In Vivo Evaluation of Different Surgical Procedures for Autologous Chondrocyte Implantation

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Marina Maréchal^{1,*}, Hadewych Van Hauwermeiren², Johan Neys¹,
Gert Vanderlinden¹, and Tom Van de Putte¹

Abstract

Objective. Autologous chondrocyte implantation (ACI) involves the application of a chondrocyte suspension into a membrane-sealed cartilage defect. Recently, “cell-seeded collagen matrix-supported” ACI has been developed wherein chondrocytes are seeded on a biomembrane. This study aimed at preclinically comparing 4 variant ACI techniques in a refined goat model: 2 traditional procedures, whereby the defect is sealed by a periosteal flap or collagen membrane, and 2 cell-seeding methods, with the collagen membrane either sutured or glued into the defect. **Design.** The efficacy of the surgical techniques was evaluated in an acute critical size chondral defect in the medial condyle of 32 skeletally mature goats, randomly assigned to 1 of the 4 aforementioned treatment groups. After 10 weeks *in vivo*, the quality of the repair was graded histologically by 2 independent, blinded readers using the “modified O’Driscoll” score. **Results.** The cell-seeding procedure whereby the membrane is sutured into the defect has a similar structural repair capacity than traditional ACI techniques. However, when the cell-seeded membrane was glued into the defect, the outcome appeared inferior. **Conclusion.** These findings indicate that optimizing the goat model and the postoperative recovery does allow preclinical evaluation of ACI-based cartilage implants in a load-bearing setting. This preclinical observation provides support to the clinical utilization of the sutured membrane-seeded (ACI-CS) technique, provided sutures, but not fibrin sealants, are used to fix the cell-seeded membrane in the defect bed.

Keywords

ACI, ACT-CS, membrane seeded, O’Driscoll, goat

Introduction

Cartilage has limited potential for healing because of its avascular nature, which restricts the ability to recruit endogenous chondroprogenitor cells. When left untreated, homeostasis of the whole joint may become affected, leading to further degeneration and joint dysfunction, and often osteoarthritis in later life. Early surgical intervention may prevent or delay this. Autologous chondrocyte implantation (ACI), the first cell-based approach in orthopedic surgery is a procedure whereby the patients’ chondrocytes are isolated from a small cartilage biopsy taken during arthroscopy and, after expansion of the cells *in vitro*, are re-implanted into the membrane-sealed chondral defect during a second surgery. This mostly results in defect filling with hyaline-like or mixed-type neocartilage that integrates with the host tissue,^{1,2} with good results in terms of durability and symptom relief.^{2–11} ACI has evolved since its first application by Brittberg in 1987.^{12,13} Periosteal membranes were initially used as a cover to physically contain the cells in the defects. However, periosteal graft hypertrophy—often

resulting in clinical symptoms such as knee crepitus, effusion, pain, and locking—is one of the most common problems encountered after traditional ACI surgery.¹⁴ To avoid hypertrophy associated with the living periosteal membrane and also to circumvent donor-site morbidity, ACI procedures have evolved toward the use of commercial type I/III collagen membranes.^{15–17} Nevertheless, incomplete water-tightness of the seal may lead to the loss of the transplanted cells and thus failure of ACI intervention. Therefore, user-friendly solutions have been developed.

¹TiGenix, Leuven, Belgium

²Medanex Clinic, Diest, Belgium

*Present address: Division of Skeletal Tissue Engineering (Prometheus), KU Leuven, Leuven, Belgium

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Corresponding Author:

Tom Van de Putte, TiGenix N.V. Researchpark Haasrode 1724, Romeinsestraat 12 bus 2, 3001 Leuven, Belgium

Email: tom.vandeputte@tigenix.com

Steinwachs and colleagues seeded the cell suspension directly onto a trimmed collagen membrane which is immediately sutured into the defect bed and sealed on top with fibrin glue.^{18,19} The first 2-year clinical follow-up results using this technique have been reported recently and appear to provide a satisfactory outcome in isolated cartilage defects.²⁰

Despite their clinical implementation, functional data that allow a direct comparison amongst different ACI procedures is still lacking and will likely come from retrospective analysis of clinical records. Prospective animal studies would thus be beneficiary. However, studies on cartilage repair in load-bearing animal models have been disappointing, likely because of specific anatomic and structural issues and the difficulty to impose appropriate immobilization procedures to protect delicate initial repair processes.²¹⁻²⁴ In particular, Driesang and Hunziker²⁵ have shown that tissue flaps in goat ACI procedures fail to survive the unrestricted load-bearing joint movements, even after a period of postoperative immobilization. Despite these well-known limitations, the goat is still cited as “a favorable animal model for cartilage repair studies due largely to their stifle size, cartilaginous thickness, availability and ease of handling” in the 2010 American Society for Testing and Materials (ASTM) standard F 2451-05 on the *in vivo* assessment of implantable devices intended to repair or regenerate articular cartilage.²⁶ This prompted us to further optimize and refine the goat as a surrogate load-bearing model for the *in vivo* efficacy assessment of implantables for cartilage repair. Simultaneously, we wanted to provide preclinical data on the biological consequences of surgical ACI options. Hereto, a comparison was made between two traditional ACI procedures (periosteal flap and Chondro-Gide membrane, Geistlich Pharma AG, Wolhusen, Switzerland) and the aforementioned cell-seeding method (whereby the membrane was either sutured or glued into a chondral defect) for their capacity to mediate cartilage repair in a goat model.

Methods

Chondrocyte Isolation and *In Vitro* Expansion

All cartilage biopsies were obtained from a non-weight-bearing area of the medial trochlear ridge of the right goat stifle joint. Chondrocytes were enzymatically released from the biopsies using collagenase and expanded *in vitro* according to procedures described by Dell’Accio and colleagues.²⁷ After 2 passages *in vitro*, cells were harvested and prepared for implantation in serum-free Hepes-buffered DMEM/F12 medium at a concentration of 10,000 cells/ μ L.

Ectopic Cartilage-Forming Assay *In Vivo*

All cell batches were evaluated in the nude mouse for their capacity to form cartilage in an ectopic location *in vivo*.

This was done by injecting 5 million cells in duplicate into the thighs of Rj:NMRI-Foxn1nu/Foxn1nu mouse according to the procedure described by Dell’Accio et al.²⁸ The implants were harvested after 3 weeks, fixed, and paraffin embedded. Five-micrometer thick histological sections throughout the implant were stained with safranin O and toluidine blue according to standard protocols. The quality of the retrieved cartilage implants was expressed as a histological score, ranging from 0 (no cartilaginous implant), 1 (fibrocartilage, undifferentiated cartilage, no or weak staining with toluidine blue), 2 (well-differentiated cartilage, strong staining with toluidine blue, weak staining with safranin O), and 3 (hyaline-like cartilage with highly sulfated proteoglycans, strong staining with toluidine blue and safranin O). Histological scoring of implants was performed by 2 blinded evaluators.

Surgical Procedures

Thirty-two female skeletally mature goats (2-3 years old), weighting on average 58 kg were obtained from commercial dairy farms. All goats were physically examined before the first surgery and body condition, muscle atrophy, stifle joint appearance, and lameness were scored at different time points. The goats were randomly assigned to the different treatment groups using the randomization function in Microsoft Excel. All surgical interventions were performed under general anesthesia whereby sedation was achieved using xylazine, followed by the induction of general anesthesia by a combination of ketamine and midazolam. Anesthesia was maintained with isoflurane and oxygen. Surgery, pain management, preventive antimicrobial treatment, postoperative and wound care was performed according to standard veterinary protocols. Articular cartilage biopsies were obtained via a small medial parapatellar arthrotomy at the right stifle joint. In a second surgery, 20 to 22 days post-biopsy, the *in vitro* expanded autologous chondrocytes were implanted into an acute chondral defect of 6 mm diameter ($\varnothing$) in the cranial part of the medial condyle of the left stifle joint. Care was taken to remove all native cartilage down to, but not including, the calcified layer. In goats, defects that extent into the subchondral bone have been shown to gradually deteriorate, resulting in increasingly large cavities and eventually collapse of the subchondral bone.²⁹

The ACI procedures were performed via a medial parapatellar arthrotomy. For the ACI procedure with the periosteal flap (ACI-P), an incision was first made on the craniomedial side of the proximal tibia of the left knee where an 8 mm $\varnothing$ periosteal flap was harvested. This was then sutured over the defect with the cambium layer facing the bottom of the defect. For the ACI procedure with a Chondro-Gide collagen membrane (ACI-C), a 6 mm $\varnothing$ piece was punched out from a dry Chondro-Gide membrane. The membrane was then wetted in saline solution (0.9% NaCl)

resulting in a slight expansion. The membrane was secured over the defect with stitches, the porous side of the membrane facing the bottom of the defect. For both “traditional” ACI techniques, one stitch was left open at the highest point of the defect. Water-tightness was checked by injecting saline through the open suture. The saline solution was then withdrawn after which 10^6 cells/cm² were injected underneath the defect sealant. The last suture was closed and fibrin glue (Tissucol) used to additionally seal the borders of the cover. For cell seeding procedures, a 5 mm Ø dry Chondro-Gide piece was punched out. The porous side of membrane was then seeded with 10^6 cells/cm², and the cells were allowed to adhere to the membrane for 10 minutes. The seeded membrane was then implanted with the porous surface facing the subchondral bone plate. For the sutured membrane-seeded (ACI-CS) procedure, the membrane was fixed into position by 4 resorbable sutures in the adjacent cartilage after which Tissucol was used as an additional sealing of the edge. For the membrane-seeded procedure with fibrin glue (ACI-CS-Fibrin), the membrane was glued into the defect by applying a drop of Tissucol on the exposed tidemark after which the seeded membrane was inserted and slight pressure was applied.

Rehabilitation

The treated stifle joint was gradually loaded over a period of 3 weeks postsurgery. During the first postoperative week, the stifle joint was fully immobilized with a sling. From the third postoperative day on, the sling was removed once a day for 5 minutes and the knee was bent several times while prohibiting load bearing. These manipulations were done to avoid ingrowth of synovial membrane in the defect site, which might happen because of close apposition between the synovial membrane and the defect as a consequence of the continuous knee flexion in the sling. This was followed by a limited but gradual increase in load bearing during the next 2 weeks: The sling was removed 1 hour per day during the second week, and 2 hours per day in the third week. After this immobilization period, the sling was permanently removed. To avoid unwanted peak loading, the mobility and possibility to jump or to stand on hind limbs was restricted until the end of the experiment.

Sacrifice and Histological Analysis

An implantation time of 10 weeks in the goat was chosen to monitor the early consequences of the different ACI techniques on the formation of neocartilage and on its integration into the native tissue. We believe that the experimental period of 10 weeks in the goat is a relevant indicator for long-term outcome since there is no scientific evidence to expect durable cartilage repair when short-term follow-up measures appear poor. After sacrifice by intravenous injection of T61 (Intervet), samples from the knees were fixed in

Table 1. Cumulative Population Doublings (CPD) *In Vitro* and “Ectopic Cartilage Forming Assay” (ECFA) Score of Cells Assigned to the Treatment Group^a

Treatment Group	CPD (<i>In Vitro</i>)	ECFA Score (Mouse)
ACI-P	4.11 [3.58-4.99] (8)	2.8 ± 0.3 (16)
ACI-C	4.08 [3.62-5.07] (8)	2.9 ± 0.2 (16)
ACI-CS	3.73 [3.25-4.11] (8)	2.9 ± 0.2 (16)
ACI-CS-Fibrin	3.10 [2.65-3.57] (8)	2.6 ± 0.7 (16)

^aAveraged CPD and the range of CPD [within square brackets], reached at implantation are shown. For the ECFA score, the average ± the standard deviation is shown. The maximal mouse ECFA score attainable is 3. (n) = number of cell batches/implants analyzed.

4% formaldehyde for 48 hours, decalcified, and paraffin embedded. Five-micrometer thick histological sections in the plane perpendicular to the cartilage surface were taken from at least seven levels throughout the defect. The first level started at the beginning of the defect including the borders of the defect and the last level reached the opposite border of the defect. Each section gives a tangential overview of the surrounding native cartilage, the defect and the subchondral bone. Histochemical staining with hematoxylin-eosin, safranin O, and toluidine blue was done according to standard protocols. The quality of the neo-tissue formation was graded using a modified O’Driscoll (MOD) score.^{30,31} Scoring was done at different levels, ensuring a representative picture through the entire articular cartilage defect. Individual MOD scores per goat were assessed by two independent readers blinded for the treatment groups.

Statistics

Statistical testing for differences between treatment groups was performed using the Wilcoxon 2-sample test using online software (http://www.fon.hum.uva.nl/Service/Statistics/Wilcoxon_Test.htm).

Results

In Vitro Cell Expansion and Intrinsic Cartilage-Forming Capacity

Cells were expanded for 2 passages *in vitro*. As cell doublings *in vitro* have been shown to have a major impact on the cartilage forming capacity,^{28,32,33} cumulative population doublings of the cells in different experimental groups was compared. At implantation time, cells had reached cumulative population doublings (CPD) between 2.62 and 5.07, with an average of 3.75. Details on the CPD within the different experimental groups are given in **Table 1**. Statistical analysis revealed no significant differences between CPD of cells used in the experimental groups. In addition, as no surrogate gene marker has been validated to directly assess

Figure 1. Overview of the 4 studied autologous chondrocyte implantation (ACI) methods after finishing the surgical application in an acute chondral defect in the cranial part of the medial condyle. **(A)** ACI using an autologous periosteal flap that is water tightly sutured over the defect. **(B)** ACI using the Chondro-Gide biomembrane (Geistlich Pharma AG, Wolhusen, Switzerland) that is water tightly sutured over the defect. **(C)** Method whereby the cell-seeded Chondro-Gide membrane is positioned in the defect and secured using 4 stitches. **(D)** Chondrocyte-seeding method whereby the Chondro-Gide membrane is stuck within the chondral defect using fibrin glue.

the quality of goat chondrocytes, the ectopic cartilage-formation assay (ECFA) in the nude mouse was used to functionally evaluate the quality (i.e., the intrinsic cartilage forming capacity) of each cell batch that was implanted in the goat. Cell injections in mice were performed on the same day and using the same cell harvest as the cell implantation in the goat. All goat chondrocytes demonstrated an excellent and comparable capacity to form hyaline-like cartilage in the nude mouse. This was evident from ectopic cartilaginous tissue with lacunae around the cells, a strong safranin O staining and an absence of vascular invasion in the implant. All averaged semiquantitative ECFA scores (maximum score = 3) were greater than 2 with an averaged overall score of 2.8 in the ACI-P, 2.9 for the ACI-C group, 2.9 for the ACI-CS group, and 2.6 for the ACI-CS-Fibrin group (**Table 1**). Statistical analysis revealed no significant difference ($P = 1$) between treatment groups indicating that the intrinsic cartilage forming capacity of all cell batches were similar in the four different treatment groups. Qualitative differences in cartilage repair should therefore be a consequence of the surgical application method.

Autologous Chondrocyte Implantation

All surgeries went smoothly and no major postoperative complications were observed. **Figure 1** shows an example of each surgery immediately after finishing the application.

Clinical Evaluation of the Stifle Joint Function

Before the two surgical interventions and at sacrifice, the left stifle joint function was scored clinically using 4 parameters: (a) evaluation of the body condition of the animal,³⁴ (b) assessment of the muscle atrophy of both hind legs, (c) palpation of both stifle joints to evaluate any sign of swelling, and (d) a lameness scoring.³⁵ No major issues that could be attributed to a particular treatment could be identified. Some minor differences observed at the time of sacrifice were: a difference in muscle atrophy between the

ACI-P and the ACI-C groups (3 out of 8 goats each) and the ACI-CS and ACI-CS-Fibrin treatment groups (1 out of 8 goats each). Another noticeable difference was a higher incidence of mild lameness in the ACI-P group (5 out of 8 goats), the ACI-CS group (4 out of 8 goats) and the ACI-CS-Fibrin (3 out of 8 goats) as compared with only 1 goat in the ACI-C group. Palpation revealed one goat in the ACI-C group with swollen bony structures and one goat in the ACI-CS-Fibrin group with a peri-articular soft swelling of the treated joint.

Macroscopic Evaluation of the Condyles

At sacrifice, the freshly opened joint was macroscopically evaluated for the nature and degree of defect filling (**Fig. 2**). Various degrees of filling were observed within and across the experimental groups. Well-filled defects were macroscopically observed to contain cartilage-like tissue or a mix between fibrous and amorphous tissue. Poorly filled or empty defects were usually partially covered by native cartilage from the defect rim that had bent centrally (cartilage flow). Native cartilage in the rest of the joint was also macroscopically inspected for features of degeneration and synovium was assessed for signs of inflammation. No obvious differences between the different experimental conditions were apparent. In most cases, native cartilage near the defect appeared fibrillated with occasional small cracks. Further away from the defect, the cartilage usually appeared unaffected. Synovitis was absent in the majority of the animals.

Histological Evaluation of the Defect Healing

Histological analysis of the treated defects revealed a variable degree of filling, ranging from no filling, filling at one side of the defect to almost complete filling. Excellent filling with neo-tissue containing sulfated glycosaminoglycans was generally associated with good lateral and basal integration. The latter was associated with tidemark resorption

Figure 2. Macroscopic appearance of freshly opened joints after 10 weeks implantation *in vivo*. The condyles with the highest modified O'Driscoll score of each experimental group are shown.

Figure 3. Histological safranin O /fast green stainings showing the treated defect area, the surrounding native cartilage, and underlying subchondral bone at 10 weeks' implantation. One tangential section from the middle of the defect of the 2 best-performing goats of every study group is shown. The ID number of each goat is shown followed by the modified O'Driscoll score as was calculated from averaging different areas in the defect.

and subchondral bone remodeling, sometimes resulting in cartilaginous tissue penetrating the subchondral bone, without however causing cracks or bone cysts. Samples with no or limited filling with fibrous tissue presented themselves with an intact tidemark. Occasionally, a damaged calcified layer and subchondral bone was seen in unfilled defects, resulting in bone cracks and bone cysts under the defect. An overview is given in **Figure 3** and supplementary **Figure S1**.

To allow group comparison, a modified O'Driscoll (MOD) score was used for semiquantitative histological grading of the repair tissue. The O'Driscoll scoring is one of the few scoring tool, developed to evaluate repair tissue in animal models, in which also "integration of the repair tissue with the surrounding native tissue" is assessed.³⁶ This is not the case in the more recent ICRS II score, specifically designed to evaluate the quality of repair tissue in human biopsies.³⁷ The following features were scored: filling of the defect, tissue morphology, matrix staining (safranin O), reconstitution of the osteochondral junction

(tidemark), lateral and basal integration, chondrocyte clustering, surface regularity and structural integrity. The MOD scoring of the treatment groups ACI-P (average MOD score 15.6 ± 3.6) and ACI-C (average MOD score 16.4 ± 3.7) appeared statistically indiscernible ($P = 0.939$). The comparison between the treatment group ACI-P and ACI-CS (average MOD score 13.7 ± 3.4) also revealed no significant difference ($P = 0.559$). The same was true for the comparison between the treatment groups ACI-C and ACI-CS ($P = 0.223$), suggesting thus a comparable quality at 10 weeks of the newly formed tissues when goat chondrocytes are injected underneath a periosteal flap, underneath a Chondro-Gide membrane or seeded onto a Chondro-Gide membrane and sutured into the chondral defect. However, the outcome of the treatment group ACI-CS-Fibrin (average MOD score 10.9 ± 2.0) appeared clearly different to that from the ACI-P group ($P = 0.005$) and the ACI-C-group ($P = 0.001$). No significant difference was found between the ACI-CS group and the

Figure 4. Modified O'Driscoll (MOD) scores at 10 weeks of the individual goats (•) and averaged MOD score (—) of each treatment group ($n=8$ in each group). The MOD score comprises 9 parameters and has a maximum score of 28.

ACI-CS-Fibrin group ($P = 0.131$). For an overview, see **Figure 4**.

Although there was a considerable variability in outcome of cartilage repair between different goats in each treatment group, we could prune back the observed differences in MOD scoring to a limited defect filling, inferior tissue parameters like structural integrity and matrix composition, poorer surface architecture and a lack of basal integration in the subchondral bone. On these individual MOD parameters, the ACI-CS-Fibrin group performed significantly poorer than the ACI-P and ACI-C groups (for all these parameters: $P < 0.05$). With regard to the degree of basal integration, the ACI-CS-Fibrin group performed significantly worse than the 3 other experimental groups, including the ACI-CS group ($P = 0.02$). Other individual MOD parameters such as chondrocyte clustering, tissue morphology, lateral integration, and the presence of the tidemark were statistically indistinguishable between the 4 groups.

Discussion

The ACI procedure, originally using a periosteal flap and noncharacterized chondrocytes underwent continuous improvement over the past 25 years, most recently with the use of cells with assured quality in combination with a bioresorbable collagen membrane that allows cell seeding, a less tedious suturing of the membrane, and at the same time prevents graft hypertrophy associated with a living periosteum cover. Since direct comparison in a clinical setting is currently lacking, we performed an evaluation of different surgical ACI procedures in an optimized goat model.

Reviewing the literature on the goat model in cartilage repair reveals diversity in locations, size and depth of the defects. Here, a partial thickness, 6-mm diameter defect was made in the medial femoral condyle and cartilage was removed down to, but not penetrating, the calcified layer. A comparable method is used by others in goats and no repair was reported in empty chondral defects of 6-mm diameter.^{23,24} Our own historical data (not shown) have confirmed a 6-mm diameter defect to be critical size when left empty. The primary aim of this study was a relative comparison between the various surgical options. Therefore, and because comparison with untreated controls would require the inclusion of a control groups for each study arm (empty defect controls, Chondro-Gide without cells, periost without cells, fibrin glue alone, and combinations thereof), untreated controls were considered to be beyond the scope of this study. The incidence of graft failure, subchondral bone damage or cysts was limited and ingrowth of the synovial membrane in the defect site prevented by an intensive immobilization and rehabilitation procedure. However using this model and rehab protocol, graft failure, likely because of delamination of tissue flaps, still occurred in all experimental groups but was explicitly present in the ACI-CS-fibrin surgical technique.

The cartilage repair tissue formed by the expanded autologous goat chondrocytes appeared very similar with respect to the degree of filling, basal and lateral integration and extracellular matrix composition, irrespective of whether the cells had been implanted underneath a periosteal flap, underneath a Chondro-Gide membrane or membrane-seeded and sutured into the defect. These findings not only justify the use of the Chondro-Gide membrane in ACI procedures to replace the periosteal flap but also support its use as a matrix for the cell-seeding membrane procedure as reported by Steinwachs.¹⁸ When Tissucol was used to maintain the cell-seeded membrane in the defect bed, cartilage repair was significantly inferior to traditional ACI. No tidemark resorption or subchondral bone remodeling was apparent that would be indicative of an initiated basal integration of the—limited amount of—neotissue. By means of the ECFA that measures the intrinsic capacity to form cartilage, it was verified that this difference was not because of a nonrandom distribution of the quality of goat chondrocytes over the four treatment groups.

Despite the noteworthy differences in human and goat condyle tissue proportions, articular cartilage thickness, and the extent of rehabilitation that can be imposed on such animals, the significantly impaired performance of the ACI-CS-Fibrin group thus calls for care when employing only fibrin glue to fix implants in chondral defects. From this experimental setup, however, we cannot conclude whether this could be due to a premature loss of the cell-seeded implant because of the failure of the adhesive in the wet and mechanically challenging goat joint environment^{38,39} or to the intrinsic properties of the fibrin clot, which may act as a

barrier between the implanted cells and the tidemark, thereby interfering with basal integration.^{40,41} A cartilage implant without reasonable integration into the underlying tissue is doomed to fail sooner or later. Interestingly, the ACI-CS fixed with sutures, though statistically indiscernible, tended to perform poorer than the traditional ACI methods. Perhaps denser suturing during the ACI-CS technique in combination with the sealing of the defect rim with fibrin, could create, much alike the standard ACI procedure, a closed bioactive chamber. This could result in an increased local concentration of beneficial cartilaginous cytokines and at the same time excluding from the defect bed proteolytic enzymes and blood components that may disturb the cartilage repair.

For the clinical application of the ACI-CS method, we are therefore strongly in favor of meticulously adhering to the consensus procedure on the ACI-CS technique as it was recently published,¹⁹ in particular where it states that the spacing and number of sutures should be such that it provides the necessary mechanical stability to ensure direct contact between the cell-seeded membrane surface and the subchondral bone and use a fibrin sealant only to seal the cartilage rim.

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Declaration of Conflicting Interests

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All authors of this article are employees or former employees of Tigenix NV. H.V.H. is founder and managing Director of Medanex Clinic b.v.b.a., which provides consultancy and services for Tigenix N.V. All authors are shareholders of Tigenix N.V.

Ethical Approval

Animal experiments were performed in accordance with the Belgian legislation under the national authorization numbers LA1210530 (mice) and LA1210576 (goats) and were approved by the Ethical Committee of the Faculty of Biomedical Sciences of the University of Leuven.

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