | None | None | 3 out of 10 | 0 | 8 weeks ± 6 months | [120] |
| Phase I/IIa | PF | AdSC (n = 24) | Allogeneic | Local (1–2x) | 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 (1–2x) | 3–6 × 10 ⁷ /cm fistula length ± 1.5 x 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 (1x) | 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 | | |
| | | Control (n = 6) | N.A. | N.A. | N.A. | None | None | 3 PF out of 9 | 0 | | |
| Pilot | PF | AdSC (n = 6) | Allogeneic | Local + FG (1x) | 1–3 × 10 ⁹ /mL | None | None | 3 out of 6 | 0 | 8 months | [127] |
| Phase 3 RCT* | PF | AdSC (n = 107) | Allogeneic | Local (1x) | 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. | N.A. | 30 (27) | 7 (7) | 36 out of 105 | 0 (15/34) | | |
| Phase I/IIa | RVF | AdSC (n = 10) | Allogeneic | Local (1–2x) | 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 (1x) | 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 (1x) | 1–2 × 10 ⁶ | None | None | 10 fistulae out of 11 | 0 | 21–37 months | [131,132] |
| Phase I | PF | SVF (n = 10) | Autologous | Local + adipose tissue (1x) | 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 (1x) | 3.5 × 10 ⁷ | None | None | 0 out of 5 | 0 | 6 months | [103] |
| RCT | PF | AdSC (n = 11) | Autologous | Local + serum (1x) | 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 | | |

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

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

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

Most studies have reported a good safety profile of local stem/stromal cells administration for the treatment of fistulizing Crohn's disease (Table 1, [102,103,114–136]). In particular, the ADMIRE-CD trial reported treatment-related adverse events in 18 out of 103 patients included in the safety analysis in the AdSC group (17%) versus 30 out of 102 in the control group (29%), including 5 cases of serious treatment-related adverse events in the AdSC group against 7 in the control, by week 24 [130]. After 52 weeks of follow-up, a total of 21 treatment-related adverse effects had been described out of 103 patients (20.4%) in the AdSC group versus 27 out of 102 (26.5%) in the control, including 7 cases of serious

treatment-related adverse events in each group [140]. One of the greatest concerns with stem/stromal cell therapy is neoplasia development. Biopsies taken from the injection area of 5 patients included in a phase II clinical trial of autologous AdSC and fibrin glue injection for the treatment of complex perianal fistulae [115] showed no evidence of neoplastic tissues up to 2 years after treatment [143] and similarly, no signs of cytologic transformation were observed in biopsies taken at 7 months and 1 year from treatment with autologous AdSCs of, respectively, an enterocutaneous fistula and a rectovaginal fistula in the initial phase I trial of 2005 [114]. However, following allogeneic BM-SCs injections for the treatment of refractory perianal fistulizing Crohn's disease, 2 cases of neoplasia were reported: a cecal adenocarcinoma with peritoneal carcinomatosis >15 months after the intervention [126] and a rectal Epstein-Barr virus-associated B-cell lymphoproliferative disease 4 years after the intervention [144]. In the first case, a family predisposition was suggested while for the rectal lymphoproliferative disease, it was imputed to Crohn's disease and prolonged immunosuppressive treatments without totally excluding a potential contribution of allogeneic BM-SC injection. Recently, a case series gave some first encouraging insights on the safety profile of SC therapy in terms of fertility and pregnancies [145].

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

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

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

Evidence from small animal models tends to indicate a potentially beneficial effect of systemic, intra-peritoneal or local stem cells-derived EVs administration for luminal Crohn's disease [52,150,151]. Thus, in view of emerging experimental data on the use of EVs for post-surgical gastrointestinal fistula treatment [57,58], we consider that EV therapy for perianal and non-perianal fistulizing Crohn's disease could constitute an interesting future preclinical research axis.

6. Limits, challenges and perspectives for clinical translation

6.1. Defining the optimal cell source, formulation and administration protocol – an ongoing challenge

As of today, the question of the optimal formulation and administration protocol for stem/stromal cell therapy products for post-surgical and/or Crohn's-associated digestive fistula treatment remains to be solved. Indeed, though clinical trials have investigated a variety of potential cell sources (adipose-derived versus bone marrow-derived, autologous versus allogeneic, with or without expansion, etc), no controlled trial has formally compared them with one another to investigate potential differences in terms of safety and/or efficacy. In the absence of such evidence, other considerations, such as availability, number and cost of procedures, should be taken into account for clinical studies or compassionate

use programs. AdSCs can usually be obtained at high yields through liposuction [14]; however, this might not be feasible in some patients with insufficient levels of adipose tissue. The procedure to harvest BM-SCs, requiring bone marrow aspiration, is classically more invasive [14]. While autologous stem/stromal cells are usually considered safer, results from clinical trials of allogeneic ones administered 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 [152]. 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 [134,135,153].

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 [154] 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 [36,155]. 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 [156]. It has shown a good safety profile but a limited efficacy as a stand-alone treatment for gastrointestinal fistulae [3,73,87,157]. 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 [87,157], 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 [154,158]. 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 (Fig. 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 [154]. 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.

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

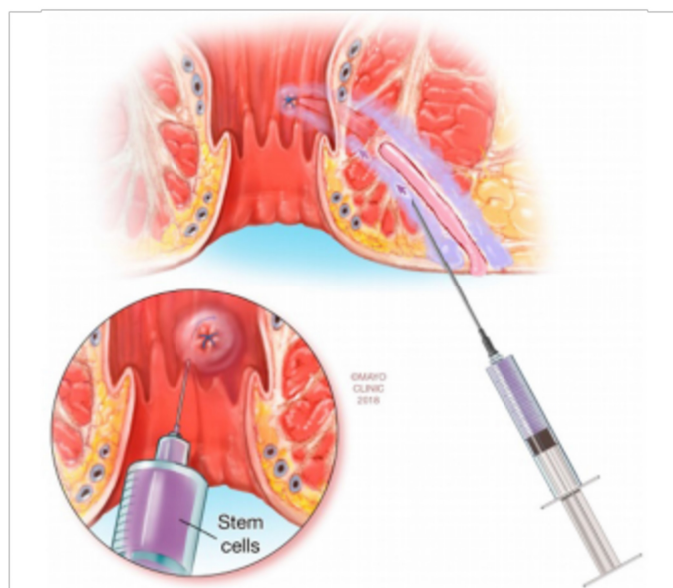


Fig. 4. Stem cell application to the internal orifice and fistula tract walls according to standardized protocols. Reprinted with permission from Springer, [152]   2018.

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 [57,159,160]. 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) [160] 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 Competing 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.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.addr.2021.113841>.

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Annexe 3 : Elise Coffin , Alice Grangier , Guillaume Perrod , Max Piffoux , Iris Marangon, Imane Boucenna, Arthur Berger et al. **Extracellular vesicles from adipose stromal cells combined with a thermoresponsive hydrogel prevent esophageal stricture after extensive endoscopic submucosal dissection in a porcine model.** Nanoscale, 2021



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Extracellular vesicles from adipose stromal cells combined with a thermoresponsive hydrogel prevent esophageal stricture after extensive endoscopic submucosal dissection in a porcine model[†]

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In this study, we investigated the combination of extracellular (nano) vesicles (EVs) from pig adipose tissue-derived stromal cells (ADSCs) and a thermoresponsive gel, Pluronic® F-127 (PF-127), to prevent stricture formation after endoscopic resection in a porcine model. ADSC EVs were produced at a liter scale by a high-yielding turbulence approach from ADSCs 3D cultured in bioreactors and characterized in terms of size, morphology and membrane markers. The thermoresponsive property of the PF-127 gel was assessed by rheology. The pro-regenerative potency of ADSC EVs was investigated *ex vivo* in esophageal biopsies under starvation. *In vivo* tests were performed in a porcine model after extended esophageal endoscopic mucosal dissection (ESD). Pigs were randomized into 3 groups: control ($n = 6$), gel ($n = 6$) or a combination of 1.45×10^{12} EVs + gel ($n = 6$). Application of gel ± EVs was performed just after ESD with a follow-up finalized on day 21 post-ESD. There was a trend towards less feeding disorder in the EV + gel group in comparison with the gel and the control groups (16.67% vs. 66.7% vs. 83.33%, respectively) but without reaching a statistically significant difference. A significant decrease in the esophageal stricture rate was confirmed by endoscopic, radiological and histological examination for the EV + gel group. A decrease in the mean fibrosis area and larger regenerated *muscularis mucosae* were observed for the EV + gel group. In summary, the application of EVs + gel after extended esophageal endoscopic resection succeeded in preventing stricture formation with an anti-fibrotic effect. This nano-therapy may be of interest to tackle an unmet medical need considering that esophageal stricture is the most challenging delayed complication after extended superficial cancer resection by endoscopy.

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Introduction

Extracellular vesicles (EVs) are membrane-enclosed sub-cellular nano-entities, mainly from 50 to 300 nm, that constitute a far-reaching intercellular communication system.¹ EVs, especially from stromal cells (SCs), are currently reported to contribute to tissue repair and homeostasis by modulating cell recruitment, proliferation, migration, and differentiation.^{2–4} ADSC EVs have been increasingly considered an alternative to cells for regenerative medicine in a paradigm shift with cell therapy evolving to sub-cellular (EV) therapy.^{5,6} Previous reports evidenced the beneficial effect of ADSC EVs in the therapy of liver, heart, kidney, skin and brain injuries.^{6–11} The SC EV therapy holds the promise to mitigate the risks of uncontrolled cell differentiation, replication, or vascular occlusion while offering storage and shelf-life gains.^{12,13} Besides, the immune-privileged status of SC EVs may enable us to consider “off-the-shelf” nano-therapy in an allogeneic approach.^{14,15}

Due to their promising features, EVs may represent innovative biogenic nano-therapies for tackling the current unmet needs in regenerative medicine. In this regard, we focus herein on esophageal stricture, which is a complication following extensive endoscopic submucosal dissection for the management of esophageal cancer. Esophageal cancer is the 6th cause of malignancy-related death in the world.¹⁶ The high morbidity/mortality rate related to surgical resection¹⁷ prompted the development of minimally invasive techniques to remove superficial cancer by the endoscopic procedure. Endoscopic submucosal dissection (ESD) represents the most recent technique allowing *en bloc* resection regardless of the lesion size.¹⁸ Stricture rates after extensive esophageal ESD range from 66% to 100%.^{19,20} Extensive ESD, defined by resection of more than $\frac{3}{4}$ of the esophageal circumference, is considered the main risk factor for esophageal stricture. Deep tumor invasion beyond the epithelium (*i.e.* T1a tumors according to the Japanese classification of superficial tumors) is the second main risk factor for a stricture occurrence.²¹

The pathogenesis of esophageal stricture is orchestrated by immune cells and myofibroblasts. The loss of the epithelium barrier function following ESD triggers immune cell recruitment, resulting in an infiltrating inflammatory process.²² In a later phase, fibroblasts and pericytes transform into α -smooth actin-expressing myofibroblasts responsible for fibrosis.²³ Fibrosis in the submucosa is related to muscularis mucosae destruction and muscularis propria infiltration, leading to esophageal strictures.²⁴

In the case of esophageal stricture after extended ESD, the first-line therapy is endoscopic balloon dilations. Despite a high success rate, there are several limitations, listed as follows: (i) a high number of balloon dilations from 6 to 20, (ii) several procedures under general anesthesia, and (iii) the risk of severe complications such as bleeding, perforation and mediastinitis.^{25–27} There are no satisfactory therapies for post-esophageal ESD stricture prevention. Oral, intravenous, and local injections of steroids were tested in this regard without satisfactory results in terms of stricture prevention,²⁸ and

severe adverse events have been reported such as abscesses.²⁹ Different endoscopic shielding techniques have been investigated and failed to prevent esophageal strictures.³⁰ Therefore, there is a strong need for an efficient esophageal stricture prevention therapy.

Regenerative medicine is a new promising field and may represent an alternative for stricture prevention therapy. For instance, a previous report showed promising results in terms of post-ESD stricture prevention after cell sheet transplantation using oral epithelial cells.³¹ Our team demonstrated that a double cell sheet of ADSCs allowed a reduction of esophageal stricture and fibrosis in a porcine model.³² In fact, SCs are competent to elicit immunosuppressive and anti-inflammatory effects, which may be mediated by a paracrine effect.³³ In this regard, many recent studies have pointed out the role of EVs in this paracrine effect.³⁴ Our aim herein was to investigate the effect of ADSC EV nano-therapy on the prevention of esophageal stricture.

To facilitate EV delivery into the esophagus, we used a thermoresponsive gel (Poloxamer 407 or Pluronic® F-127, PF-127)³⁵ containing EVs. This thermoresponsive gel composed of nano-micelles is expected to allow minimally invasive injection in the liquid state at <20 °C and gelation *in situ* at body temperature to favor EV residence time in the site of interest.³⁶ Besides being a delivery vehicle, PF-127 seems to be beneficial for wound healing by recruiting some factors like VEGF and TGF β 1 and increasing the density of micro-vessels, collagen level and rate of formation of granulation tissue.³⁷ Our team previously evidenced the therapeutic effect of ADSC EVs and PF-127 gel for esophageal fistula healing in a porcine model.³⁸ The aim of this study was to evaluate the effect of allogeneic porcine ADSC EVs in combination with the PF-127 gel to prevent esophageal strictures after an extensive ESD in a clinically relevant porcine model. ADSC EVs were produced at a liter scale by a high-yielding turbulence approach in bioreactors³⁸ from cells in 3D culture and administered with the gel by endoscopy in a minimally invasive strategy (Fig. 1). In this study, endoscopic, radiological and histological analyses were performed. The degree of esophageal stenosis on day 21 (D21) assessed by a blinded radiological examination was considered the primary endpoint.

Experimental section

Production and characterization of thermoresponsive PF-127 gels

Preparation of PF-127 gels. PF-127 was dissolved at a concentration of 20% (weight/weight) in a saline solution under stirring for 24 hours at 4 °C. Then, sterilizing filtration (0.22 μ m filter) was carried out at 4 °C.

Characterization of PF-127 gels. Rheological studies were performed for the PF-127 hydrogels with a Physica RheoCompass MCR 302 (Anton Paar) using a cone and plate geometry (diameter = 50 mm, cone angle = 1°) and a solvent trap to prevent evaporation. The measurements of G' and G'' ,



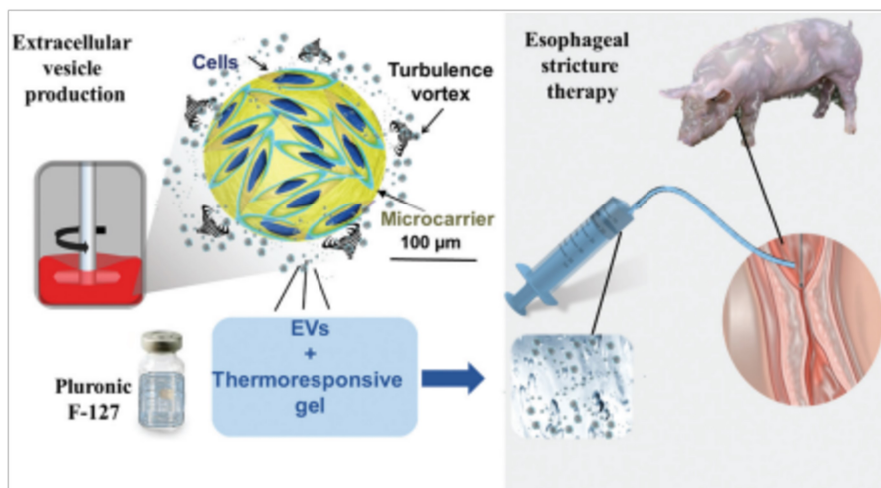


Fig. 1 Schematic representation of the experimental approach consisting of producing EVs by turbulence stimulation from swine ADSCs cultured on microcarriers in bioreactors. ADSC EVs were combined with the Pluronic F-127 thermoresponsive hydrogel and administered at $<19\text{ }^{\circ}\text{C}$ (below the gel transition temperature) at post-resection esophageal lesion gelling *in situ* at body temperature.

the elastic and viscous moduli, respectively, as a function of temperature, were performed. The temperature was gradually increased by $5\text{ }^{\circ}\text{C min}^{-1}$ starting from $5\text{ }^{\circ}\text{C}$ to $40\text{ }^{\circ}\text{C}$. The sol-gel transition temperature, cross-over between G' and G'' , was investigated.

ADSC culture, EV production and EV characterization

ADSC culture in 2D flasks. EVs were produced from pig ADSCs (CD105+, CD90+, CD45-, CD31- from ABCell-Bio, Paris, France). First, ADSCs were grown in 2D flasks under standard culture conditions ($37\text{ }^{\circ}\text{C}$ and 5% CO_2) in a culture medium comprising alpha modified Eagle's medium (alpha MEM), GlutaMax, 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin (PS). When cells had reached about 80% confluence, they were detached with PBS and trypsin and then centrifuged for 5 min at 300g. After centrifugation, the supernatant was removed and the cells were resuspended in 50 ml of complete medium.

ADSC EV production in 3D cell culture by turbulence stimulation. A 3D cell culture was performed in a 1 L spinner flask bioreactor containing Cytodex 3 beads of about $200\text{ }\mu\text{m}$ diameter, to which complete alpha MEM GlutaMax culture medium was added. Cells were inoculated at a ratio of 5:1 cells per bead and subjected to 24 cycles of 45 min, followed by 3 min of agitation within the bioreactor (at 30 rotations per minute) to allow a homogeneous distribution of cells on beads. Cells were left in culture until they were confluent (approximately 7 days) with a daily check of cell distribution on beads and cell density. In total, 30 to 70% of the culture medium was changed every 2–3 days according to the level of confluence. Four washes with serum-free DMEM were then performed, and then cells on beads were placed in serum-free DMEM + 1% PS without phenol red. The spinner flask bioreactor containing a magnetic impeller was then placed on a magnetic stirring plate. The stirring intensity was set to obtain

a diameter of the smallest vortex of $35\text{ }\mu\text{m}$ for 4 hours to generate turbulence shear stress stimulation.³⁹ After that, cellular debris of the supernatant was removed by centrifugation at 2000g for 10 min. EVs were then ultracentrifuged at 110 000g for 1 h, 10 min to obtain an EV pellet.

Cell viability and detachment analyses. A Nucleocounter NC-200 (Chemometec A/S, Allerød, Denmark) device was used for cell viability and detachment analyses with calibrated cassettes from the supplier containing acridine orange and DAPI to quantify dead and live cells, respectively. The software NucleoView NC-200 version 1.3.0.0 was used. The cell viability and detachment on beads were determined following the manufacturer's instructions using the supplied A and B solutions for cell lysis. Briefly, all cells were lysed and stained with DAPI in the first cassette to count the total number of nuclei. Then, in the second cassette, only cells in the sample supernatant (after bead sedimentation) were stained with acridine orange and DAPI. Considering that all the cells on the beads are alive, the percentages of viable and detached cells were deduced.

Nanoparticle tracking analysis. The size distribution and concentration measurements of ADSC EVs were performed with a Nanosight (NS300, Malvern, UK). Before measurements, samples were diluted to the appropriate concentration (between 7×10^7 parts per ml and 5×10^8 parts per ml) in sterile PBS. A total of 5 movies of 60 s each were recorded for each sample. The acquisition was performed with the following parameters: camera level = 16 and screen gain = 1. Processing was performed at a gain of 10 and a detection threshold of 5.

Cryo-transmission electron microscopy. A volume of 2–7 μl of ADSC EVs was transferred to an electron microscopy grid featuring a perforated carbon film coating (Ted Pella, Redding, CA, USA). The liquid in excess was blotted off with filter paper, and the grid was then quickly plunged into liquid ethane. Analysis was performed using a MET Jeol 2100 (LaB6) at 80 kV.



Imaging flow cytometry. ADSC EVs were analyzed by flow cytometry imaging (Amnis® ImageStream®) using human anti-CD63 phycoerythrin (PE) and anti-CD81 allophycocyanin (APC) antibodies (BioLegend) and anti-CD9 fluorescein isothiocyanate (FITC) antibodies (CliniSciences). Briefly, 3.3 μL of anti-CD9 (3.3 μL of anti-CD63 and 2.5 μL of anti-CD81) were mixed with 5 μL of EVs (1.6×10^{12} particles per mL) in a final volume of 50 μL , completed with PBS. Each antibody was used alone before performing multiple staining. Incubation was carried out for 15 min in the dark. A total of 100 000 events were analyzed. Images were collected in the bright field, dark field, a green channel for FITC, a yellow channel for PE and a red channel for APC fluorophores simultaneously. Speed beads were used for calibration (Amnis® SpeedBeads®). The analysis was first carried out by plotting the side scatter intensity (SSC) versus the fluorescence intensity corresponding to CD9 FITC, CD63 PE or CD81 APC. Gates corresponding to the apoptotic bodies (AB), large vesicles (IEVs) and small vesicles (sEV) were set according to their SSCs. In the second step, events in the sEV and IEV gates, for each antibody, were analyzed by plotting fluorescence intensities for CD9 FITC versus CD63 PE; CD81 APC versus CD9 FITC; and CD81 APC versus CD63 PE. Amnis IDEAS® software was used for data analysis.

Ex vivo pro-survival tests in pig esophageal biopsies. The pro-survival effect of ADSC EVs on pig esophageal biopsies was evaluated *ex vivo* via an Alamar test. Pig esophageal biopsies were collected under general anesthesia (10 mg kg⁻¹ intramuscular ketamine and 8 mg kg⁻¹ intravenous propofol). A radial jaw was used to collect biopsies, which were then transferred to DMEM containing 100 U mL⁻¹ penicillin and 100 U mL⁻¹ streptomycin. Biopsies were individually placed in 96-well plates (one biopsy per well). The Alamar blue test (Invitrogen) was performed for all wells individually and used as the baseline. The test was carried out according to the supplier's instructions. Biopsies were then incubated for 72 h with ADSC EVs in a serum-free DMEM containing 2.5×10^{10} or 1.25×10^{11} EVs mL⁻¹. The serum-free medium and complete medium were used as negative and positive controls, respectively. The Alamar test was carried out again at 24 h and 72 h time points. The obtained data were normalized for each biopsy individually using the baseline values.

In vivo studies. This protocol was conducted at the Biosurgical Research Laboratory of the Alain Carpentier Foundation in Paris and the National Institute for Agricultural Research (INRA) in Val de Loire Center. It was approved by the local ethics committee of Descartes University (no. 14099). A total of 18 female pigs between 30 and 50 kg were randomized into 3 groups: a control group ($n = 6$), a gel group ($n = 6$), and an EV + gel group ($n = 6$).

ESD technique. ESD under general anesthesia was performed on D0 in all pigs. Animals received 2 mg kg⁻¹ tiletamine-zolazepam and 4 mg kg⁻¹ propofol IV for anesthesia induction. Anesthesia was maintained through 2% isoflurane. Animals were fasted 24 hours before the procedure for solid food and 8 hours before the procedure for liquid ingestion. ESD was performed in all pigs on D0 using a standard adult

gastroscope (EG-530WR Fujifilm, Japan) and a Flushknife 1.5 BT (Fujifilm, Japan). An electrosurgical unit ERBE ICC 350 (ERBE Technology, Germany) and a pulsed pressure pump JW-2 (Fujifilm, Japan) were also used. After marking a virtual esophageal tumor, saline was injected into the sub-mucosa to facilitate dissection. The ESD strategy was to make a tunnel (proximal and distal incision and then submucosal dissection) 5 cm long and $\frac{3}{4}$ circumferential between 50 and 55 cm of the dental arch. The next step was lateral incision (left and right), and final dissection allowed a complete *en bloc* resection. All resected pieces were retrieved, pinned on a dedicated plate and measured.

PF-127 gel application. In the gel group, just after ESD, PF-127 (8 mL) was applied to the scar using an endoscopic plastic catheter (Liguory 7 Fr, Cook Medical, USA). PF-127 was injected in the liquid form at a temperature <19 °C. In order to prevent gel transition and catheter clogging, the catheter was kept on ice. Gel transition took place in contact with the lesion at body temperature.

ADSC EV application with PF-127 gel. EVs stored in a -80 °C freezer were thawed and diluted in saline or PF-127 at 20% just before ESD. For EV administration, a total of 1.45×10^{12} ADSC EVs were divided into three fractions and applied into the lesions after ESD in the following sequence:

- (1) 1/3 of EVs were dispersed in 10 mL of saline and sprayed on the scar with a spray catheter (Alton, China);
- (2) 1/3 of EVs were dispersed in 10 mL of saline and injected into the edges of the scar (needle Interject, Boston Scientific, USA);
- (3) 1/3 of EVs were dispersed in 8 mL of the gel and applied to uniformly cover the scar using a catheter (Liguory 7Fr, Cook Medical, USA).

PF-127 gel application

For the gel group, only 8 mL of PF-127 gel was applied uniformly to cover the scar using a catheter (Liguory 7Fr, Cook Medical, USA).

Follow-up

Clinical evaluation. Daily examination of the pigs was performed as follows: assessment of pain, behavior, food intake, regurgitation, and vomiting. Dysphagia was scored based on the Mellow-Pinkas score (0: normal swallowing, 1: unable to swallow a proportion of the solid diet, 2: able to swallow a semi-solid diet, 3: able to swallow liquids only, 4: complete dysphagia including saliva).⁴⁰

Radiological evaluation. The primary endpoint was the degree of esophageal stenosis on D21 assessed by a blinded radiological examination. A fluoroscopy was performed (2 orthogonal incidences) using Arcadis (Siemens, Germany) with a contrast agent (Omnipaque 300, Guerbet, France) injected into the operator channel to perform esophagography. The most relevant radiological incidence (anteroposterior or lateral) was kept, and we calculated a degree of stenosis as follows: $[1 - (\text{length of the short axis inside stenosis}/\text{length of the normal axis under stenosis}) \times (100)]$.



Endoscopic evaluation. Endoscopic evaluation was performed on D21. The following two criteria were noted: (i) the possibility to pass with the gastroscope (diameter of 9.2 mm) through the esophageal lumen; and (ii) the esophageal lumen diameter estimated using close or open biopsy forceps (measuring respectively 2 mm and 7 mm).

Histological evaluation. On D21, pigs were euthanized and the entire esophagus including the stricture area was surgically resected. Specimens were stored in 4% paraformaldehyde for 24 hours before changing the medium to 70% alcohol. Between 5 and 7 blocks from the stricture area were created and included in paraffin. Then, slides of 4 μm thickness were stained using hematoxylin and eosin and Sirius red. Finally, all the slides were digitally scanned (Digitiser Hamamatsu Photonics®, France), and dedicated software (NDP view software®, France) was used for histological analysis.

The following criteria were measured: the maximal surface of fibrosis, maximal length of muscularis propria, and maximal length of defect of epithelium.

Statistical analysis. For comparison of qualitative variables between the different groups, an exact Fischer test was performed (expected values less than 5). Non-normal quantitative variables were compared using the non-parametric Kruskal–Wallis test. A difference was considered statistically significant if $p < 0.05$. If there was a significant overall difference, a Mann–Whitney test compared one group to another. Statistical analysis was performed using GraphPadPrism software (Graphpad Software, La Jolla, Calif., USA) for non-parametric Kruskal–Wallis tests, and Fisher's exact tests were performed via statistical software R.

Results

Characterization of viscoelastic properties of PF-127 for the thermo-controlled EV delivery strategy

The PF-127 gel at 20% featured a transition temperature of 19.3 $^{\circ}\text{C}$ (Fig. 2A). At 24.7 $^{\circ}\text{C}$, the storage modulus reached a plateau, imparting stability to the gel. At 37 $^{\circ}\text{C}$, the elastic modulus was 2×10^4 Pa and the loss modulus was 9×10^2 Pa. Therefore, at the body temperature of pigs, PF-127 is a gel and its viscoelastic properties are expected to confer good mechanical strength to be retained on the esophageal scar after endoscopic resection.

Allogeneic ADSC EVs produced in high yield in bioreactors at a liter scale by the turbulence approach

ADSCs were first cultured in 2D in T-flasks with a viability >90% (ESI Fig. 1 and Table 1†). In a scalable approach, cells were cultured in 3D on the surface of Cytodex 3 beads in 1 L spinner flask bioreactors with a viability of >90% (ESI Fig. 2A–D and Table 2†). The EV production was triggered by turbulence stimulation by controlling the stirring speed of the bioreactor in order to reach a Kolmogorov length of 35 μm in 4 hours.³⁹ The producer cell viability after turbulence stimulation was >81% (ESI Fig. 2E–G and Table 3†).

The turbulence approach enabled the production of 62 700 particles per ADSC in 4 hours, for instance. Starvation in 2D flasks for 72 h (with a post-production cell viability of 61.7%) provided a particle yield of 14 550 per cell, which was about 4 times less and 18 times longer (results not shown). ADSC EVs were isolated by centrifugation/ultracentrifugation steps and characterized by NTA (Fig. 2B and C). For instance, a total of 3

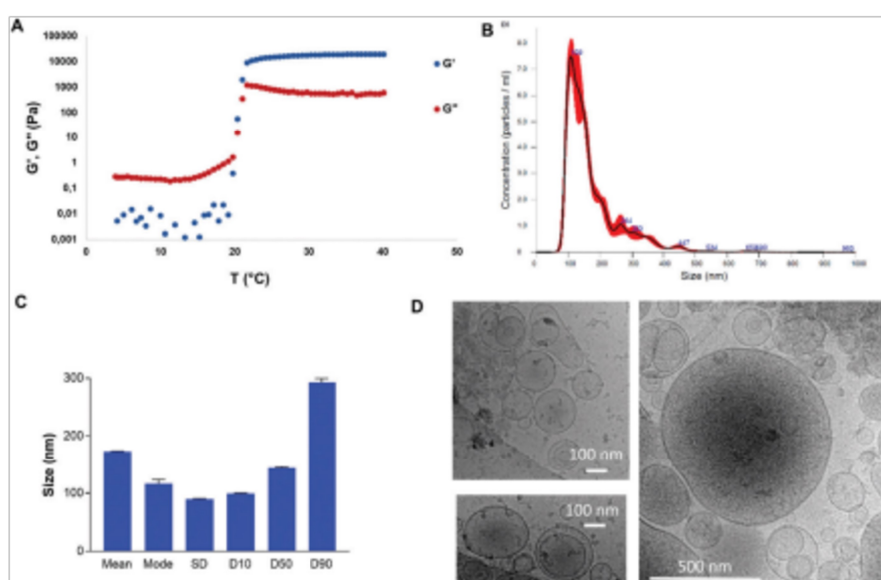


Fig. 2 Characterization of Pluronic F-127 by rheological analysis and ADSC EV characterization by nanoparticle tracking analysis and cryogenic transmission electron microscopy. Evolution of dynamic storage modulus (G') and loss modulus (G'') of Pluronic F-127 at 20% and a heating rate of 5 $^{\circ}\text{C min}^{-1}$ from 5 $^{\circ}\text{C}$ to 40 $^{\circ}\text{C}$ for the determination of the sol–gel transition temperature (G' and G'' cross over) (A). Size distribution of ADSC EVs, averaged on 5 recorded videos (B). Mean, mode, standard deviation and percentiles of the size of EV samples, represented as the mean \pm SEM of three independent batches by nanoparticle tracking analysis (C). Cryogenic transmission electron microscopy images of ADSC EVs, zoom $\times 10\,000$ (D).



$\times 10^{12}$ EVs were obtained from 250 million ADSCs in 1 L bioreactors with a volume of 380 mL of medium after purification. The size distribution and concentration of EVs were analyzed by NTA. The sample featured characteristic EV size distribution that mainly ranged from 100 to 250 nm. The mean size and mode were 172.8 nm and 116.3 nm, respectively, with a size distribution standard deviation of 89.3 nm. For this sample, 90% of particles were smaller than 293.1 nm, 50% of particles were smaller than 143.7 nm, and 10% of particles were smaller than 99 nm. Cryo-microscopy analysis showed characteristic round-shaped membrane-delimited structures with high size polydispersity between 100 and 500 nm (Fig. 2D).

Allogeneic ADSC EVs produced by turbulence featured classical EV markers according to image-based high-throughput multispectral analysis

EVs were characterized using multispectral imaging flow cytometry (Fig. 3). This technique merges high-throughput flow cytometry with high statistical power for the visualization advantages of confocal microscopy on a single event basis. Images of 100 000 events per sample were collected in the bright field, dark field, as well as in a green fluorescence channel for FITC (CD9 antibody detection), a yellow one for PE (CD63 antibody detection) and a red one for APC (CD81 antibody detection). Events were gated as small EVs (sEVs) and large EVs (lEVs) when plotting side scatter (SSC) intensity *versus* fluorescence intensity corresponding to CD9 FITC, CD63 PE or CD81 APC (Fig. 3A–C): 971, 314 and 1240 events, respectively. The majority of them were sEVs. For instance, sEVs represented more than 65% of the EV events concerning CD63 PE and more than 85% of the EV events concerning CD9 FITC or CD81 APC fluorescence intensities (Fig. 3D). Representative images of sEVs and lEVs (Fig. 3E and F, respectively) indicated some single- and double-positive events.

sEV and lEV gate events from the SSC *versus* CD9 FITC fluorescence intensity (Fig. 3A) were further plotted for CD81 APC *versus* CD63 PE fluorescence (Fig. 3G and H, respectively) to evaluate single CD9+ (gate CD63–CD81–), double CD81+CD9+ only (gate CD63–CD81+), double CD63+CD9+ only (gate CD63+CD81–) and triple CD63+CD81+CD9+ (gate CD63+CD81+) populations. These biparametric dot plots and their bar graph representation (Fig. 3I) indicated that ~92% of sEVs were single CD9+ and ~7% of them were double CD63+CD9+.

Considering the reduced event number (314), EV gate events from SSC *versus* CD63 PE fluorescence intensity (Fig. 3B) were not further analyzed.

EV gate events from the SSC *versus* CD81 APC fluorescence intensity (Fig. 3C) were further plotted for CD63 PE *versus* CD9 FITC fluorescence (Fig. 3J and K for sEVs and lEVs, respectively) to evaluate single CD81+ (gate CD9–CD63–), double CD63+CD81+ only (gate CD9–CD63+), double CD9+CD81+ only (gate CD9+CD63–) and triple CD9+CD63+CD81+ (gate CD9+CD63+) populations. These biparametric dot plots and their bar graph representation (Fig. 3L) indicated that ~99% of sEV population was single CD81+ with a negligible double or

triple positive population. Concerning lEVs, a negligible percentage of the population was CD9+CD81+ only, ~8% were double CD63+CD81+ only, ~26% were triple CD9+CD63+CD81+ and 65% were single CD81+.

The apoptotic body (AB) gate was constituted of events featuring important bright- and dark-field signals, but the events were devoid of fluorescence signals (ESI Fig. 3†). Therefore, events in the AB gate were not considered as EVs herein.

Allogeneic ADSC EVs produced by turbulence elicit pro-survival effect *ex vivo* on the pig esophageal biopsies under starvation

The cell survival of the pig esophageal biopsies under starvation for 24 and 72 h incubated with or without ADSC EVs was tested by the Alamar blue assay. The pig esophageal biopsies incubated in complete medium and serum-depleted medium (starvation) were used as positive and negative controls, respectively. This test was carried out on a single biopsy basis with one biopsy per well (Fig. 4A). Cell survival was assessed at T0, T24 and T72 h. Normalization to the T0 value was performed to reduce inter-biopsy variability. At 24 h incubation, ADSC EVs slightly increased the biopsy survival under starvation compared to the negative control (Fig. 4B). At 72 h incubation, ADSC EVs significantly increased the biopsy survival at the highest dose of 1.25×10^{11} EVs mL⁻¹ compared to the negative control (Fig. 4C).

Allogeneic ADSC EVs produced by turbulence combined with the PF-127 gel prevent stricture formation with an anti-fibrotic effect following extended esophageal endoscopic resection in pigs

Both ESD and therapy were carried out on D0, and *in vivo* evaluation was finalized on day 21 (Fig. 5). All ESDs were performed without complications. The mean procedure duration was 30 ± 10 minutes. Pieces of dissection were removed and measured. The lesion's size was comparable between the three groups ($p = 0.40$ for the height of the piece and $p = 0.75$ for the width).

Clinical evaluation after ESD

Eating disorders such as regurgitation and vomiting were observed from day 12 in 55.5% of pigs. There was a trend towards a therapeutic benefit for the EV + gel group, but no significant difference was observed comparing control, gel and EV + gel groups (respectively, 83.33%, 66.7, and 16.67%, $p = 0.11$). These results are shown in Table 1. Dysphagia score means were respectively 2.66, 2.50, and 1.50 for control, gel and EV + gel groups.

Endoscopic evaluation at D21

The passage of the gastroscop through esophageal stricture was possible for only one pig, and it was in the EV + gel group. The passage of the closed biopsies forceps (2 mm in diameter) through the esophageal stricture was possible in all cases. The passage of open forceps (7 mm diameter) was more frequently feasible in the EV + gel group compared to the gel and control ones (66.7% vs. 33.3% and 0%, $p = 0.08$) (Fig. 6A and Table 1).



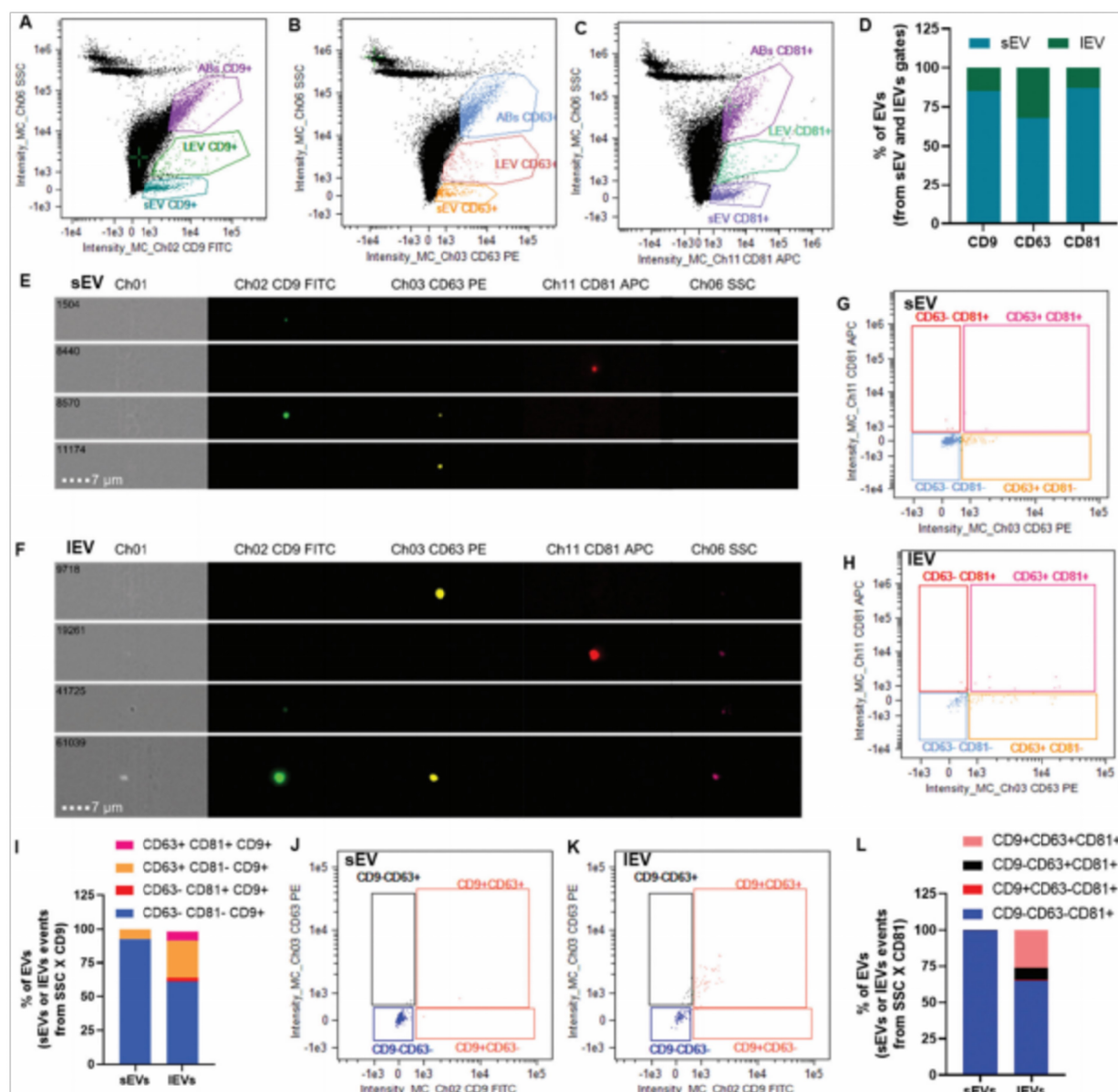


Fig. 3 EV analysis by multispectral imaging flow cytometry. Biparametric dot plots of the side scatter intensity (SSC) versus fluorescence intensity corresponding to CD9 FITC, CD63 PE and CD81 APC (A, B and C, respectively). The percentages of small EVs (sEVs) and large EVs (IEVs) considering the sum of them for each of these three biparametric dot plots were calculated (D). Images were acquired for single EVs in the bright field, fluorescence channels for FITC (CD9), PE (CD63) and APC (CD81) as well as in the dark field for sEVs (E) and IEVs (F). The events in the EV gates for the SSC versus CD9 FITC fluorescence intensity were further plotted for CD81 APC versus CD63 PE fluorescence intensities to investigate single, double and triple positiveness for sEVs (G) and IEVs (H). The quantitative bar graph of this plot indicated the % of single CD9+, double CD81+CD9+, double CD63+CD9+ and triple CD63+CD81+CD9+ (I). The events in the EV gate for the SSC versus CD81 APC fluorescence intensity were further plotted for CD63 PE versus CD9 FITC intensities to investigate single, double and triple positiveness for sEVs (J) and IEVs (K). The quantitative bar graph of this plot indicated the percentages of single CD81+, double CD63+CD81+, double CD9+CD81+ and triple CD9+CD63+CD81+ (L).

Radiological evaluation

One pig (control group) was excluded from the radiological analysis because the stricture reached the cardia and it was not possible to measure the post-stenotic esophagus diameter. Esophagography analysis indicated that the degree of stricture was significantly lower in the EV + gel group (67.9%) in comparison with the control group (92.5%) ($p = 0.04$). This rep-

resents the primary outcome of the *in vivo* study. The difference was not significant when comparing the gel group (83.4%) to control or to the EV + gel group (Fig. 6B and C).

Histological evaluation

Histological analysis for the EV + gel group evidenced that (i) the minimal length of muscularis mucosae was signifi-



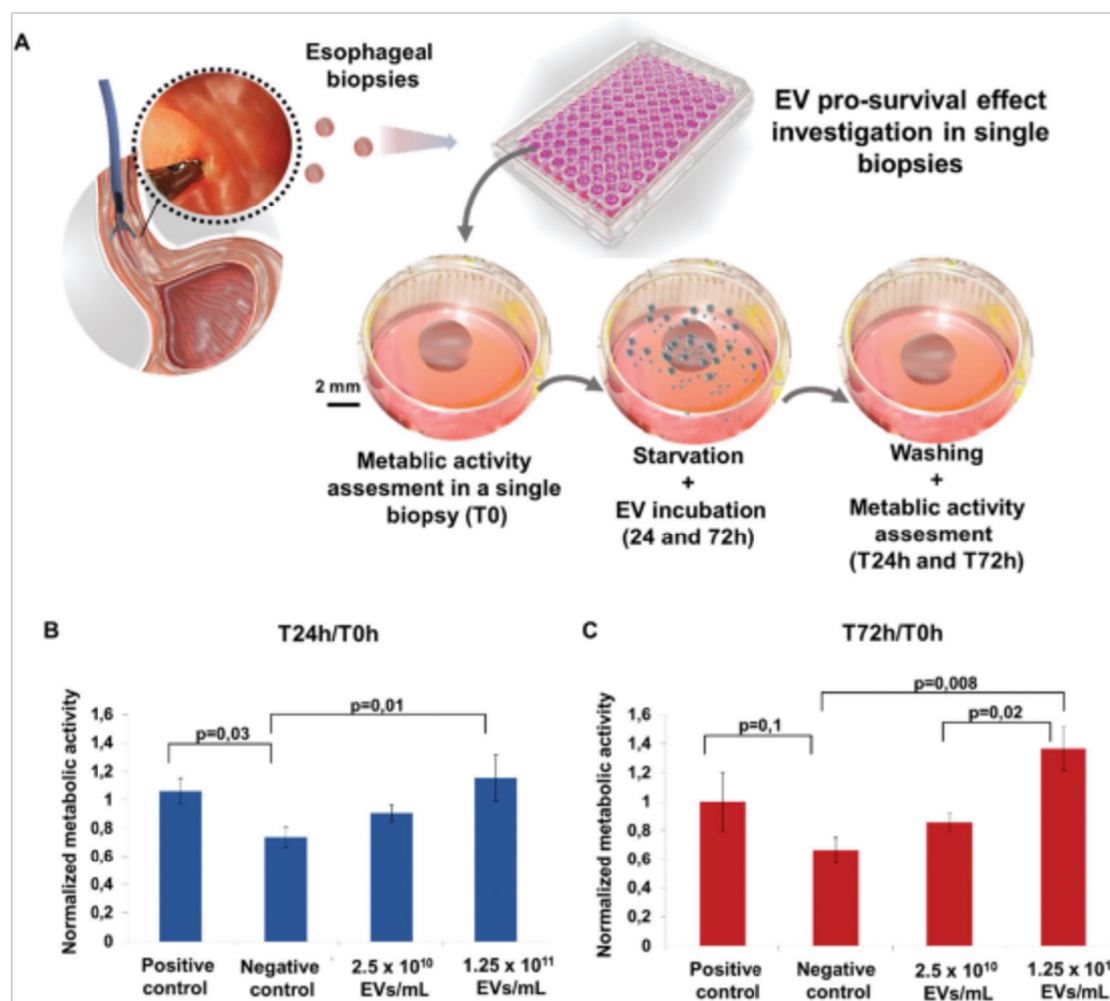


Fig. 4 The potency evaluation *ex vivo* of ADSC EVs by a single-biopsy pro-survival test. Schematic representation of the assay from biopsy sampling of the esophagus from pigs by endoscopy to single biopsy metabolic activity assessment in individual wells under starvation before and after incubation with ADSC EVs (A). Metabolic activity of single biopsies normalized to T0 values after incubation with 2.5×10^{10} or 1.25×10^{11} ADSC EVs mL^{-1} under starvation in comparison with a negative (starvation) control and a positive (complete medium) control at 24 h (B) and 72 h (C).

cantly higher compared to that of the control group (Fig. 6D); (ii) there was a significantly smaller fibrosis area compared to the gel group (Fig. 6E) (statistically significant difference compared to the control group was not observed in a sample of 6 animals per group due to the wide dispersion of control values); (iii) there was superficial fibrosis without infiltration into the muscularis propria in comparison with the control group and also to the gel one (both control and gel groups feature thick fibrosis with a dilapidated fibrosis infiltration within the muscularis propria) (Fig. 6F).

Histological analysis is further reported in ESI Table 4.† There was no significant difference between the groups in terms of the maximal length of defect of epithelium neither in terms of the maximal thickness of fibrosis. In the EV + gel group, the length of muscularis mucosae was higher when compared to those in the gel and control groups.

Discussion

We evaluated herein an innovative minimally invasive nanotherapy to prevent esophageal stricture in pigs based on endoscopic injection of ADSC EVs and delivery in a thermo-responsive PF-127 gel. Neither EV-based esophageal stricture prevention nor the thermo-actuated delivery strategy has been explored before for this disorder.

Thermoresponsive injectable hydrogels offer the advantage of administration in a liquid state at a temperature below 19 °C. This facilitates the delivery to the gastrointestinal mucosal by using an appropriate injection device; in our case, a catheter is introduced *via* an endoscope. Such an administration strategy allowed us to cover the wide extent of the resection lesion with EVs embedded into the PF-127 gel. Once the composition comes into contact with the resection lesion at body temperature, there is gel transition characterized by a viscosity increase. This has the advantage of enabling the compo-



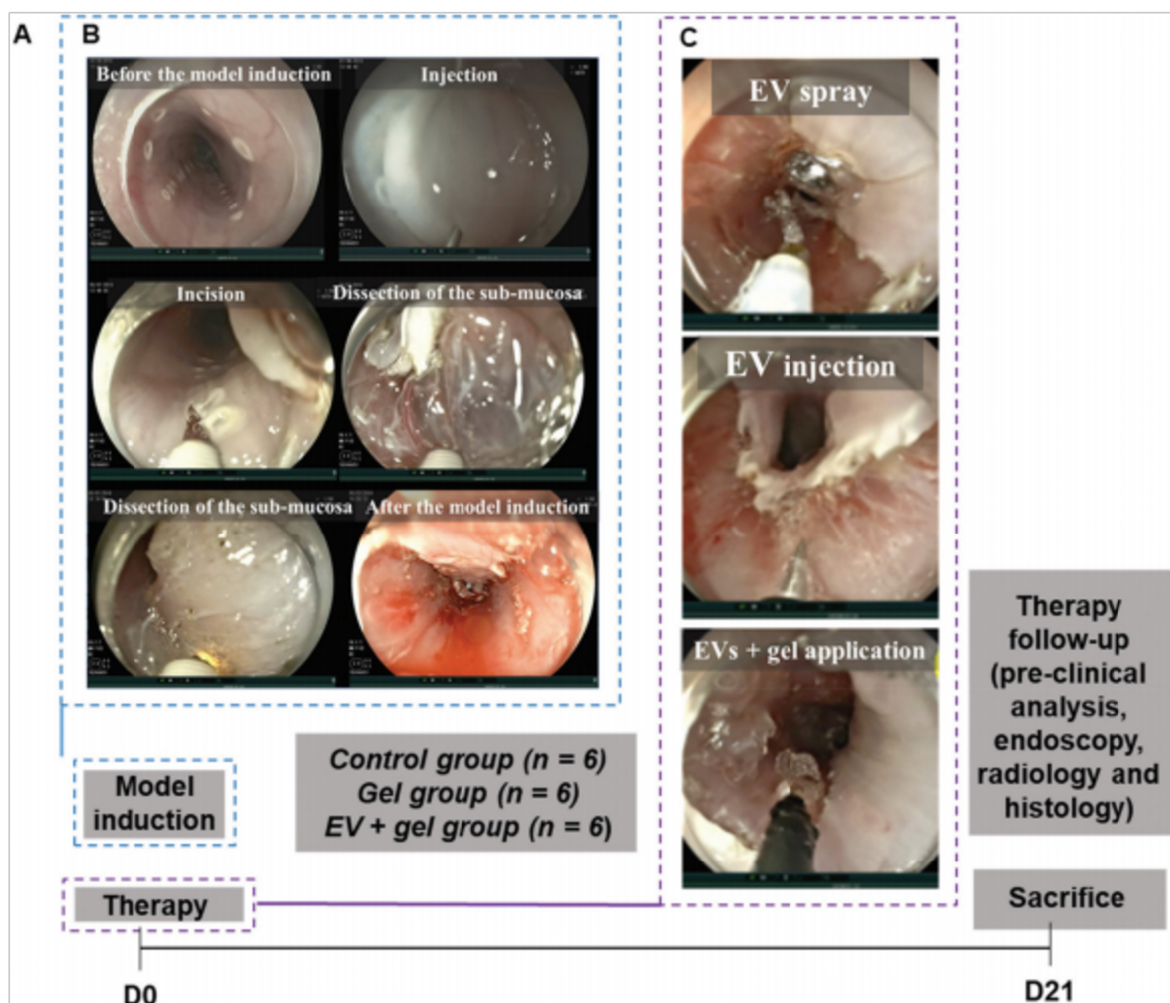


Fig. 5 Design of the *in vivo* study and overview of the main steps. *In vivo* study timeline and sample size per group (A). Endoscopic procedure for the esophageal stricture model induction from saline injection and incision to dissection (B). Endoscopic views of the therapy procedure for the EV + gel group in three steps: (i) ADSC EV spraying, (ii) ADSC EV injection and (iii) application of the ADSC EVs plus gel in the esophageal resection lesions (C). Note that for the gel group, there was only this third step with the application of the gel alone.

Table 1 Clinical and endoscopic evaluation parameters for control, gel and EV + gel groups

| Parameter | Control group (n = 6) | Gel group (n = 6) | EV + gel group (n = 6) | p |
|--|-----------------------|-------------------|------------------------|--------|
| Eating disorder cases (%) | 4 (66.67%) | 5 (83.33%) | 1 (16.67%) | 0.11 |
| Cases with feasible passage of the endoscope (%) | 0 (0%) | 0 (0%) | 1 (16.67%) | 1 (NS) |
| Cases with feasible full opening of the biopsy forceps (%) | 0 (0%) | 2 (33.3%) | 4 (66.67%) | 0.08 |

sition to remain on the surface of the affected area facilitating the contact and the residence time, transiently retaining EVs in the site of interest. Endoscopic administration of EVs into the PF-127 gel was previously tested in the therapy of esophageal fistulas by our team.³⁸ In that study, we evidenced by rheological analysis that the integration of EVs into the PF-127 gel did not alter its thermoresponsive properties. The EV integration into the gel also seemed to have a minor impact on the integrity of EVs. Besides, we evidenced that the EV release was expected to be driven by the saliva flow exerting a dilution effect. We formerly showed that gel dilution to a concentration exceeding the

threshold of about 13% notably induced a viscosity decrease, releasing EVs.³⁸ Indeed, NTA experiments indicated that EVs were entrapped in the PF-127 gel when its concentration was 20%, which was evidenced by the absence of Brownian motion. Such a trapping effect was reduced by gel dilution by a factor 2. To a greater extent, dilution by a factor of 10 intensively released EVs featuring Brownian motion. The EV release after gel dilution is expected to render EVs able to interact with recipient cells/tissues.³⁸

In addition to the ADSC EV delivery embedded into the gel, a previous step was the administration of ADSC EVs in saline



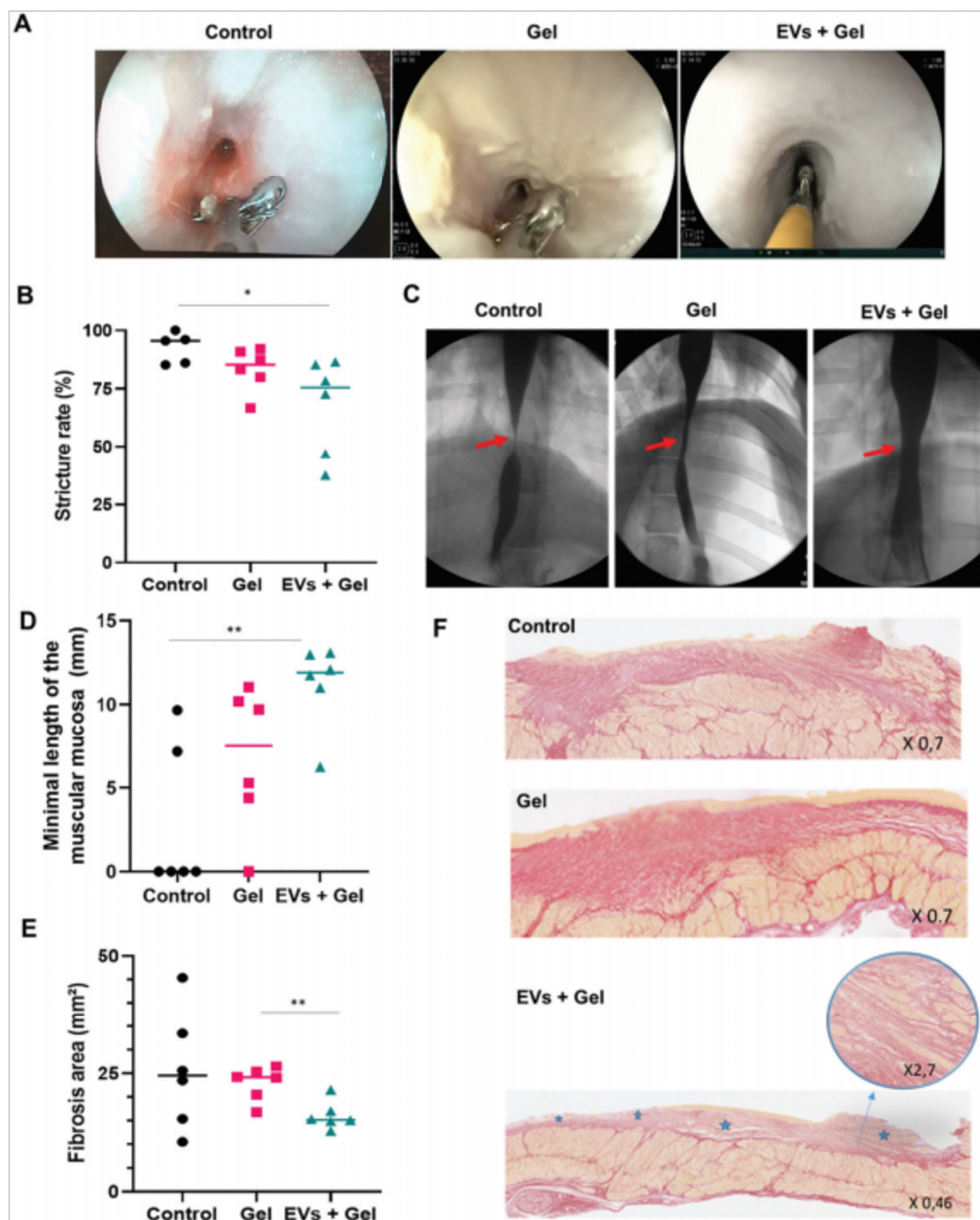


Fig. 6 The potency evaluation *in vivo* for ADSC EVs by a multi-modal approach in pigs treated with the gel or ADSC EVs + gel in comparison with untreated negative control. Therapy follow-up by endoscopic evaluation assessing the feasibility of an open biopsy forceps (7 mm in diameter) passage through the esophageal stricture (A). Therapy follow-up by radiologic evaluation assessing the stricture rate (B) calculated from esophagography (C). The minimal length of the muscular mucosa (D) and the fibrosis area (E) quantified by histological analysis. Histological sections, colored by Sirius red for fibrosis evaluation. The upper, middle and bottom panels correspond to histological sections from the control, gel and EV + gel groups (respectively). For the latter, the inset shows a zoom-in of the muscularis mucosa and stars correspond to areas where this layer is preserved (F).

over the entire surface of the esophageal resection lesion using a spray catheter. This thin aqueous layer enabled a fraction of EVs to be in direct contact with the tissue in complement to the thicker gel layer embedding EVs administered later.

Additionally, EVs were injected into the edges of lesions in order to maximize the healing effect. Indeed, "leader" cells and follower ones originating from the lesion borders play an important role in wound healing.⁴¹ We consider that EV



administration at this strategic site may be beneficial for favoring the healing process. In this regard, it has been reported that EV trails left behind the migrating leader cells would be able to promote chemotaxis of the follower ones.^{42,43}

On day 21 post-therapy, a multimodal follow-up was performed. Endoscopy, radiology and histology data consistently indicated a significantly reduced stricture rate and fibrosis area and an increased mucosal muscle width by the combined action of EVs and the thermoresponsive hydrogel. The therapeutic outcome with PF-127 seemed better than that with the control group. Indeed, there was a trend towards the reduction of the stricture rate and an increase in the minimal length of the muscular mucosa compared to the control. However, in both cases, a statistically significant difference was not reached, possibly due to the number of animals per group. Previous studies reported that the topical application of the PF-127 gel promoted cutaneous wound healing in rats and increased microvessel density.³⁷ In particular, increased expression of the vascular endothelial growth factor (VEGF) and transforming growth factor-beta1 (TGF- β 1) was observed. These effectors probably played an important role in the recruitment of the inflammatory cells, angiogenesis and granulation tissue formation since histopathological analyses revealed more leukocyte infiltration accompanied by well-formed granulation tissue and fibroblast proliferation in the group treated with the PF-127 gel.³⁷ A moderate therapeutic effect in fistula healing was also evidenced in pigs after the administration of the gel alone, including a trend towards the reduction of the inflammation score compared to control.³⁸ Therefore, the PF-127 gel may be considered more than a carrier vehicle, contributing by itself to some extent to the therapeutic effect observed in the pig stricture model reported herein. Importantly, the PF-127 gel effect in the prevention of esophageal stricture was markedly enhanced by the combination with ADSC EVs. These EVs are expected to carry pro-regenerative signaling to help in restoring tissue homeostasis. Importantly, in an *ex vivo* setting, we evidenced the pro-survival effect of our ADSC EVs in esophageal tissue single biopsies under starvation. To our knowledge, this is the first time that pro-survival EV potency is assessed on a single biopsy basis. We had previously performed pro-survival potency tests in monolayers of cells isolated from esophageal biopsies following an enzymatic tissue dissociation treatment to obtain individual cells.³⁸ We are aware of all the limitations of the monolayer system, and we believe that alternative approaches should be investigated to bridge the gap between *in vitro* and *in vivo* studies. Our single biopsy strategy herein may be of interest in this regard.

Concerning the obtained *in vivo* data, we showed that the degree of esophageal stricture was lower in the EV + gel group compared with those in the gel and control ones in this porcine model of esophageal stricture induced by extensive ESD. The endoscopic evaluation indicated a less severe stricture in the EV + gel group. In the control group, it is interesting to note the occurrence of infiltrative fibrosis reaching the muscularis propria. This is in agreement with a study evaluat-

ing the role of fibrosis after mucosectomy in dogs, in which the fibrosis-mediated destruction of the muscularis mucosae was shown.²⁴ In the same study, fibrosis induced atrophy of the muscularis propria fibers. EVs plus gel induced an anti-fibrotic effect, also inhibiting muscularis mucosae atrophy. Indeed, animals from the EV + gel group featured a higher length of muscularis mucosae compared to the other groups. The fibrosis was more superficial and did not invade the muscularis propria in the EV + gel group. These results indicating the combined action of ADSC EVs plus PF-127 gel leading to a morphological tissue switch towards a less fibrotic and less atrophic state in the muscularis mucosae are very promising. Previous reports evidenced the anti-fibrotic action of ADSC EVs, including in ureteral stricture rat models.⁴⁴

Our study concerned a single EV dose administration. Other authors showed the interest of multiple-dose administration in order to obtain an enhanced therapeutic effect.⁷ Undoubtedly, the possibility of obtaining greater effects by repeated doses may be considered of interest. In particular, limitations in the gel residence time due to esophageal peristalsis, swallowing movements and saliva dilution could be counterbalanced by repeated applications.

In this study, we used EVs from porcine ADSCs. These cells have many advantages, and some of them are their ease of harvesting without an invasive intervention compared to SCs from bone marrow and the highest SC yield obtained.⁴⁵ As far as we know, this is the first time that ADSC-EVs have been investigated to prevent esophageal strictures as an alternative to cell therapy. SCs, as whole cells, administered *via* an endoscopic procedure have been previously investigated for this therapeutic indication by others in a canine ESD lesion model.⁴⁶ In a previous report of our team, SCs were organized in double cell sheets, and we showed a significant improvement in the rate of esophageal strictures in pigs.³² However, the manufacturing of cell sheets requires particular expertise and needs to be planned several days before resection.⁴⁷ Several advantages emerge when using EV-based nano-therapies with respect to the whole cells, among which EVs are safer and easier to handle due to their smaller size and the absence of the nucleus, thus avoiding self-replication and highly reducing the cell endogenous tumorigenic potential.¹² Moreover, EVs, as carriers for bioactive molecules, may be exploited to overcome tissue barriers to reach specific cell populations. Additionally, EVs can be stored in a freezer for several months, offering a banking possibility.¹³ Besides, it is interesting to note that allogeneic transplantation of ADSCs is well-tolerated because these cells have an immunosuppressive role, they do not express class II MHC or co-activating molecules of lymphocyte response and EVs from MSC probably have the same immunotolerance status.⁴⁸ This immune-privileged status of ADSC EVs in an allogeneic setting and the banking possibilities may open the way towards an "off-the-shelf" therapeutic modality.

ADSC EVs were produced herein in a scalable and high yield approach in bioreactors, which contributes to the translational potential of our investigation. Compared to the starvation approach, the turbulence approach in 4 h enabled a



more than 5-fold increase in the EV production yield compared to 48 h of starvation and more than a 4-fold increase in 72 h of starvation, which means an 18 times shorter time for the latter. We previously evidenced that the turbulence approach was scalable based on turbulence physical laws and enabled the production of EVs featuring markers, molecular content and potency equivalent to state-of-the-art starvation EVs.³⁹ Advantageously, our turbulence approach is directly integrated into the scalable cell culture process in stirred tank bioreactors. Its originality is to go beyond the bioreactor use as a cell culture device. Indeed, we use the bioreactor stirring system in a dual manner, for homogenization (its primary purpose) and for triggering massive EV production via the generated turbulence shear stress. For instance, the required dose to treat 2 pigs was obtained from 250 million ADSCs in 1 L bioreactors with a volume of 380 mL of medium. The high-yield turbulence approach enabled us to produce EVs efficiently in the human dose range in a sufficient amount to be tested in a clinically relevant setting using large animals.

Conclusions

One of the major contributions of our study is that we investigated the proposed nano-therapy in a porcine model, allowing us to ascertain esophageal stricture disease, administration modalities as well as the required therapeutic doses relevant to humans in a translational approach. Another strength of our study is the association of nanoscale EVs with a thermo-responsive biomaterial constituted of nano-micelles^{36,38} to prevent esophageal strictures. Our results strengthen the view that a combination of EVs and a biomaterial may improve the biomaterial therapeutic effect while facilitating EV administration. We have shown for the first time that ADSC EV-based treatment combined with endoscopic injection of a thermo-responsive gel of Pluronic® F-127 prevented the esophageal stricture in a porcine model by an anti-fibrotic and regenerative effect on the muscular mucosae. The confirmation of this preventive effect in a clinical study could provide real hope for patients tackling current unmet needs.

Author contributions

Conception, design, and study supervision: Amanda Karine Andriola Silva and Gabriel Rahmi. Acquisition of data: Elise Coffin, Guillaume Perrod, Iris Marangon, Max Piffoux, Alice Grangier, Arthur Berger, Imane Boucenna, Leila M'Harzi, Jessica Assouline, and Thierry Lecomte. Analysis and interpretation of data: Elise Coffin, Amanda Karine Andriola Silva, Gabriel Rahmi, Olivier Clément, and Christophe Cellier. Writing, review, and/or revision of the manuscript: Amanda Karine Andriola Silva, Gabriel Rahmi, Claire Wilhelm, Florence Gazeau, and Imane Boucenna.

Conflicts 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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