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(54) Title: CD34+ cells for use in treating osteoarthritis

(57) Abstract: Provided are populations of CD34+ cells for use in methods of treating osteoarthritis and related methods.

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CD34+ cells for use in treating osteoarthritis

Technical Field

The disclosure relates to methods of treating osteoarthritis using populations of CD34+ cells and populations of CD34+ cells for use in such methods.

Background

CD34 is a cell surface marker used to identify and isolate hematopoietic stem/progenitor cells (HSPCs). CD34+ cells can be isolated from blood samples using immunomagnetic techniques. CD34+ cells can differentiate into all types of blood cells as well as endothelial cells.

CD34+ cells can be collected via leukapheresis. Leukapheresis comprises removing blood from the body and then separating the white blood cells before returning the blood to the body. Where leukapheresis is used to collect CD34+ cells, typically a subject is first administered a hematopoietic growth factor. Alternatively, CD34+ cells can be expanded from a whole blood sample. An automated device (StemXpand®) that allows stem cell expansion after granulocyte colony-stimulating factor (G-CSF) mobilisation has been developed and shown to provide CD34+ cells numbers at least equivalent to those collected during leukapheresis (Saucourt, et al. 2019). Characteristics (CD34+ - cell numbers, purity/impurity profile, and viability) and safety (sterility, pyrogen, and mycoplasma content) of the expanded cells have been assessed and the functionality of expanded cells (eCD34+) has been demonstrated in an *in vivo* preclinical study in rats (Saucourt et al., 2019).

Osteoarthritis is the most prevalent rheumatic disease, causing pain, and reducing mobility and quality of life (Heidari, 2011). Osteoarthritis is characterized by cartilage degradation, subchondral bone sclerosis, calcification of ligaments and synovial inflammation (Li et al., 2013). The main risks factors are age, obesity, genetics and injuries following traumas (Blagojevic et al., 2010). The prevalence of osteoarthritis is constantly increasing and enormous public health resources are devoted to the treatment of this disease (Cross et al., 2014). Current treatments are limited to use of antalgics and non-steroid anti-inflammatory drugs to alleviate symptoms and delay end-stage treatment of joint replacement by a prosthesis (da Costa et al., 2017). This irreversible surgical intervention is only considered as the very last choice, largely due to the limited durability of the implant (Rönn et al., 2011).

New therapeutic strategies including cell-based therapies are being developed. Different cell therapies have been tested for the treatment of knee osteoarthritis with promising results (Zhao et al., 2018). The potential of this approach was demonstrated by autologous chondrocyte implantation, one of the first cell therapy approaches in knee osteoarthritis (Davies and Kuiper, 2019). More recently, mesenchymal stromal cells (MSCs) are increasingly being considered as a promising alternative approach to target osteoarthritis (Song et al., 2020). Indeed, MSCs isolated from different sources have been shown to secrete many bioactive trophic factors that exert both immunomodulatory and regenerative actions

(Bernardo et al., 2009; Dabrowska et al., 2020). The isolation of stromal cells is however a surgical strategy that can cause donor site morbidity and infection (Mastrolia et al., 2019).

There is therefore a need for improved therapies for treatment of osteoarthritis.

Summary

The invention provides a population of CD34+ cells for use in a method of treating osteoarthritis.

Aspects of the invention further provide a population of CD34+ cells for use in a method of:

(a) increasing the expression of chondrogenic markers by chondrocytes in a subject, and/or

(b) decreasing the expression of inflammatory and/or degrading markers by chondrocytes in a subject, and/or

(c) inducing pro-anabolic effects on chondrocytes in a subject, and/or

(d) stimulating chondrogenesis in a subject, and/or

(e) modulating expression of inflammatory and/or pro-anabolic genes by chondrocytes in a subject.

The CD34+ cells may be obtained from whole blood or umbilical cord blood.

The CD34+ cells express CD44. The CD34+ cells express one or more paracrine factors.

The CD34+ cells may express or VEGF. The amount of VEGF expressed by the CD34+ cells into a culture medium may be at least about 150 pg/ml, and/or at least about $2x10^{-3}$ pg/cell.

The CD34+ cells express one or more or each of miR126, miR130a, miR21, miR26a, miR378a, miR146a, miR21, miR199a, miR590, miR133a, miR-24, miR29b, and miR132.

The CD34+ cell viability may be at least about 95%. The CD34+ cell purity may be at least about 80%.

The CD34+ cells may be a cultured population. The cells may be an expanded population (eCD34+). The CD34+ cells may be purified. The CD34+ cells may be provided as an isolated population.

The CD34+ cell population may comprise:

(i) at least about 80% CD34+ cells, and/or

(ii) about 15% monocytes or lower, and/or

(iii) about 5% granulocytes or lower, and/or

(iv) about 3% lymphocytes or lower.

The CD34+ cells may be provided as a sterile suspension. The population may be provided in the form of a pharmaceutical composition. The pharmaceutical composition may comprise buffer.

The method may further comprise the steps of:

i. administering a hematopoietic growth factor to a subject,
ii. collecting a peripheral blood sample from the subject,
iii. isolating and/or expanding CD34+ cells from the blood sample, to obtain a population of CD34+ cells, and/or
iv. administering the population of CD34+ cells to the subject

In some embodiments, one dose of cells is administered.

The method may comprise administering at least $8x10^6$ CD34+ cells. The volume administered per dose may be about 15 ml.

The CD34+ cells may increase the expression of collagen II, aggrecan, and/or Sox9.

The CD34+ cells may decrease the expression of IL1β, IL8, TNF, ADAMTS-4, MMP-1, and/or MMP-13.

The CD34+ cells may be for use in treating a patient with grade 2 or grade 3 osteoarthritis according to the Kellgren-Lawrence scale.

Brief Description of the Drawings

Figure 1. Viability assessment of ProtheraCytes® in OA synovial fluid. The viability of Protheracytes® in 2% HSA/PBS buffer was assessed after incubation at 37°C, 5% CO_2 with different concentrations (0, 10, 20, 50%) of OA synovial fluid at different timepoints (0, 1, 3, 6, 24, and 96h). Values are mean ± SD of measurements from 3 replicates.

Figure 2. Assessment of ProtheraCytes® and OA chondrocyte survival after coculture at different timepoints. (A) Percentage of dead ProtheraCytes® (PC) estimated by flow cytometry by counting the number of PC DAPI positive cells, after 6h, 24h and 48h of monoculture (PC (0.25M) only) or co-culture with OA chondrocytes at 0.1M or 0.25M cells/insert (PC (0.1M) / OA Ch and PC (0.25M) / OA Ch, respectively) in the absence (-INFL) or presence (+INFL) of inflammatory cytokines. Values are mean ± SD of measurements from 3 replicates * $p<0.05$. (B) Number of viable OA Chondrocytes after 6h, 24h and 48h of monoculture (OA Ch only) or co-culture with 0.1M or 0.25M ProtheraCytes®/well (+0.1M ProtheraCytes® and +0.25M ProtheraCytes®, respectively) in the absence (-INFL) or presence (+INFL) of inflammatory cytokines. Values are mean ± SD of measurements from 2 replicates.

Figure 3. Expression levels of chondrogenic genes in OA chondrocytes at different timepoints. Real-time reverse transcriptase–polymerase chain reaction of OA Chondrocytes after 6h, 24h and 48h of monoculture (OA Ch only) or co-culture with 0.1M or 0.25M ProtheraCytes®/well (+0.1M PC and +0.25M PC, respectively) in the absence (-INFL) or presence (+INFL) of inflammatory cytokines. Values are mean ± SD of measurements from 4 independent donors in 3 replicates. *= $P<0.05$, ** = $P<0.01$, *** = $P<0.001$. # = $P<0.05$, ## = $P<0.01$, ### = $P<0.001$, difference between -INFL and +INFL at identical conditions.

Figure 4. Expression levels of pro-inflammatory genes in OA chondrocytes at different timepoints. Real-time reverse transcriptase–polymerase chain reaction of OA Chondrocytes after 6h, 24h and 48h of monoculture (OA Ch only) or co-culture with 0.1M or 0.25M ProtheraCytes®/well (+0.1M PC and +0.25M PC, respectively) in the absence (-INFL) or presence (+INFL) of inflammatory cytokines. Values are mean ± SD of measurements from 4 independent donors in 3 replicates. *= P<0.05, ** = P<0.01. # = P<0.05, ## = P<0.01, difference between -INFL and +INFL at identical conditions.

Figure 5. Expression levels of ECM-degrading genes in OA chondrocytes at different timepoints. Real-time reverse transcriptase–polymerase chain reaction of OA Chondrocytes after 6h, 24h and 48h of monoculture (OA Ch only) or co-culture with 0.1M or 0.25M ProtheraCytes®/well (+0.1M PC and +0.25M PC, respectively) in the absence (-INFL) or presence (+INFL) of inflammatory cytokines. Values are mean ± SD of measurements from 4 independent donors in 3 replicates.

Figure 6. Assessment of ProtheraCytes® and OA chondrocyte survival after 24h. (A) Percentage of dead ProtheraCytes® (PC) estimated cytoflurimetrically by evaluating the number of DAPI positive cells, after 24h of monoculture (PC (0.25M) only) or co-culture at 0.1M or 0.25M cells/insert with OA chondrocytes (PC (0.1M) / OA Ch and PC (0.25M) / OA Ch, respectively) in the absence (-INFL) or presence (+INFL) of inflammatory cytokines. **(B)** Number of viable OA chondrocytes after 24h of monoculture (OA Ch only) or co-culture with 0.1M or 0.25M PC/well (+0.1M PC and +0.25M PC, respectively) in the absence (-INFL) or presence (+INFL) of inflammatory cytokines. Values are mean ± SD of measurements from 4 independent experiment with 3 replicates/experiment.

Figure 7. Expression levels of chondrogenic genes in OA chondrocytes after 24h. Real-time reverse transcriptase–polymerase chain reaction of OA Chondrocytes after 24h of monoculture (OA Ch only) or co-culture with 0.1M or 0.25M ProtheraCytes® (PC)/well (+0.1M PC and +0.25M PC, respectively) in the absence (-INFL) or presence (+INFL) of inflammatory cytokines. Values are mean ± SD of measurements from 4 independent experiment with 3 replicates/experiment. Each replicate was analysed in duplicate.

Figure 8. Expression levels of pro-inflammatory and ECM-degrading genes in OA chondrocytes after 24h. Real-time reverse transcriptase–polymerase chain reaction of OA Chondrocytes after 6h, 24h and 48h of monoculture (OA Ch only) or co-culture with 0.1M or 0.25M ProtheraCytes®/well (+0.1M PC and +0.25M PC, respectively) in the absence (-INFL) or presence (+INFL) of inflammatory cytokines. Values are mean ± SD of measurements from 4 independent donors in 3 replicates. *= P<0.05, **** = P<0.0001. # = P<0.05, ## = P<0.01, ### = P<0.001, #### = P<0.0001 difference between -INFL and +INFL at identical conditions.

Figure 9. Secretome analysis after 24h culture. Secretome of monocultured OA chondrocytes (OA Ch only), monocultured ProtheraCytes® (PC only) and OA chondrocytes co-cultured with 0.1M or 0.25M ProtheraCytes®/well in the presence (+IFLM) of inflammatory cytokines, quantified by multiplex immunoassays. The effect of factors secreted by ProtheraCytes® on OA chondrocytes is displayed as the

relative concentration (measured over expected concentrations). Values are mean ± SD of measurements from 3 independent experiment with 2 replicates/experiment. * = P<0.05 difference, *** = P<0.001 from OA Ch only.

Figure 10. Analysis of the impact on ProtheraCytes® after needle injection. The viability, purity, and CD34+ cell number of ProtheraCytes® was analysed before and after injection through 20G needle and no significant differences were observed for any of the parameters. The same results were obtained with a 21G needle.

Figure 11. Biodistribution of ProtheraCytes® after intra-articular administration 8 days after collagen-induced OA lesion. Representative histological sections of knee joints injected with ProtheraCytes® at day 8 and sampled at day 9. Histological sections of knee joints showing the presence of a moderate number of ProtheraCytes® in the synovial membrane (A, C) and muscle (B) (green arrows). Femurs are localized in the upper part and tibias on the lower part.

Figure 12: Biodistribution of ProtheraCytes® after intra-articular administration 29 days after collagen-induced OA lesion. Representative histological sections of knee joints injected with ProtheraCytes® at day 29 and sampled at day 30. Histological sections of knee joint showing the presence of a low number of human cells in the synovial membrane (A) and a high number of human cells in the muscle (B) (green arrows). Femurs are localized in the upper part and tibias on the lower.

Figure 13: VEGF as a potency test (A, B) Concentration of Vascular Endothelial Growth Factor (VEGF) in cell culture supernatants after 9 days of CD34+ cell expansion from four healthy donors and 16 patients with acute myocardial infarction (EXCELLENT study). No significant difference observed when VEGF concentration was compared between patients and healthy donors, but a significant difference was observed between patients and StemFeed® (p=0.0007) and healthy donors and StemFeed® (p=0.0087). (C, D) VEGF concentration and CD34+ cells after expansion: significant correlation between VEGF concentration and the number of CD34+ cells after expansion (Pearson correlation coefficient= 0.7484, p value =0.0009)

Figure 14: Proangiogenic, anti-apoptotic, anti-fibrosis and cardiac miRNAs relative expression by ProtheraCytes® and their secreted exosomes. (A) Total RNA from ProtheraCytes® (Cells) and their exosomes (Exosomes) collected from AMI patients (n=7) were subjected to real time PCR and normalized to small RNA (let-7a). (B) Comparative fold change expression of exosomes versus cells representation of the indicated miRNA collected from AMI patients (n=7).

Detailed Description

Definitions

“CD34+ cells” are hematopoietic stem/progenitor cells (HSPCs) that can differentiate into all types of blood cells as well as endothelial cells. CD34+ cells can be mobilized from the bone marrow into the

peripheral blood by the administration of hematopoietic growth factor, such as G-CSF. Total CD34+ cells represent approximately 0.5–1% of total bone marrow derived mononuclear cells. CD34+ cells comprise hematopoietic stem/progenitor cells (HSPCs) as well as endothelial progenitor cells (EPCs). HSPCs can differentiate into all blood cell types and EPCs can differentiate into endothelial cells. CD34+ cells grow in suspension cultures. CD34+ cells may also be obtained from umbilical cord blood.

"VEGF" (vascular endothelial growth factor) is a potent proangiogenic growth factor known to stimulate the formation of new blood vessels.

"ProtheraCytes®" is the trade name for the applicant's isolated CD34+ cells for use in therapeutic applications. ProtheraCytes® are human autologous CD34+ cells expanded according to a GMP automated manufacturing process designed for large clinical scale production. ProtheraCytes® are registered as an ATMP – Advanced Therapy Medicinal Product – within the classification of Tissue Engineered product by the European Medicines Agency. A process for making ProtheraCytes® is set out in Example 1.

"StemXpand®" is the trade name for an incubator system for the automated expansion of cells, such as CD34+ cells. The StemXpand® system is described in US10676705B2.

"StemFeed®" is the trade name for a proprietary medium for expansion of CD34+ cells comprising basal IMDM medium, human plasma and a mix of cytokines.

"microRNAs" (miRNAs) are small non-coding RNAs that influence gene expression.

Methods of treating of osteoarthritis

Osteoarthritis is the most prevalent rheumatic disease, causing pain, and reducing mobility and quality of life (Heidari, 2011). Osteoarthritis is characterized by cartilage degradation, subchondral bone sclerosis, calcification of ligaments and synovial inflammation (Li et al., 2013). The main risks factors are age, obesity, genetics and injuries following traumas (Blagojevic et al., 2010).

The populations of CD34+ cells described herein are provided for use in a method of treating osteoarthritis. The population of cells may be administered to an area of a subject that is affected by osteoarthritis. The present invention can be used to treat any joint affected by osteoarthritis, including but not limited to the knee, finger, thumb, spine, hips, and toe.

Also provided herein are methods of treating osteoarthritis comprising administering a population of CD34+ cells described herein to a subject. The population of cells may be administered to an area of the subject that is affected by osteoarthritis.

As discussed elsewhere herein, the CD34+ cells may be obtained from the subject that is to be treated for osteoarthritis, i.e. the treatment may involve autologous transplantation of CD34+ cells. Alternatively,

the CD34+ cells may be obtained from a source other than the subject to be treated, such as umbilical cord blood, i.e. the treatment may involve allogeneic transplantation of CD34+ cells.

The method of treating osteoarthritis may further comprise one or more of the following steps:

- administering a hematopoietic growth factor, such as G-CSF, to a subject,
- collecting a peripheral blood sample from the subject,
- isolating and/or expanding CD34+ cells from the blood sample to obtain a population of CD34+ cells, and/or
- administering the CD34+ cells to the subject to treat osteoarthritis.

The population of CD34+ cells may be for use in alleviating one or more symptoms of osteoarthritis. Symptoms of osteoarthritis that can be treated according to the invention include joint pain, joint swelling, joint stiffness and reduced joint range of motion.

Without wishing to be bound by any particular theory, the data presented herein demonstrate that populations of CD34+ cells increase the expression of chondrogenic markers (collagen II and Sox9) and decrease the expression of inflammatory/degrading (IL1β, IL1β, IL-8, ADAMTS-4, TNF, MMP-1 and MMP-13) markers at gene or protein levels. These effects are most likely due to the secretion of paracrine factors by CD34+ cells, such as exosomes containing pro-angiogenic miRNAs (126, 130a, 378, 26a) and anti-apoptotic miRNAs (21 and 146a) and growth factors that promote angiogenesis such as VEGF. CD34+ cells might also prevent the formation of fibrocartilage as they secrete exosomes containing anti-fibrotic miRNAs (133a, 24, 29b, 132).

The population of CD34+ cells of the invention may express CD44. CD44 is a receptor of hyaluronic acid, which is abundantly present in the synovial membrane. This increases the chances of CD34+ cell engraftment in the arthritic joint and therefore increases their therapeutic efficacy.

The population of CD34+ cells may be for use in a method of treating osteoarthritis, wherein the CD34+ cells increase the expression of chondrogenic markers by chondrocytes. The chondrogenic markers may comprise or consist of collagen II, aggrecan, and/or Sox9.

The population of CD34+ cells may be for use in a method of treating osteoarthritis, wherein the CD34+ cells decrease the expression of inflammatory and/or degrading markers by chondrocytes. The inflammatory and/or degrading markers may comprise or consist of any one of IL1β, IL-8, ADAMTS-4, TNF, MMP-1, and/or MMP-13 or any combination thereof.

The population of CD34+ cells may be for use in a method of treating osteoarthritis, wherein the CD34+ cells increase the expression of chondrogenic markers and/or decrease the expression of inflammatory and/or degrading markers by chondrocytes. The chondrogenic markers comprise or consist of collagen II and/or Sox9. The inflammatory and/or degrading markers may comprise or consist of any one of IL1β, IL-8, ADAMTS-4, TNF, MMP-1, and/or MMP-13 or any combination thereof. The increase or decrease of expression of the markers disclosed herein may be at the gene and/or protein level.

Also provided is a population of CD34+ cells for use in a method of increasing the expression of chondrogenic markers by chondrocytes in a subject. The method may comprise administering CD34+ cells to a subject in an area populated by chondrocytes. The chondrogenic markers may comprise or consist of collagen II, aggrecan, and/or Sox9. The CD34+ cells may be administered to a subject in an area affected by osteoarthritis.

Also provided is a population of CD34+ cells for use in a method of decreasing the expression of inflammatory and/or degrading markers by chondrocytes in a subject. The inflammatory and/or degrading markers may comprise or consist of any one of IL1β, IL-8, ADAMTS-4, TNF, MMP-1 and/or MMP-13 or any combination thereof. The CD34+ cells may be administered to a subject in an area affected by osteoarthritis.

Also provided is a population of CD34+ cells for use in a method of increasing the expression of chondrogenic markers and/or decreasing the expression of inflammatory and/or degrading markers by chondrocytes in a subject. The chondrogenic markers comprise or consist of collagen II and/or Sox9. The inflammatory and/or degrading markers may comprise or consist of any one of IL1β, IL-8, ADAMTS-4, TNF, MMP-1, and/or MMP-13 or any combination thereof. The CD34+ cells may be administered to a subject in an area affected by osteoarthritis.

Also provided is a population of CD34+ cells for use in a method of inducing pro-anabolic effects on chondrocytes in a subject. The method may comprise administering CD34+ cells to a subject in an area populated by chondrocytes. The CD34+ cells may be administered to a subject in an area affected by osteoarthritis.

Also provided is a population of CD34+ cells for use in a method of treating osteoarthritis, wherein the CD34+ cells are administered to a subject in an area affected by osteoarthritis, wherein the CD34+ cells secrete paracrine factors in said area thereby treating osteoarthritis. Paracrine factors may include one or more of pro-angiogenic miRNAs (e.g. miRNA 126, 130a, 378, and/or 26a), anti-apoptotic miRNAs (e.g. miRNA 21 and/or 146a), anti-fibrotic miRNAs (133a, 24, 29b, 132), and growth factors such as VEGF.

Also provided is a population of CD34+ cells for use in a method of treating osteoarthritis, wherein the CD34+ cells are administered to a subject in an area affected by osteoarthritis, wherein the CD34+ cells promote chondrogenesis in said area thereby treating osteoarthritis.

Also provided is a population of CD34+ cells for use in a method of stimulating chondrogenesis in a subject. The method may comprise administering CD34+ cells to a subject in an area populated by chondrocytes. The CD34+ cells may be administered to a subject in an area affected by osteoarthritis.

Also provided is a population of CD34+ cells for use in a method of modulating expression of inflammatory and/or pro-anabolic genes by chondrocytes in a subject. The method may comprise

administering CD34+ cells to a subject in an area populated by chondrocytes. The CD34+ cells may be administered to a subject in an area affected by osteoarthritis.

Any of the methods disclosed herein may comprise one or more of the following steps:

- administering a hematopoietic growth factor, such as G-CSF, to a subject,
- collecting a peripheral blood sample from the subject,
- isolating and/or expanding CD34+ cells from the blood sample to obtain a population of CD34+ cells, and/or
- administering the population of CD34+ cells to the subject.

The population of CD34+ cells for use in the invention may be an isolated and/or purified/ and/or cultured and/or expanded population of CD34+ cells (eCD34+).

Patient groups

The population of CD34+ cells for use in the methods disclosed herein are administered to a subject. The subject may be in need of treatment for osteoarthritis. The subject may be a mammalian subject, such as human, horse, dog, cat. The subject may be a human.

The Kellgren-Lawrence scale (as described in Kohn et al.) is a method of classifying the severity of osteoarthritis using five grades:

- Grade 0 (none)
- Grade 1 (doubtful)
- Grade 2 (minimal)
- Grade 3 (moderate)
- Grade 4 (severe)

The CD34+ cells may be for use in a method of treating grade 1, grade 2, grade 3 or grade 4 osteoarthritis according to the Kellgren-Lawrence scale. In a preferred embodiment, the CD34+ cells are for use in a method of treating grade 2 or grade 3 osteoarthritis according to the Kellgren-Lawrence scale.

The population of CD34+ cells may be for use in a method of treating osteoarthritis in a patient aged 30 years or older, aged 40 years or older, aged 50 years or older, aged 60 years or older, aged 70 years or older or aged 80 years or older. In a preferred embodiment, the population of CD34+ cells is for use in a method of treating osteoarthritis in a patient aged 40 years or older.

Isolation of CD34+ cells

The CD34+ cells of the invention may be autologous or allogeneic CD34+ cells. Preferably, the CD34+ cells are autologous. The CD34+ cells are preferably obtained from a whole blood sample. The whole

blood sample may have a volume in the range 250-440 mL ± 10 mL. Alternatively, the CD34+ cells may also be obtained from umbilical cord blood. The whole blood sample may be obtained from a donor subject. The donor subject may be a subject to be treated with a population of CD34+ cells according to the invention. The donor subject may be a human. The donor subject may be a human in need of treatment. The donor subject may be a human in need of treatment for osteoarthritis.

The whole blood sample may be obtained following granulocyte-colony stimulating factor (G-CSF) mobilisation. The whole blood sample may be subjected to red blood cell sedimentation. The whole blood sample may be subjected to total nuclear cell isolation. Total nuclear cell isolation may follow the gelatin method whereby the whole blood sample is mixed with a gelatin solution and hung for a period of time to facilitate red blood cell sedimentation. The red blood cells remaining in the pellet may be mixed with gelatin and hung for a second period of time. Following sedimentation, the supernatant may be centrifuged to pellet the total nuclear cells. Following centrifugation, the CD34+ cells may be purified by immunoselection. Immunoselection may be performed by any known method, for example with the CliniMACS system (Magnetic-Activated Cell Sorting).

Where allogeneic CD34+ cells are used in the invention, the cells may be genetically modified in order to reduce, minimise or avoid host rejection. For example, cells may be modified by techniques and tools known in the art, such as CRISPR- based systems, TALEN, or ZFN, to inhibit the expression of proteins that play a role in transplant rejection, such as HLA/MHC and related proteins

Where allogeneic CD34+ cells are used in the invention, further therapeutic agents may be administered in combination with the CD34+ cells. For example, the CD34+ cells may be administered with an immunosuppressive agent, such as cyclosporin A.

Culture or Expansion of CD34+ cells

The purified CD34+ cells may be cultured and/or expanded. The CD34+ cells may be cultured and/or expanded for 5 to 12 days. The CD34+ cells may be cultured and//or expanded for 5, 6, 7, 8, 9, 10, 11, or 12 days. The CD34+ cells may be cultured and/or expanded for 9 days. The CD34+ cells may be cultured and/or expanded at 37°C. The CD34+ cells may be cultured and/or expanded in a 5% CO_2 controlled atmosphere. The CD34+ cells may be cultured and/or expanded in a culture medium comprising cytokines such as interleukin 6 (IL6), interleukin 3 (IL3), Stem Cell Factor, ThromboPoietin, and Fms-Like Tyrosin kinase 3 Ligand at various concentrations. Cells may be cultured at any suitable concentration, for example 2.5 x 10^5 cells/mL.

The invention may be carried out using human autologous or allogeneic CD34+ cells expanded according to a GMP automated manufacturing process designed for large clinical scale production, e.g. ProtheraCytes®.

The CD34+ cells for use in the invention may be a cultured and/or expanded cell population. The cultured or expanded CD34+ cell population may be produced by the methods disclosed herein.

Additional processing steps

Following the culture or expansion of CD34+ cells as described above, the CD34+ cells may undergo further processing steps, such as, for example, purification and/or immunoselection.

The CD34+ cells may be immunoselected for example using magnetic activated cell sorting. The immunoselected CD34+ cells may then be resuspended in a buffer, for example 15ml. The buffer may comprise albumin and/or saline and/or PBS. The buffer may comprise or consist of 2%, 3% or 4% albumin in saline. In an embodiment, the buffer is 4% albumin in saline. The buffer may be phosphate buffered saline (PBS)/2% human serum albumin (HSA). The cells may be resuspended in 15 ml PBS/2% HSA. The cell suspension may be conditioned in multiple syringes. For example, 15ml suspension may be conditioned in three syringes of 5ml each. The CD34+ cell population for use according to the present invention may be provided as a population of cells suspended in buffer. The population of CD34+ cells for use in the invention may be provided as a suspension in, for example, PBS/2% human serum albumin.

Said processing may be to form a product suitable for use in the invention. For example, ProtheraCytes® are CD34+ cells which have been processed to form a product suitable for use in a method of treatment. ProtheraCytes® are registered as an ATMP (Advanced Therapy Medicinal Product) within the classification of Tissue Engineered product by the European Medicines Agency. A process for making ProtheraCytes® is set out in Example 1.

Populations of CD34+ cells for use in the invention

The population of CD34+ cells for use in the invention may be isolated from a blood sample. A population of CD34+ cells that has been isolated from a blood sample may be referred to as an “isolated population of CD34+ cells”.

The population of CD34+ cells for use in the invention may be a cultured and/or expanded and/or purified population of CD34+ cells. Methods for culturing, expanding and purifying CD34+ cells are disclosed herein.

The population of CD34+ cells for use in the invention may have a CD34+ cell viability of at least about 70%, 75%, 80%, 85%, 90%, 95% or 99%. In a preferred embodiment the population of CD34+ cells for use in the methods of the invention have a CD34+ cell viability of at least about 95%.

The population of CD34+ cells for use in the invention may have a CD34+ cell purity of at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99%. In a preferred embodiment the population of CD34+ cells for use in the methods of the invention have a CD34+ cell purity of at least about 80%.

The population of CD34+ cells for use in the invention may comprise less than or equal to about 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 3%, 2% or 1% monocytes. In a preferred embodiment the population of CD34+ cells for use in the methods of the invention comprise less than or equal to about 15% monocytes.

The population of CD34+ cells for use in the invention may comprise less than or equal to about 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 3%, 2% or 1% granulocytes. In a preferred embodiment the population of CD34+ cells for use in the invention comprises about 5% or less granulocytes.

The population of CD34+ cells for use in the invention may comprise less than or equal to about 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 3%, 2% or 1% lymphocytes. In a preferred embodiment the population of CD34+ cells for use in the invention comprises about 3% or less lymphocytes.

The population of CD34+ cells for use in the invention may comprise at least about $2x10^6$ CD34+ cells, at least about $3x10^6$ CD34+ cells, at least about $4x10^6$ CD34+ cells, at least about $5x10^6$ CD34+ cells, at least about $6x10^6$ CD34+ cells, at least about $7x10^6$ CD34+ cells, at least about $8x10^6$ CD34+ cells, at least about $9x10^6$ CD34+ cells, or at least about $10x10^6$ CD34+ cells. Preferably, the population of CD34+ cells for use in the invention comprises at least about $8x10^6$ CD34+ cells.

The population of CD34+ cells for use in the invention may comprise about $2x10^6$ CD34+ cells, about $3x10^6$ CD34+ cells, about $4x10^6$ CD34+ cells, about $5x10^6$ CD34+ cells, about $6x10^6$ CD34+ cells, about $7x10^6$ CD34+ cells, at least about $8x10^6$ CD34+ cells, about $9x10^6$ CD34+ cells, or about $10x10^6$ CD34+ cells. Preferably, the population of CD34+ cells for use in the invention comprises about $8x10^6$ CD34+ cells.

In an embodiment, the CD34+ cell population for use in the invention has one or more of the following features:

(a) about $8x10^6$ CD34+ cells,
(b) a CD34+ cell viability of at least about 95%,
(c) a CD34+ cell purity of at least about 80%,
(d) about 15% monocytes or lower,
(e) about 5% granulocytes or lower, and
(f) about 3% lymphocytes or lower.

In an embodiment the CD34+ cell population for use in the invention has all of features (a)-(f) listed above.

CD34+ cell viability and purity can be determined by any suitable method known in the art. For example, cell viability and purity can be determined via flow cytometry. Kits enabling reliable enumeration of CD34+ stem cells are commercially available, for example the BD® Stem Cell Enumeration Kit (BD Biosciences). Cell counts may be performed at any stage of the manufacturing process, for example, on WB, before a first round of immunoselection, before expansion, before a second round of

immunoselection, at the end of the process as in-process controls or any combination thereof. Cell counts may be performed using the Stem Cell Enumeration kit and the Stem Cell Control kit (both from BD Biosciences, San Jose, CA) and analyzed with a Fluorescence Activated Cell Sorting (FACS) Canto II analyzer (BD Biosciences) and FACS DIVA software following the manufacturer's instructions and ISHAGE guideline (Gratama et al). The proportion of immature CD34+ cells can be determined by analysing CD133+ coexpression as described in Saucourt et al. The percentage of cell impurities may be determined as described in Saucourt et al.

Expression characteristics of populations of CD34+ cells

The population of CD34+ cells for use in the invention may express VEGF in a culture medium in an amount indicative of biological and/or therapeutic activity or efficacy as described below. The CD34+ for use in the methods of the invention may express miRNAs indicative of biological and/or therapeutic activity as described below. The CD34+ cells for use in the methods of the invention may express both VEGF in a culture medium in an amount indicative of biological and/or therapeutic activity or efficacy and miRNAs indicative of biological and/or therapeutic activity as described below.

The population of CD34+ cells for use in the invention may express CD44.

Determination of VEGF expression

The population of CD34+ cells of the invention may be assayed to determine VEGF expression levels. The amount of VEGF expressed by a population of CD34+ cells may be determined by any known means, for example, by western blot, enzyme-linked immunosorbent assay (ELISA), fluorescence-linked immunosorbent assay (FLISA), competition assay, radioimmunoassay, lateral flow immunoassay, flowthrough immunoassay, electrochemiluminescent assay, nephelometric-based assays, turbidometric-based assay, or fluorescence activated cell sorting (FACS)-based assays. Determination of the amount of VEGF may be by mass spectrometry. Determination of the amount of VEGF may be by radioimmunoassay. Preferably, determination of the amount of VEGF is by ELISA. For example, determining the amount of VEGF expressed by a population of CD34+ cells may involve one or more of the following:

- collecting supernatant from CD34+ cell cultures;
- storing the supernatant;
- measuring VEGF using an ELISA kit e.g. QuantiGlo ELISA Kit (R&D Systems, MN, USA) according to the manufacturer's instructions, for example with the SpectraMax L (Molecular Devices, San Jose, CA USA).

A negative control may be used, for example, a culture medium such as StemFeed® medium. A positive control may be used, for example, Immunoassay Control Set 732 for Human VEGF (R&D Systems). The amount of VEGF expressed by a population of CD34+ cells may be determined by measuring the concentration of VEGF released by the cells into a cell culture medium. The amount of VEGF expressed

by a population of CD34+ cells may be determined by measuring the concentration of VEGF contained in CD34+ cell derived exosomes. The CD34+ cell culture or a portion thereof may be centrifuged and the concentration of VEGF present in the supernatant determined. For example, approximately 50 mL of supernatant may be obtained and frozen as smaller aliquots. Typically, 50 µL of sample per well in the ELISA assay may be used.

VEGF expression of CD34+ cells

The population of CD34+ cells for use in the methods of the invention may express VEGF in a culture medium in an amount indicative of biological and/or therapeutic activity or efficacy. For example, CD34+ cells for use in the methods of the invention may express VEGF in a culture medium or supernatant of a CD34+ cell culture in an amount of about 1, 5, 10, 20, 25, 50, 75, 100, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195 or 200 pg/ml. The CD34+ cells for use in the methods of the invention may express VEGF in a culture medium or supernatant of a CD34+ cell culture in an amount of at least 25 pg/ml. The CD34+ cells for use in the methods of the invention may express VEGF in a culture medium or supernatant of a CD34+ cell culture in an amount of at least 50 pg/ml. The CD34+ cells for use in the methods of the invention may express VEGF in a culture medium or supernatant of a CD34+ cell culture in an amount of at least 150 pg/ml. If the concentration of VEGF contained in CD34+ cell derived exosomes is measured, the CD34+ cells may express VEGF in the exosomes at any threshold value disclosed herein.

The amount of VEGF expressed by a population of CD34+ cells may be determined by measuring an amount of VEGF expressed as an amount per cell. The CD34+ cell culture or a portion thereof may be centrifuged and the amount of VEGF present in the supernatant determined. The number of CD34+ cells may be counted by any known method, for example flow cytometry, and the amount of VEGF may be expressed as an amount per cell. The CD34+ cells for use in the invention may express VEGF in an amount per cell of at least about $0.5x10^{-3}$ pg, at least about $1x10^{-3}$ pg, at least about $1.5x10^{-3}$ pg, at least about $2x10^{-3}$ pg, at least about $2.5x10^{-3}$ pg, or at least about $3x10^{-3}$ pg. The CD34+ cells for use in the invention may express VEGF in an amount per cell of at least about $2x10^{-3}$ pg.

Detection of miRNA expression

The population of CD34+ cells of the invention may be assayed to determine miRNA expression. The expression of miRNA in a population of CD34+ cells may be detected by measuring miRNA expression in CD34+ cells and/or in CD34+ cell derived exosomes. miRNA may be isolated from exosomes and/or cells and measured.

Exosomes may be purified by centrifugation of CD34+ cells to remove the cells and cell debris. The resulting supernatant may then be centrifuged again to pellet the exosomes. For example, 50 mL of culture supernatant may be collected, and may be frozen into smaller aliquots. Smaller volumes, such as, 200 µL of sample, may be used for extracting miRNA from the exosomes. The miRNA may then be extracted from the exosomes by any known method, such as the use of a commercially available miRNA extraction kit. miRNA may be extracted from CD34+ cells by any known method, such as the use of a

commercially available miRNA extraction kit. One example of a commercially available miRNA extraction kit is the Qiagen® miRNeasy® kit.

The miRNAs may be detected and/or quantified by any known method. For example, the miRNAs may be detected and quantified by Quantitative real-time PCR (RT-qPCR), digital PCR, microarray, and/or high-throughput small RNA-sequencing. One example of a commercially available kit for miRNA detection and quantification is the Qiagen® miRCURY LNA miRNA PCR kit. Suitable primers available from Qiagen® include YP00204230 (miR-21-5p), YP00206023 (miR-26a-5p), YP00204227 (miR-126-3p), YP002046658 (miR-130a-3p), YP00204788 (miR-133a-3p), YP00204688 (miR146a-5p), YP00204536 (miR-199a-3p), YP00205946 (miR-378a-3p), and YP00205448 (miR-590-3p). The qPCR data may be normalized to miR-let7a-5p (YP00205727) values. Relative miRNA expressions may be calculated using the $2^{-\Delta\Delta Ct}$ method.

The miRNAs disclosed herein may be detected by, for example, detecting either a 3p and/or a 5p miRNA strand. For example, the miRNAs disclosed herein may be detected via any of the following strands:

miR126-3p UCGUACCGUGAGUAAUAAUGCG (SEQ ID NO: 1)
miR126-5p CAUUAUUACUUUUGGUACGCG (SEQ ID NO: 10)
miR130a-3p CAGUGCAAUGUUAAAAGGGCAU (SEQ ID NO: 2)
miR130a-5p GCUCUUUUCACAUUGUGCUACU (SEQ ID NO: 11)
miR21-3p CAACACCAGUCGAUGGGCUGU (SEQ ID NO: 16)
miR21-5p UAGCUUAUCAGACUGAUGUUGA (SEQ ID NO: 3)
miR26a-3p CCUAUUCUUGGUUACUUGCACG (SEQ ID NO: 17)
miR26a-5p UUCAAGUAAUCCAGGAUAGGCU (SEQ ID NO: 4)
miR378a-3p ACUGGACUUGGAGUCAGAAGGC (SEQ ID NO: 5)
miR378a-5p CCUCCUGACUCCAGGUCCUGUGU (SEQ ID NO: 12)
miR146a-3p CCUCUGAAAUUCAGUUCUUCAG (SEQ ID NO: 18)
miR146a-5p UGAGAACUGAAUUCCAUGGGUU (SEQ ID NO: 6)
miR199a-3p ACAGUAGUCUGCACAUUGGUUA (SEQ ID NO: 7)
miR199a-5p CCCAGUGUUCAGACUACCUGUUC (SEQ ID NO: 13)
miR590-3p UAAUUUUAUGUAUAAGCUAGU (SEQ ID NO: 8)
miR590-5p GAGCUUAUUCAUAAAAGUGCAG (SEQ ID NO: 14)
miR133a-3p UUUGGUCCCCUUCAACCAGCUG (SEQ ID NO: 9)
miR133a-5p AGCUGGUAAAAUGGAACCAAAU (SEQ ID NO: 15)
miR24-1-5p UGCCUACUGAGCUGAUAUCAGU (SEQ ID NO: 19)
miR24-2-5p UGCCUACUGAGCUGAAACACAG (SEQ ID NO: 20)
miR24-3p UGGCUCAGUUCAGCAGGAACAG (SEQ ID NO: 21)
miR132-5p ACCGUGGCUUUCGAUUGUUACU (SEQ ID NO: 22)
miR132-3p UAACAGUCUACAGCCAUGGUCG (SEQ ID NO: 23)
miR29b-1-5p GCUGGUUUCAUAUGGUGGUUUAGA (SEQ ID NO: 24)
miR29b-2-5p CUGGUUUCACAUGGUGGCUUAG (SEQ ID NO: 25)

miR29b-3p UAGCACCAUUUGAAAUCAGUGUU (SEQ ID NO: 26)

miRNA expression of CD34+ cells for use in the methods of the invention

The population of CD34+ cells for use in the method of the invention may express miRNA indicative of biological and/or therapeutic activity or efficacy. For example, the population of CD34+ cells for use in the method of the invention may express one or more or each of miR126, miR130a, miR21, miR26a, miR378a, miR146a, miR199a, miR590, miR133a, miR-24, miR29b, and miR132.

The population of CD34+ cells for use in the invention may express one or more or each of miR126, miR130a, miR21, miR26a, and miR378a.

The population of CD34+ cells for use in the invention may express one or more or each of miR21, miR26a, and miR378a. The population of CD34+ cells for use in invention may express miR146a and/or miR21. The population of CD34+ cells for use in the invention may express miR199a and/or miR590. The population of CD34+ cells for use in the invention may express miR133a. The miRNA may be expressed in the cell and/or in exosomes. CD34+ cells for use in the invention may secrete exosomes containing one or more (for example, all) of the miRNAs disclosed herein.

Delivery, doses and dosage regimes

The population of CD34+ cells for use in the invention may be provided as a sterile suspension of cells. The sterile suspension is preferably aqueous. The sterile suspension is preferably isotonic. The sterile suspension may further comprise pharmaceutically acceptable carriers, diluents or wetting agents. The population of CD34+ cells may be provided as a pharmaceutical composition comprising one or more additional agents such as pharmaceutically acceptable carriers, buffers, diluents or wetting agents. Pharmaceutical compositions disclosed herein may include a population of CD34+ cells and a buffer. The buffer may comprise albumin and/or saline and/or PBS. The buffer may comprise or consist of 2%, 3% or 4% albumin in saline. In an embodiment, the buffer is 4% albumin in saline. The buffer may be phosphate buffered saline (PBS)/2% human serum albumin (HSA). The CD34+ cell population for use according to the present invention may be provided as a population of cells suspended in buffer. The population of CD34+ cells for use in the invention may be provided as a suspension in, for example, PBS/2% human serum albumin.

The population of CD34+ cells for use in the invention may be administered to a subject by injection. The injection may be into the joint affected by osteoarthritis. The injection may be intraarticular. Cells may be administered at a single injection site in the joint. The injection site may be the supero-external corner of the lateropatellar region of the knee joint.

For example, an injection protocol may comprise one or more, for example all, of the following steps:

1. Connect a sterile 18 -20 gauge steel needle to a syringe,

2. Introduce the needle into the joint. In the case of the knee joint, the injection site may be the supero-external corner of the lateropatellar region of the knee joint;
3. Remove synovial fluid if necessary;
4. Connect successively syringes (for example 3 syringes) containing CD34+ cells to smoothly perform their intra-articular injection;
5. Compress the injection point with a sterile compress for about 1-5 minutes, while gently and passively performing several flexion/ extension exercises of the joint;
6. Place on the injection site an adhesive bandage (e.g. sticking plaster) to be kept in place for 24 hours;

Keep the patient at rest for ½ hour to allow a good diffusion of the cells into the whole joint.

The number of CD34+ cells administered per dose may be about $2x10^6$, about $3x10^6$, about $4x10^6$, about $5x10^6$, about $6x10^6$, about $7x10^6$, about $8x10^6$, about $9x10^6$, or about $10x10^6$. Preferably, the number of CD34+ cells administered per dose is about $8x10^6$. Cells may be counted by any suitable method, such as flow cytometry. Kits enabling reliable enumeration of CD34+ stem cells are commercially available, for example the BD® Stem Cell Enumeration Kit (BD Biosciences).

The number of CD34+ cells administered per dose may be at least about $2x10^6$, at least about $3x10^6$, at least about $4x10^6$, at least about $5x10^6$, at least about $6x10^6$, at least about $7x10^6$, at least about $8x10^6$, at least about $9x10^6$, or at least about $10x10^6$. Preferably, the number of CD34+ cells administered per dose is at least about $8x10^6$.

The volume administered per dose may be about 1 ml, about 2 ml, about 3 ml, about 4 ml, about 5 ml, about 10 ml, about 15 ml, about 20 ml about 25 ml or about 30 ml. In a preferred embodiment, the volume administered per dose is about 15 ml. The 15 ml may be conditioned in three syringes of 5 ml each.

One or more doses of isolated CD34+ cells may be given to a subject. For example, 1 dose, 2 doses, 3 doses, 4 doses, or 5 doses of isolated CD34+ cells may be given to a subject. In a preferred embodiment, 1 dose of isolated CD34+ cells is given to a subject. Successive doses may be separated by at least one month, for example 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, or longer.

Incorporation by reference

All documents cited herein are incorporated by reference to the fullest extent permitted by law.

Examples

Example 1: Obtaining CD34+ cells

Patient Samples, Healthy Donors and Cell-Production Centers

AMI patients and healthy male volunteers were enrolled in this study after approval by the French regulatory agency Agence Nationale de Sécurité du Médicament et des produits de santé and the regional ethics committee. All participants provided signed informed consent. Each participant first underwent daily subcutaneous (s.c.) administration of 10 µg/kg per day G-CSF (Lenograstim) for 4 days. A whole blood (WB) sample of 250-440 mL ± 10 mL was withdrawn in the morning of the fifth day by simple venous puncture and collected in a blood bag and immediately shipped at ambient temperature to the Cell Production Centre. The manufacturing process was started on the sixth day, after overnight storage of the WB sample at 4°C-8°C C, using the StemXpand® automated integrated system and StemPack® disposable kits developed by CellProthera®.

ProtheraCytes® Preparation

Starting from the initial WB sample, red blood cell (RBC) sedimentation was performed for total nuclear cell (TNC) isolation using the gelatin method. Briefly, 250-440 mL of WB/phosphate-buffered saline 1:1 solution (PBS; Macopharma, Mouvaux, France) was mixed with the same volume of (250-440 mL) of 4% gelatin (Gelofusine, BBraun, Melsungen, Germany) in two 600-ml transfer bags, which were hung for 20 minutes to facilitate RBC sedimentation. RBCs remaining in the pellet were again mixed with 4% gelatin for a second 20-minute sedimentation period. The two supernatants were pooled and centrifuged at 400g for 10 minutes at room temperature to pellet the TNC, from which basal (b)-CD34+ stem cells (SCs) were purified using the CliniMACS system (Magnetic-Activated Cell Sorting, Miltenyi Biotec, Bergisch Gladbach, Germany). The bag containing purified b-CD34+ SC suspension or thawed frozen healthy donor (FHD) CD34+ cells (Lonza), was immediately connected to the machine kit to undergo a 9-day culture period in our proprietary StemFeed® medium into the StemXpand® incubator, in which the expansion steps are automatically programmed and controlled: first, predetermined volumes of StemFeed® culture medium, cytokine mix (composed of interleukin IL6, IL3, Stem Cell Factor, ThromboPoietin, and Fms-Like Tyrosin kinase 3 Ligand at various concentrations), and the CD34+ SCs were successively distributed into the dedicated culture bag placed on the agitator contained in the device incubator. The bag was then gently agitated for 30 seconds to disperse the cell mixture, which was then incubated at 37°C in a 5% CO_2-controlled atmosphere for a 9-day cell expansion period, without any further intervention. At the end of incubation, the cell suspension was dispersed by gentle agitation, followed by adjustment of the agitator tray to an 80° inclination to facilitate distribution of the cell suspension into two collection bags in equal volumes. Samples were collected at day 0 and day 7 to analyze sterility after dispersing the cell suspension and 50° inclination of the agitator tray.

At the end of the 9-day period, the culture product was collected, centrifuged, and immunoselected using the CliniMACS system for the purification of expanded (e)CD34+ SC, which constituted the final product (ProtheraCytes®) once resuspended in 15 ml PBS/2% human serum albumin (HSA) and conditioned in three syringes of 5 ml each.

Example 2: *in vitro* and murine cartilage models

In this study, we decided to investigate the therapeutic potential of ProtheraCytes® in an *in vitro* OA cartilage model. We co-cultured ProtheraCytes® with human OA chondrocytes in medium containing pro-inflammatory cytokines (Acevedo Rua et al., 2021) to investigate the survival of ProtheraCytes® and the capacity of ProtheraCytes® to modulate the inflammatory/degrading state of the chondrocytes. We also evaluated the survival and engraftment of ProtheraCytes® after intra-articular (IA) injection in the murine model of collagenase-induced osteoarthritis (CIOA) to determine the best timing of cell injection for future therapeutic efficacy studies in this animal model.

Materials and Methods

ProtheraCytes® culture

ProtheraCytes® were obtained after expansion of mobilized CD34+ cells from frozen healthy donors (Lonza, NC, USA) as previously described (Saucourt et al., 2019). ProtheraCytes® were conditioned in 2% human serum albumin (HSA) in phosphate buffered saline (PBS) in sterile prelabelled syringes and shipped the same day in a refrigerated box to the Cartilage Engineering laboratory at the University Hospital Basel for analysis.

Coculture of ProtheraCytes® with OA synovial fluid

ProtheraCytes® resuspended in 2% HSA/PBS were incubated with four different concentrations (0, 10, 20 and 50%) of synovial fluid from six OA patients (donor 1: female, 73y; donor 2, male, 70y; donor 3, male, 79y; donor 4, male, 67y; donor 5, female, 70y; donor 6, male, 69y) and for five different timepoints (1, 3, 6, 24, and 96 hours). The synovial fluid samples were provided by Dr. S. Lefebvre, MD from the Mulhouse Institute of the Musculoskeletal System after signed informed consent. Cell number and viability were assessed with the Stem Cell Enumeration kit (BD) via flow cytometry with FACS Canto II and FACSDiva software (BD Biosciences).

Cartilage sample collection

Macroscopically fibrillated human articular cartilage was obtained from the knee joints of 4 donors (donor 1: male, 63y; donor 2: male, 52y; donor 3: female, 56y; donor 4: male, 76y) with clinical history of OA who were undergoing total knee replacement, after informed consent from patients and in accordance with the Institutional Ethics Committee (University Hospital Basel, Switzerland).

Chondrocyte isolation and expansion

OA chondrocytes were isolated from native tissues (cartilage samples) after 22 hour enzymatic digestion in 1.5 mg/mL collagenase as previously described (Barbero et al., 2004). Isolated cells were resuspended in Complete medium consisting of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 10 mM Hepes buffer, 1mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.29 mg/ml L-glutamine (all from Gibco) and seeded at a density of 10,000 cells/cm^2

and cultured in complete medium supplemented with 1 ng/ml Transforming Growth Factor-β1 (TGF-β1) and 5 ng/ml Fibroblast Growth Factor-2 (FGF-2). When cells were approximately 80% confluent, first passage (P1) cells were detached using 0.05% trypsin/0.53 mM EDTA and frozen in complete medium containing 10% dimethylsulfoxide.

Chondrocyte/ ProtheraCytes® co-culture experiment

Passage 1 OA chondrocytes were thawed and seeded in 24 well plates at a density of 10,000 cells/cm^2 and cultured one additional week in complete medium. ProtheraCytes® sent to the Cartilage Engineering laboratory of the University Hospital Basel were centrifuged, counted, and seeded onto 0.4 µm pore size polycarbonate Transwell filters (Corning B.V. Life Science) at a density of 100,000 (0.1M) and 250,000 (0.25M) cells/insert. Control groups consisted of only OA chondrocytes (in the well) or ProtheraCytes® (in the insert). In the first experiment with OA chondrocytes from one donor (donor 1) and one ProtheraCytes® batch, cells were co-cultured for 6h, 24h, and 48h in complete medium containing 5% FBS in the absence (-INFL) or presence of inflammatory cytokines (+INFL: 50 pg/ml Interleukin (IL)-1β, 100 pg/ml IL-6 and 50 pg/ml tumour necrosis factor (TNFα) (Acevedo Rua et al., 2021). In the second experiment, OA chondrocytes from three additional donors (donor 2-4) and two ProtheraCytes® batches were co-cultured only for 24h, after this timepoint was selected from the first experiment. Three replicates/group were analysed. At the end of the experiments, supernatants were collected and stored at -80°C for subsequent quantification of cytokines; OA chondrocytes were counted with trypan blue and processed for RT-PCR analyses, and ProtheraCytes® were assessed by flow cytometry.

Flow cytometry analysis

ProtheraCytes® were washed with PBS and stained with 0.1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (ThermoFisher) in PBS containing 0.1% bovine serum albumin (BSA) and 2 mM EDTA. ProtheraCytes® were analyzed on the Cytoflex flow analyzer (BD), and the frequency of dead cells was measured.

Quantitative real-time RT-PCR

RNA was extracted from OA chondrocytes using the Quick RNA mini prep kit (Zymo), according to the manufacturer's instructions. Complementary DNA (cDNA) was generated from the maximum amount of RNA possible for each sample (from 200-1000 ng) by using 500 µg/ml random hexamers (Promega, Madison, WI) and 0.5 µl of 200 units/ml Reverse transcriptase superscript III (Invitrogen), in the presence of dNTPs and DTT. The PCR was based on TaqMan reaction using the TaqMan mix (Thermo Fisher scientific). cDNA samples (2 µl, for a total volume of 10 µl per reaction) were analysed both for gene of interest ACAN (Hs00153936), Col1A1 (Hs00164004), Col2A1 (Hs00264051), Col10A1 (Hs00166657), MMP-1 (Hs00233958), MMP-13 (Hs00233992), SOX9 (Hs00165814), CXCL8 (Hs00174103), IL1β (Hs01555410), IL6 (Hs00985639), ADAMTS-4 (Hs00192708), ADAMTS-5 (Hs00199841) and for the housekeeping gene GAPDH (Hs2758991) (all from Applied Biosystems). For each cDNA sample, the threshold cycle (Ct) value of each target sequence was subtracted from the Ct value of the reference

gene, to derive ΔCt. The level of expression of each target gene was then calculated as 2ΔCt. Each sample was assessed at least in duplicate for each gene of interest.

Quantification of secreted proteins

Supernatants collected after the coculture experiments were centrifuged for 4 min at 16,000xg to remove cellular debris. The concentrations of IL1β, IL6, IL8/CXCL8, RANTES/CCL5, TNFα and VEGF were quantified by Luminex Magnetic Assay (R&D Systems), while the concentrations of ADAMTS-4 and ADAMTS-5, MMP-1 and MMP-13 were measured using DuoSet ELISA systems, according to manufacturer's instruction.

In vitro assessment of ProtheraCytes® after delivery with a needle for intra-articular injection

We evaluated the viability, purity, and number of ProtheraCytes® before and after delivery with a needle for knee injection in humans (21G, 2" BD301155) and mice (20G, 25 mm BD304827). These parameters were evaluated with the Stem Cell Enumeration kit (BD) via flow cytometry with FACS Canto II and FACSDiva software (BD Biosciences).

Murine model of collagenase-induced osteoarthritis

The collagenase-induced osteoarthritis (CIOA) model was induced by collagenase injection (type VII collagenase from Clostridium histolyticum; 1 U in 5 µL saline solution) in the intra-articular space of knee joints of severe combined immunodeficient (SCID) Beige immunodeficient male mice at 8 weeks of age, at day 0 and day 2. This treatment induces ligament laxity and knee instability leading to osteoarthritic like lesions. ProtheraCytes® were injected intra-articularly in the knee joint of mice at day 8 or day 29 after OA induction. ProtheraCytes® were injected at the dose of 250,000 cells in 5 µL of 5% HSA in physiological serum. Mice were euthanised 1 day, 7 days or 21 days after cell injection. Therefore the mice were divided in six groups with four mice per group: Group 1 (collagenase + ProtheraCytes® at day 8, euthanasia at day 9); Group 2 (collagenase + ProtheraCytes® at day 8, euthanasia at day 15); Group 3 (collagenase + ProtheraCytes® at day 8, euthanasia at day 29); Group 4 (collagenase + ProtheraCytes® at day 29, euthanasia at day 30); Group 5 (collagenase + ProtheraCytes® at day 29, euthanasia at day 36); Group 6 (collagenase + ProtheraCytes® at day 29, euthanasia at day 50). Mice were housed in cages with unlimited access to food and water in a specific pathogen-free animal facility under controlled atmosphere. At euthanasia, hind paws were collected and fixed in 3.7% formaldehyde for 4 days at ambient temperature. Hind paws were then rinsed twice in PBS and stored at 4°C in PBS until histological processing.

Immunohistochemistry staining of ProtheraCytes®

Hind paws were decalcified in TBD-2 solution (Fisher Scientific) for 24 hours at room temperature and then rinsed in PBS before inclusion in paraffin. After paraffin inclusion of samples, three frontal sections of 7 µm in thickness and spaced by 100 µm were recovered from each sample. Sections were then immunostained using the Alu Positive Control Probe II from Ventana (Roche). This Alu probe consists in

a cocktail of oligonucleotide probes labelled with dinitrophenol (DNP) that are specific for human DNA. Analysis of immunostaining was performed by attributing an arbitrary score from "-" (absence of immunostaining) to "+++" (strong immunostaining) for the 3 frontal sections of each sample. Slides were scanned using the Nanozoomer 2.0 Hamamatsu in order to illustrate the immunostaining scoring.

Statistical analysis

All data are presented as mean values ± SD. Using the statistical analysis software GraphPad Prism, Mann Whitney U testing or Kruskal-Wallis for nonparametric unpaired sample sets were performed. For each donor and experimental group, technical duplicates or triplicates were performed. Unilateral P values < 0.05 were considered significant.

Results

OA synovial fluid supports the survival of ProtheraCytes®

When Protheracytes® in 2% HSA/PBS buffer were incubated at 37°C, 5% CO_2 with different concentrations (0, 10, 20, 50%) of OA synovial fluid at different timepoints (0, 1, 3, 6, 24, and 96h), we observed that cell viability varied little from 99.0% to 96.2% from 0 to 6 hours but decreased to 84.4% after 24 hours only for the 0% synovial fluid condition (Figure 1). However, at 96 hours of incubation, cell viabilities decreased sharply to 16.9% in 0% synovial fluid, 56.6% in 10% synovial fluid, 89.7% in 20% synovial fluid but there was no decrease in the 50% synovial fluid condition with a viability of 98.7% (Figure 1).

ProtheraCytes® can modulate the expression of some inflammatory and pro-anabolic genes in OA chondrocytes

To optimize the experimental conditions, we performed a first coculture experiment with OA chondrocytes and different numbers of ProtheraCytes®/insert (0, 0.1, 0.25 million cells/insert), different timepoints (6, 24, and 48 hours), and in the presence (+INFL) or absence (-INFL) of inflammatory cytokines. We observed that the percentage of dead ProtheraCytes® (PC) was similar in the inflammatory and non-inflammatory conditions and increased from 6 to 24 hours (up to 5%) remaining unchanged at 48 hours of culture (Figure 2A). The highest cell death was observed when ProtheraCytes® were cocultured at the lowest density (0.1M cells/insert) with OA chondrocytes (Figure 2A).

We also assessed the number of viable OA chondrocytes and at 6 hours, no difference could be observed in any of the co-culture conditions performed in the absence or presence on inflammatory cytokines (Figure 2B). At 24 hours, a slight reduction in the number of viable OA chondrocytes cocultured with the highest dose of ProtheraCytes® (0.25M cells/insert) was observed in the inflammatory condition. At 48 hours, the number of viable OA chondrocytes decreased when cocultured with ProtheraCytes® in the absence of inflammatory cytokines (Figure 2B).

Gene expression analysis was performed to assess possible pro-anabolic or anti-inflammatory/catabolic effect of the ProtheraCytes® on OA chondrocytes. We observed a decreased expression of collagen II (Col II) and increased expression of Col X when OA chondrocytes were exposed to inflammatory conditions (Figure 3). Aggrecan (ACAN) and Col II were significantly modulated by the presence of ProtheraCytes® in the absence of inflammatory cytokines at different time points but no significant change was observed on the other chondrogenic genes (Figure 3). All the analysed pro-inflammatory (IL-1β, IL-6 and IL8/CXCL8) and the pro-degrading A Disintegrin And MetalloProteinase with Thrombospondin motifs 4 (ADAMTS-4), ADAMTS-5, Matrix MetalloProteinase-1 (MMP-1), and MMP-13 genes were more highly expressed in the inflammatory condition (Figures 4 and 5). ProtheraCytes® showed a trend for the down-regulation of the expression of IL-1β following 24 hours of co-culture, but upregulated the expression of IL-8 at 48 hours and the expression of IL-6 at 24 hours of co-culture (Figure 4). ProtheraCytes® also showed a trend for the down-regulation of the expression of ADAMTS-4 and MMP-13 at 24 hours of co-culture in inflammatory conditions but no significant changes were observed in extracellular matrix (ECM)-degrading genes (Figure 5). Results from this first experiment showed that ProtheraCytes® survived well when cocultured with OA chondrocytes in the presence of inflammatory cytokines and can modulate the expression of some inflammatory and pro-anabolic genes by the OA chondrocytes.

A second experiment was then performed with OA chondrocytes from three different donors (donors 2-4) cocultured for 24 hours with two other batches of ProtheraCytes® to corroborate the results obtained in the first experiment. We observed similar high viability of ProtheraCytes® exposed or not to inflammatory conditions and slightly increased cell death in the lowest density group (0.1M cells/ml) (Figure 6A). The number of viable OA chondrocytes did not significantly change when exposed to inflammatory conditions (Figure 6B).

There was a reduction of ACAN and Col II expression and increase of Col X expression by OA chondrocytes when cultured in inflammatory conditions (Figure 7). We observed a trend of increased Sox9 and Col II expression when OA chondrocytes were cocultured with ProtheraCytes® (Figure 7). As observed in the first experiment, the pro-inflammatory and pro-degrading genes were more highly expressed by OA chondrocytes in the inflammatory condition (Figure 8). ProtheraCytes® at the higher density significantly downregulated the expression of IL1β and MMP13 in the inflammatory condition but upregulated ADAMTS5 and MMP-1 in the non-inflammatory condition (Figure 8).

The proteins secreted by OA chondrocytes when cocultured with ProtheraCytes® were quantified in the supernatant by multiplex immunoassays. The release of the majority of the analysed proteins by OA chondrocytes was increased in the inflammatory conditions, with more than 10-fold increase in RANTES/CCL5, IL1β, IL6, and IL8/CXCL8 (Table 1). To assess the modulatory effects of ProtheraCytes® on OA chondrocytes in the inflammatory condition, we compared the measured levels to the expected concentrations, calculated as the sum of the amount secreted by OA chondrocytes plus the amount defined in the OA chondrocytes/ProtheraCytes® co-cultures. Generally, ProtheraCytes® at the highest dose (0.25M cells/insert) had more pronounced anti-inflammatory/degrading effects on OA chondrocytes than the lower dose (0.1M cells/insert) (Figure 9). In particular ProtheraCytes® at the low

dose significantly reduced the secretion of only ADAMTS-4 (1.6-fold) and MMP-13 (5.5-fold), whereas at the highest dose, significantly reduced the secretion of TNF (1.9-fold), ADAMTS-4 (1.8-fold), MMP-1 (2.3-fold) and MMP-13 (2.0-fold) by OA chondrocytes (Figure 9).

	-INFL				+INFL			
	OA Ch only	**PC (0.25M) only**	**OA Ch/PC (0.1M)**	**OA Ch/PC (0.25M)**	**OA Ch only**	**PC (0.25M) only**	**OA Ch/PC (0.1M)**	**OA Ch/PC (0.25M)**
IL1β	0.37 ± 0.024	0.61 ± 0.05	0.49 ± 0.05	0.82 ± 0.09	18.2 ± 2.52 ####	17.2 ± 2.05	19.4 ± 1.26 #	18.8 ± 2.2
IL6	292 ± 23.9	0 ± 0	305 ± 44.9	466 ± 83.6	5763 ± 9500 ##	0 ± 0	45543 ± 5266 #	48489 ± 5786
IL8/CXCL8	136 ± 40.2	237 ± 29.1	147 ± 32.4	286 ± 43.6	59490 ± 8255 ####	346 ± 35.8	67763 ± 8168 ####	60918 ± 6212 ##
TNFα	0 ± 0	0 ± 0	0 ± 0	0 ± 0	22.4 ± 1.52 ####	10.1 ± 1.15	22.4 ± 1.52 ####	17.2 ± 0.94 #
RANTES/CCL5	2.21 ± 1.28	0.06 ± 0.006	2.25 ± 1.08	3.36 ± 1.22	342 ± 139 ###	0 ± 0	195 ± 60.4 #	195 ± 70.8 #
ADAMTS4	124.7 ± 39.23	116.5 ± 34.8	79.61 ± 3.027	58.98 ± 14.6 ****	89.26 ± 13.41	90.32 ± 10.22	73.68 ± 11.91	96.46 ± 10.43
ADAMTS5	2623 ± 1036	576 ± 285.9	3249 ± 1816	1037 ± 747	1699 ± 1454	798.2 ± 321.8	2983 ± 1620	2175 ± 1144
MMP1	6476 ± 1619	4275 ± 1950	185 ± 56.8	317 ± 140	11649 ± 222	12703 ± 196	7866 ± 1193	10439 ± 1568 ###
MMP13	2422 ± 651	2938 ± 1111	2866 ± 591	597 ± 185	7734 ± 726 #	7534 ± 726 #	1963 ± 1025 **	7727 ± 304 ####
VEGF	774 ± 188	5.12 ± 1.27	708 ± 197	103.4 ± 18.62	1120 ± 191	1.74 ± 0.39	1049 ± 122	1006 ± 155

Table 1. Secretome of monocultured OA chondrocytes (OA Ch only), monocultured ProtheraCytes® (PC only) and OA chondrocytes co-cultured with 0.1M (OA Ch / PC (0.1M)) or 0.25M ProtheraCytes®/well in the absence (-INFL) or presence (+INFL) of inflammatory cytokines, quantified by multiplex immunoassays. Values are mean ± SD of measurements from 3 independent experiment with 2 replicates/experiment. * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001 difference from OA Ch only, and # = P<0.05, ## = P<0.01, ### = P<0.001, #### = P<0.0001 difference between -INFL and +INFL at identical conditions defined by Mann-Whitney-U analyses.

Delivery with a needle for intra-articular injection does not impact ProtheraCytes®

Before conducting preclinical studies, we wanted to test if the injection through a needle for intra-articular injection would alter the viability, purity, and CD34+ cell number of ProtheraCytes®. We tested two needle diameters for knee injection in humans and mice and showed no significant difference before and after injection for viability, CD34+ cell purity and number (Figure 10).

Biodistribution of ProtheraCytes® after intra-articular injection in OA murine model

In order to determine the optimal timing for cell injection, we performed a pilot study to evaluate the survival and biodistribution of ProtheraCytes® after intra-articular injection in the knee joint of a collagen-induced osteoarthritis (CIOA) immunodeficient mouse model. ProtheraCytes® were injected at day 8 after CIOA and mice were euthanised at day 9, 15, and 29. We also injected ProtheraCytes® at day 29 after CIOA and euthanised mice at day 30, 36, and 50. In the group of mice injected with ProtheraCytes® at day 8, immunohistochemical analysis revealed the presence of a low to moderate number of human cells in 3/4 mice in each group sacrificed at day 9, 15 and 29. ProtheraCytes® were located in the synovial membrane along the femur and the patella or in the muscle for one mouse sacrificed at day 9 (Figure 11). In the group of mice injected with ProtheraCytes® at day 29, we observed the presence of low to moderate numbers of human cells in 2/4 mice sacrificed at day 30 and day 36 and no human cells were detected in animals sacrificed at day 50. For the animals euthanised at day 30 and 36, ProtheraCytes® were detected in the synovial membrane and muscle (Figure 12).

Discussion

Osteoarthritis is the most common form of arthritis and a leading cause of disability worldwide, affecting millions of people (Litwic et al., 2013). OA is a degenerative joint disease that can lead to immobility, difficulty with daily activities, and disability (Clynes et al., 2019; McDonough and Jette, 2010; Neogi, 2013). OA is not simply a process of wear and tear but rather abnormal remodeling of joint tissues driven by a host of inflammatory mediators within the affected joint, resulting in pain, deformity and loss of function (Loeser et al., 2012). Unfortunately, there is no cure for OA but stem cell-based therapies have the potential to promote the repair of damaged joints by modulating the immune response, transplanting stem cell-derived chondrocytes, or stimulating the patient's own cells for regeneration (Medvedeva et al., 2018; Pers et al., 2018, 2016). In this study, we wanted to evaluate the potential of ProtheraCytes® for the treatment of OA.

First, we showed that synovial fluid from OA patients is not toxic to ProtheraCytes® and maintains their viability when incubated for 96 hours at 37°C, 5% CO_2. It is possible that factors present in the synovial fluid such as hyaluronan and proteoglycan 4 (Tamer, 2013), might be responsible for the protection for ProtheraCytes®. This is encouraging data showing that synovial fluid would support the survival of ProtheraCytes® if they were injected in the knee joint of OA patients.

We then evaluated the performance of ProtheraCytes® using a relatively simple *in vitro* model consisting on their co-culture with human OA chondrocytes in separate layers of Transwells, thus allowing communication between the two cell types through soluble factors. Such type of communication is supposed to occur if ProtheraCytes® were injected in the synovial joint cavity of OA patients. To mimic the low-grade inflammatory milieu of the OA joint, we supplemented the culture medium with a cocktail of selected proinflammatory cytokines that have been described to play a pivotal role in OA (TNFα, IL1β, and IL6) (Mathiessen and Conaghan, 2017; Wang and He, 2018), at low concentrations (Acevedo Rua et al., 2021).

Clearly, the inflammatory condition used here exacerbated the inflammatory/degenerated traits of OA chondrocytes with the consequent up-regulation of the expression of the inflammatory (RANTES/CCL5, IL1b, IL6, IL8/CXCL8) and degrading (ADAMTS-4 and-5, MMP-1 and -13) markers at protein and/or mRNA level. The inflammatory condition also promoted the down-regulation of the expression of the cartilage genes collagen type II and aggrecan and the up-regulation of the expression of the hypertrophic marker collagen type X.

We observed that ProtheraCytes® remained highly viable once cultured for up to 48 hours in such OA mimicking environment. Still, up to 20% of ProtheraCytes® died upon co-culture with OA chondrocytes. Lower cell mortality is expected to occur in a joint environment that is rich in synovial fluid. Indeed, based on the results of previous experiment, ProtheraCytes® demonstrated high viability when exposed to synovial fluids from OA patients.

Importantly, our experiments demonstrated that ProtheraCytes® reduce the expression of not only the pro-inflammatory factor TNF by OA chondrocytes but also of key degenerative markers (ADAMTS-4, MMP-1 and MMP-13) known to play a significant role in aggrecan and collagen depletion in osteoarthritic cartilage (Malemud, 2019). Even if ProtheraCytes® exerted more pronounced anti-inflammatory/degrading effects at the highest dose (0.25M cells/insert), still, lower doses of ProtheraCytes® (0.1M cells/insert) induced OA chondrocytes to significantly reduce the expression of ADAMTS-4 and MMP-13.

We investigated whether ProtheraCytes® can induce pro-anabolic effects on OA chondrocytes. Our results showed that ProtheraCytes® at the highest dose in the absence of inflammation induced OA chondrocytes to up-regulate the expression of Sox9 and type II collagen. It is likely that ProtheraCytes® induce these effects on OA chondrocytes via the secretion of paracrine factors, including exosomes containing anti-apoptotic miRNAs (21 and 146a) (Huang et al., 2016; Lu and Lu, 2020).

We then evaluated the delivery of ProtheraCytes® with the needle used for intra-articular injection to determine if it would modify the cells before conducting the *in vivo* studies. We observed that delivery through the intra-articular injection needle did not change the viability, CD34+ cell purity and number of ProtheraCytes®.

The survival and biodistribution of ProtheraCytes® was then evaluated after intra-articular injection in the CIOA murine model in order to determine the best timing of administration in OA mice. Histological analysis of knee joints revealed that ProtheraCytes® were detected in 75% of mice injected with cells at day 8 and sacrificed at days 9, 15, and 29. The number of detected human cells was higher in mice sacrificed at day 9 than at days 15 and 29. ProtheraCytes® were always located in the synovial membrane no matter the day of mouse euthanasia, and we did not observe ProtheraCytes® in the cartilage. This might be due to the fact that CD34+ cells are known to express CD44 (a receptor of hyaluronic acid, HA) (Legras et al., 1997) and thus can preferentially adhere to the synovial membrane, a tissue containing high amounts of HA (Revell et al., 1995) instead of cartilage or other joint tissues. Mesenchymal stromal cells (MSCs) also express CD44 and have been shown to home to the synovial membrane after injection in the knee joints of CIOA mice (Toupet et al., 2015). The synovial membrane plays an important role in maintaining tissue homeostasis within the intra-articular joint and producing the synovial fluid that nourishes the cartilage. The synovial membrane also constitutes a niche for MSCs (Kurth et al., 2011) and might be a favourable environment for exogenous MSCs and CD34+ cells.

When ProtheraCytes® were injected at day 29 after CIOA, human cells were only found in 50% of the mice sacrificed at days 30 and 36 and in 0% of the mice sacrificed at day 50. This is in line with the results obtained when MSCs are injected in the knee joints of CIOA mice (Toupet et al., 2015). ProtheraCytes® were mostly located in the synovial membrane and muscle. The muscle location might be attributed to the migration of cells but most likely to the difficulty of precise cell injection in the intra-articular space of the dislocated knee joints at day 29. Altogether, this study indicates that ProtheraCytes® survive at least 20 days in the knee joint of CIOA immunodeficient mice when they are injected at an early stage of OA (day 9). Survival is limited to 7 days when cells are injected at day 29, when OA has progressed. These results are indicative of the survival of ProtheraCytes® in an OA joint environment but must be interpreted with caution since only 4 mice were included in each group and 3 sections of the entire joint were examined. These results need to be confirmed in larger preclinical studies that also evaluate the therapeutic efficacy of ProtheraCytes® in this animal model.

Example 3: Exploratory Study

Study design

This Example describes a single-arm, open-label, two-stage, exploratory study in two cohorts of patients with moderate to severe chronic osteoarthritis.

Approximately 10 eligible patients with either grade II or III (moderate) OA on the KL scale and symptomatic in at least one knee are enrolled to receive ProtheraCytes® as single intraarticular injection.

The first Stage (I) includes Cohort 1 with N=5 patients. After evaluation of preliminary safety and efficacy of Cohort 1 (including one month follow up), Cohort 2 (N=5) is included for Stage II. No formal statistical interim analysis is performed.

After signing informed consent and confirmation of eligibility, all patients in Cohort 1 and Cohort 2 are treated with Granocyte® for five days, and once with intraarticular injection of ProtheraCytes®:

- Granocyte® treatment: all patients are initially treated with Granocyte® (lenograstim, a G-CSF compound), about l0 µg/kg injected subcutaneously once daily for five days to stimulate CD34+ stem cell mobilization. On the sixth day, whole blood is harvested from the patient and sent to a dedicated cell therapy center (CTC) for a nine-day CD34+ stem cell expansion processing.
- ProtheraCytes® injection: Within the 37 hours following the end of the cell production processing, ProtheraCytes® are injected intraarticularly on D16 in the patient's index knee during an outpatient procedure.

All patients are followed for 24 months. Study assessments take place at Screening, Baseline (i.e., day of intraarticular injection of ProtheraCytes®), 1-, 6-, 12-, 18-, and 24-months post baseline. The visit at 24-months is also the End of Study visit.

Study objectives

Primary Objectives:

- To assess the safety of the intraarticular injection of ProtheraCytes® in patients with OA

Secondary Objectives:

- To assess preliminary efficacy data on pain and physical exercise capacity in patients with OA

Exploratory Objectives:

- To assess prevention of cartilage degradation after ProtheraCytes® intraarticular injection of patient with symptomatic knee OA assessed by knee MRI.
- To retrospectively assess dose-effectiveness

Investigational product

ProtheraCytes® are a GMP-expanded autologous CD34+ cells characterized as an ATMP, injected intraarticularly with the following specifications:

- Total number of CD34+ cells ≥ $8x10^6$;
- CD34+ cell viability ≥ 95%;
- CD34+ cell purity ≥ 80%;
- Presence of monocytes ≤ 15%;
- Presence of granulocytes ≤ 5%;
- Presence of lymphocytes ≤ 3%.

Main evaluation criteria

Primary evaluation criteria:

- Incidence, seriousness, relatedness, and severity of adverse events at 1 month

Secondary evaluation criteria:

- Incidence, seriousness, relatedness, and severity of adverse events at Days 2, 3, 7, and Months 1, 3, 6, 12, 18, and 24 (EOS).
- Change from baseline on quantitative MRI at 12 and 24 months
- Change from baseline on Patient Reported Outcomes (PROs), especially global pain and function using Visual Analog Scale (VAS) and WOMAC questionnaires at 3, 6, 12, 18, and 24 months.
- Response rate as per OMERACT-OARSI criteria
- Change from baseline in Quality-of-Life score using the SF-36 Questionnaire at 3, 6, 12, 18, and 24 months.

Exploratory evaluation criteria:

- The cartilage formation via MOCART and WORMS is assessed from baseline to 1 year, and 2 years after treatment
- Correlation of dose and effectiveness in order to determine the optimal dose range

Inclusion criteria

Patients are included if they meet all of the following criteria:

1. Age at screening ≥ 40 years old
2. Body mass index (BMI) of < 35 kg/m^2.
3. Pain score of 4 or more on the Numeric Pain Rating Scale (NPRS)
4. Ability to comply with requirements of study visits.
5. Be willing and capable of giving written informed consent to participate.
6. Be willing and capable of complying with study related requirements, procedures, and visits.
7. Females of childbearing potential (those who are not surgically sterilized or post-menopausal for at least 2 years) are excluded from participation in the study unless they agree to use adequate contraception.
8. Moderate grade II or III OA on the KL scale and symptomatic (pain, swelling, oedema, or warmth) in only one knee.

Exclusion criteria

Patients must be excluded if they meet any of the following criteria:

1. Hb<10 g/dL and platelets <1011/L at screening
2. Patients who have taken any pain medication (except Paracetamol) within 1 week prior to screening.
3. Patients who use anticoagulants, have a substance abuse history, and/or fail to agree not to take any knee symptom-modifying opioid drugs during the course of the study without discussing and reporting the use to the site principal investigator and study team.
4. Severe instability / history of past surgery on the index knee within 12 months prior to screening.
5. Symptomatic (pain, swelling, oedema, or warmth) in both knees, requiring treatment of both knees.

6. Strong malalignment, i.e., varus or valgus exceeding 5°.

7. Mechanical knee symptoms (locking of joints)

8. Patients with intra-articular injection of any drug including corticosteroids and viscosupplementation in the index knee in the last 3 months

9. Traumatic injury to the index knee within the last 3 months

10. Planned elective surgery during the course of the study (Cohort 1) or planned elective surgery before 6 months of follow-up (Cohort 2)

11. History of organ or hematologic transplantation, rheumatoid arthritis, or other autoimmune disorders

12. Patients on immunosuppressive medications / treatment

13. Patients who participated in another clinical trial or treatment with any investigational product within the last 30 days prior to the inclusion in the study

14. Contraindications to radiography or MRI imaging

15. Serious neurological, psychological, or psychiatric disorders

16. Other medical condition determined by the site principal investigator as interfering with the study

17. Injury or disability claims under current litigation or pending or approved workers' compensation claims

18. History of hematopoietic growth factor administrations

19. History of liver cirrhosis or hepatic severe failure

20. Constitutional or acquired coagulopathy

21. Haemodialysis or renal severe failure (creatinine clearance < 30ml/min)

22. History of Phenylketonuria

23. History of Splenomegaly

24. Prior malignancies <5 years (never in case of myeloid leukaemia in complete remission) except non-melanoma skin cancer or adequately treated in situ cervical cancer

25. Chronic immunomodulatory or cytotoxic drug treatment intake

26. History of HIV1-2, HTLV1-2, HCV, Syphilis or active HBV

27. History of Iron-Dextran or murine protein allergy

Discontinuation criteria

Patients must discontinue the study if they meet any of the following criteria:

1. Hb<10 g/dL and platelets <1011/L at the time of blood harvest

2. Blood transfusion within 3 days before the first Granocyte® injection

3. Adverse Event. The subject experiences an AE that imposes an unacceptable risk to the subject's health, or the subject is unwilling to continue because of an AE.

4. Non-compliance with Study Procedures. The subject fails to adhere to other study requirements.

5. Lost to Follow-up. The subject does not return to the clinic for scheduled assessments and does not respond to the site's attempt to contact.

6. Withdrawal by Subject. The subject wishes to withdraw from the study. The site should attempt to determine the underlying reason for voluntary withdrawal and record it on the eCRF; if the underlying reason is AE or lack of efficacy, that category should be recorded and not withdraw of consent.

7. Study Terminated by Sponsor. The Sponsor, IRB/IEC or regulatory agency terminates the study.

Study duration

Per patient: 3 weeks screening, 5 to 6 day treatment with G-CSF, blood harvest on the 6th day, cell processing for 9 days, injection with IMP on day 17th, followed by 24 months follow up.
Total study duration: 30 months (6 months patient recruitment + 24 months follow up)

Interim Analysis

No formal statistical interim analysis is performed. An interim analysis for safety (AEs, SAEs, lab abnormalities) is performed on Cohort 1 after the last patient of Cohort 1 has reached the 1 month time point. The interim analysis is performed by the Principal Investigator (PI), key members of the Scientific Advisory Board (SAB), and key representatives of the Sponsor.

Statistics

Cohort 1: 5 patients; Cohort 2: 5 patients
This is an exploratory safety and feasibility study. Therefore, no formal sample size calculation is performed. These sample sizes are normal and acceptable for these kinds of exploratory studies.

Efficacy assessments

Numeric Pain Rating Scale (NPRS)

Patients indicate pain intensity by selecting a number between 0 and 10, with 0 indicating no pain and 10 indicating the worst pain. A quadruple NPRS (Q-NPRS) that determines pain intensity using four questions: pain at present, average pain over 24 h, pain when it is at its worst, pain when it is at its best is administered. Each question has the same scaling structure, 0 to 10, used in the single-question NPRS. The responses across the four questions are averaged to obtain the Q-NPRS score. The NPRS is deemed to be reliable and valid in individuals with advanced knee OA.

Knee Injury and Osteoarthritis Outcome Score (KOOS)

The seven-item Knee Injury and Osteoarthritis Outcome Score—Physical Function Shortform (KOOS-PS) is a self-reported measure that was developed from the full-length KOOS with the aim of reducing administrative burden and item redundancy while preserving sound measurement properties in assessing physical functions in patients with advanced knee OA.

Each of the seven items of the KOOS-PS is scored on a scale of 0 to 4, with 0 indicating no difficulty and 4 indicating extreme difficulty in completing the functional task. The total score across the seven items is converted into an adjusted score of 0 to 100, with 0 indicating complete functional impairment and 100 indicating no impairment. The KOOS-PS has been validated as a tool for examining physical functions in multiple linguistic and cultural contexts.

Outcome Measures in Rheumatology (OMERACT) committee and Osteoarthritis Research Society International (OARSI) Standing Committee for Clinical Trials Response Criteria Initiative

The OMERACT-OARSI initiative has resulted in the development of a uniform core set of outcome measures for osteoarthritis (OA). The symptomatic variables selected by both the OMERACT and OARSI

societies were: pain, functional impairment and patient's global assessment. The main characteristics of the sets of criteria are the following:

They cover three domains: pain, function and patient's global assessment. For each of these domains, a response is defined by both a relative and an absolute change. The cut-off that defines a relevant change differs with regard to:

- OA localization (e.g. hip vs knee)
- evaluated study drug (e.g. NSAIDs vs specific anti-OA drug)
- route of administration (e.g. per os vs intra-articular)
- specific domain (pain, function, patient's global assessment).

Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC)

The WOMAC evaluates 3 dimensions: pain, stiffness, and physical function with 5, 2, and 17 questions, respectively. The Likert version of the WOMAC is rated on an ordinal scale of 0 to 4, with lower scores indicating lower levels of symptoms or physical disability. Each subscale is summated to a maximum score of 20, 8, and 68, respectively. There is also an index score or global score, which is most commonly calculated by summating the scores for the 3 subscales (4). A visual analog scale (VAS) version of the WOMAC is also available. The questionnaire is self-administered and takes 5 to 10 minutes to complete.

Short Form-36 Health Survey Questionnaire (SF-36)

The Short Form 36 Health Survey Questionnaire (SF-36) is used to indicate the health status of particular populations, to help with service planning and to measure the impact of clinical and social interventions.

X-Ray for Kellgren-Lawrence (KL) Score

Osteoarthritis is a slowly progressive degenerative disease characterized by a gradual loss of articular cartilage. Radiography is the standard for assessment of the progression of osteoarthritis, and osteoarthritis severity is graded from radiographic images according to the Kellgren-Lawrence (KL) score. The KL score has been also adopted by the World Health Organization as the reference standard for cross-sectional and longitudinal epidemiologic studies. However, the radiographic staging of the joint is limited in that the soft tissues (cartilage, ligaments, and joint capsule) that are commonly affected by osteoarthritis are not visible on radiographs. In addition, the sensitivity for observation of features, such as osteophytes, bone eburnation, and other subchondral bone abnormalities, is limited because radiographs are a 2D composite of complex 3D structures.

2D Magnetic Resonance Observation of Cartilage Repair Tissue (MOCART)

MOCART is an exploratory Magnetic resonance imaging (MRI) assessment. MRI will be used to evaluate postoperative lesion characteristics per the MOCART scale (magnetic resonance observation of cartilage repair tissue). The size, location, lesion stability, traumatic aetiology, and skeletal maturity will be recorded.

Whole-organ MRI scoring method (WORMS)

WORMS is an exploratory MRI assessment. MR images will be scored with respect to 14 independent articular features: cartilage signal and morphology, subarticular bone marrow abnormality, subarticular cysts, subarticular bone attrition, marginal osteophytes, medial and lateral meniscal integrity, anterior and posterior cruciate ligament integrity, medial and lateral collateral ligament integrity, synovitis, loose bodies and periarticular cysts/bursae.

Efficacy analyses

Statistics for the following analyses:

1. Change from baseline on quantitative MRI at 12 and 24 months
2. Change from baseline on Patient Reported Outcomes (PROs), especially global pain and function using Visual Analog Scale (VAS) and WOMAC questionnaires at 3, 6, 12, 18, and 24 months.
3. Response rate as per OMERACT-OARSI criteria
4. Change from baseline in Quality-of-Life score using the SF-36 Questionnaire at 3, 6, 12, 18, and 24 months.
5. Change from baseline on cartilage formation via MOCART and WORMS 1 year and 2 years.

Exploration of any correlation of dose and effectiveness in order to determine the optimal dose is performed.

Example 4: Measurement of VEGF expression and exosomal miRNA expression by ProtheraCytes®

Methods

Cell culture

ProtheraCytes® were obtained after expansion of mobilized $CD34^+$ cells from AMI patients (EXCELLENT Phase I/IIb clinical trial NCT02669810) and from Frozen Healthy Donors (FHD) $CD34^+$ purified cells (Lonza, NC, USA) as previously described (Saucourt et al., 2019).

VEGF quantification

Supernatants from AMI patients and FHD ProtheraCytes® were collected after 9 days of expansion in StemFeed® cell culture medium (Eurobio, France) and stored at -80°C until analysis. Supernatants were thawed and VEGF levels were measured in using the Human VEGF QuantiGlo ELISA Kit (R&D Systems, MN, USA) according to the manufacturer's instructions with the SpectraMax L (Molecular Devices, San Jose, CA USA). The Immunoassay Control Set 732 for Human VEGF (R&D Systems) was used as a positive control and StemFeed® medium as a negative control.

Exosome isolation

ProtheraCytes® were cultured at the concentration of $2.5x10^5$ cells/mL in StemSpan-AOF (StemCell Technologies, BC Canada) supplemented with cytokines for 40 hours. Then, cells were collected by

centrifugation at 400*g* for 10 minutes; and exosomes were purified from the supernatant by precipitation using the ExoQuick-TC™ kit (System Biosciences, CA, USA) according to the manufacturer's instructions. After isolation, exosomes were characterized by flow cytometry using the ExoStep™ kit with a bead-bound anti-CD63 capture, anti-CD81 and anti-CD34 antibodies (ImmunoStep, Spain) confirming the identity of the exosomes secreted by ProtheraCytes®.

MicroRNA quantification

Total RNA from ProtheraCytes® and their secreted exosomes collected from 7 AMI patients were isolated respectively using a miRNeasy Tissue/cells Advanced MiniKit and a miRNeasy Serum/Plasma Advanced Kit (QiAGEN, France) according to the manufacturer's protocols. RNA isolated from exosomes and cells was reverse transcribed to cDNA using the miCURY LNA RT Kit (QIAGEN, France). UniSp6 RNA spike-in controls were added during cDNA synthesis to ensure the quality of the experiment. Real-time qPCR amplifications were performed for each RT reaction. Reactions were performed according to the manufacturers' instructions using a miRCURY LNA miRNA SYBR Green PCR Kit (QIAGEN, France) with the Bio-Rad CFX96™ Real time PCR Detection System (BioRad Laboratories, France). All primer sets were custom designed by the supplier. Primers used were miR-21-5p (YP00204230), miR-26a-5p (YP00206023), miR-126-3p (YP00204227), miR-130a-3p (YP002046658), miR-133a-3p (YP00204788), miR146a-5p (YP00204688), miR-199a-3p (YP00204536), miR-378a-3p (YP00205946), and miR-590-3p (YP00205448). The qPCR data were normalized to miR-let7a-5p (YP00205727) values. Relative miRNA expressions were calculated using the $2^{-\Delta\Delta Ct}$ method.

Results

Secretion of VEGF as a Potency test

Human $CD34^+$ cells have been shown to secrete VEGF (Bautz et al., 2000) and we wanted to determine the level of VEGF secretion by $CD34^+$ cells after expansion of 16 ProtheraCytes® batches manufactured from AMI patients from the EXCELLENT Phase I/IIb clinical trial, as well as 4 batches manufactured from healthy donors. The quantification of VEGF concentration by ELISA showed that the culture supernatants of ProtheraCytes® from patients ranged from 185.6 pg/mL to 1032.4 pg/mL with a mean value of 596.2±242.3 pg/mL, and the VEGF concentration from healthy donor cells ranged from 315.3 pg/mL to 718.3 pg/mL with a mean value of 526.2±208.1 pg/mL (Figure 13A). No significant difference was observed between the VEGF concentrations of patients and healthy donors (Figure 13A and B). Conversely, the concentration of VEGF observed in the StemFeed® culture medium (negative control) ranged from 2.7 pg/mL to 3.0 pg/mL with a mean value of 2.8±0.2 pg/mL, which was significantly lower than the VEGF concentration of patients (p=0.0007) and healthy donors (p= 0.087) (Figure 13B). Furthermore, the concentration of VEGF in the culture supernatant of the expanded $CD34^+$ cells from AMI patients was significantly correlated with the number of $CD34^+$ cells obtained after expansion (Figure 13C and D) (Pearson correlation coefficient r = 0.7484; p-value = 0.0009).

miRNA expression in ProtheraCytes®-derived exosomes (CD34Exo)

To investigate the proangiogenic activity of ProtheraCytes® derived exosomes (CD34Exo), we first isolated exosomes and characterized them by flow cytometry. We then isolated RNA from CD34Exo and

determined the expression levels of miRNAs that have been reported to be key positive regulators of the angiogenesis processes. We assessed the expression levels of miR-126, miR-130a, miR-21, miR-378, and miR-26a, (Sahoo et al., 2011; Mathiyalagan et al., 2017; Templin et al., 2017; Mcneill et al., 2019; Li et al., 2021; Chang et al., 2022) in ProtheraCytes® and their exosomes (CD34Exo). Exosomes were found to be significantly enriched in miR-130a, miR-126, miR-378, miR-26a, and miR-21, compared to ProtheraCytes® (Figure 14A). The miR-130a and miR-21 were the two most enriched miRNAs in CD34Exo and their expression was 6.9 and 12.1 fold higher, respectively, than in ProtheraCytes® (Figure 14B). Similarly, we observed significantly higher expression of miR-126 by 4.4-fold, and miR-378 and miR-26a by 3.2-fold in ExoCD34$^+$ compared to ProtheraCytes® (Figure 14B).

These results indicate that ProtheraCytes® are able to secrete exosomes containing proangiogenic miRNAs which might lead to the induction of angiogenesis and contribute to the vascular repair process. We also investigated the expression level of miR-146a, which has been shown to attenuate apoptosis (Huang et al., 2016; Scărlătescu et al., 2021) and found that it was also enriched in ExoCD34$^+$ by 3.5-fold compared to ProtheraCytes® (Figure 14B).

Expression of both miR-199a and miR-590 were detected at low levels but were significantly higher in ExoCD34$^+$ compared to ProtheraCytes® (Figure 14A). Interestingly, miR590 was one of the most enriched miRNAs in ExoCD34+with 13.5-fold higher expression and miR-199a was 4.6 times higher in ExoCD34+ than in cells (Figure 14B).

Finally, we further examined the expression of miR-133a, miR-24, miR-29b, and miR-132, known to have anti-fibrotic activity (Xiao et al., 2019). As shown in Figure 14A and B, miR-29b, miR-24, and miR-132 were significantly enriched in Exo CD34+ and their expression was 11.7, 5.8 and 4.3-fold higher, respectively, than in ProtheraCytes®. On the other hand, there was no significant difference in the expression of miR133 between ExoCD34$^+$ and ProtheraCytes®.

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Claims

1. A population of CD34+ cells for use in a method of treating osteoarthritis.

2. A population of CD34+ cells for use in a method of:
(a) increasing the expression of chondrogenic markers by chondrocytes in a subject, and/or
(b) decreasing the expression of inflammatory and/or degrading markers by chondrocytes in a subject, and/or
(c) inducing pro-anabolic effects on chondrocytes in a subject, and/or
(d) stimulating chondrogenesis in a subject, and/or
(e) modulating expression of inflammatory and/or pro-anabolic genes by chondrocytes in a subject.

3. The population of CD34+ cells for use of claim 1 or claim 2, wherein the CD34+ cells are obtained from whole blood or umbilical cord blood.

4. The population of CD34+ cells for use of any preceding claim, wherein the CD34+ cells express CD44.

5. The population of CD34+ cells for use of any preceding claim, wherein the CD34+ cells express one or more paracrine factors, and/ or VEGF.

6. The population of CD34+ cells for use of claim 5, wherein the amount of VEGF expressed by the CD34+ cells into a culture medium is at least about 150 pg/ml, or wherein the amount of VEGF expressed by the CD34+ cells into a culture medium is at least about $2x10^{-3}$ pg/cell.

7. The population of CD34+ cells for use of any preceding claim, wherein the CD34+ cells express one or more or each of miR126, miR130a, miR21, miR26a, miR378a, miR146a, miR21, miR199a, miR590, miR133a, miR-24, miR29b, and miR132.

8. The population of CD34+ cells for use of any preceding claim, wherein the CD34+ cell viability is at least about 95%, and/or wherein the CD34+ cell purity is at least about 80%.

9. The population of CD34+ cells for use of any preceding claim, wherein the CD34+ cells are a cultured and/or expanded population, and/or purified, and/or, wherein the CD34+ cells are an isolated population.

10. The population of CD34+ cells for use of any preceding claim, wherein the population comprises
(i) at least about 80% CD34+ cells, and/or
(ii) about 15% monocytes or lower, and/or
(iii) about 5% granulocytes or lower, and/or

(iv) about 3% lymphocytes or lower.

11. The population of CD34+ cells for use of any preceding claim, wherein the CD34+ cells are provided as a sterile suspension, and/or wherein the population is provided in the form of a pharmaceutical composition, optionally comprising buffer.

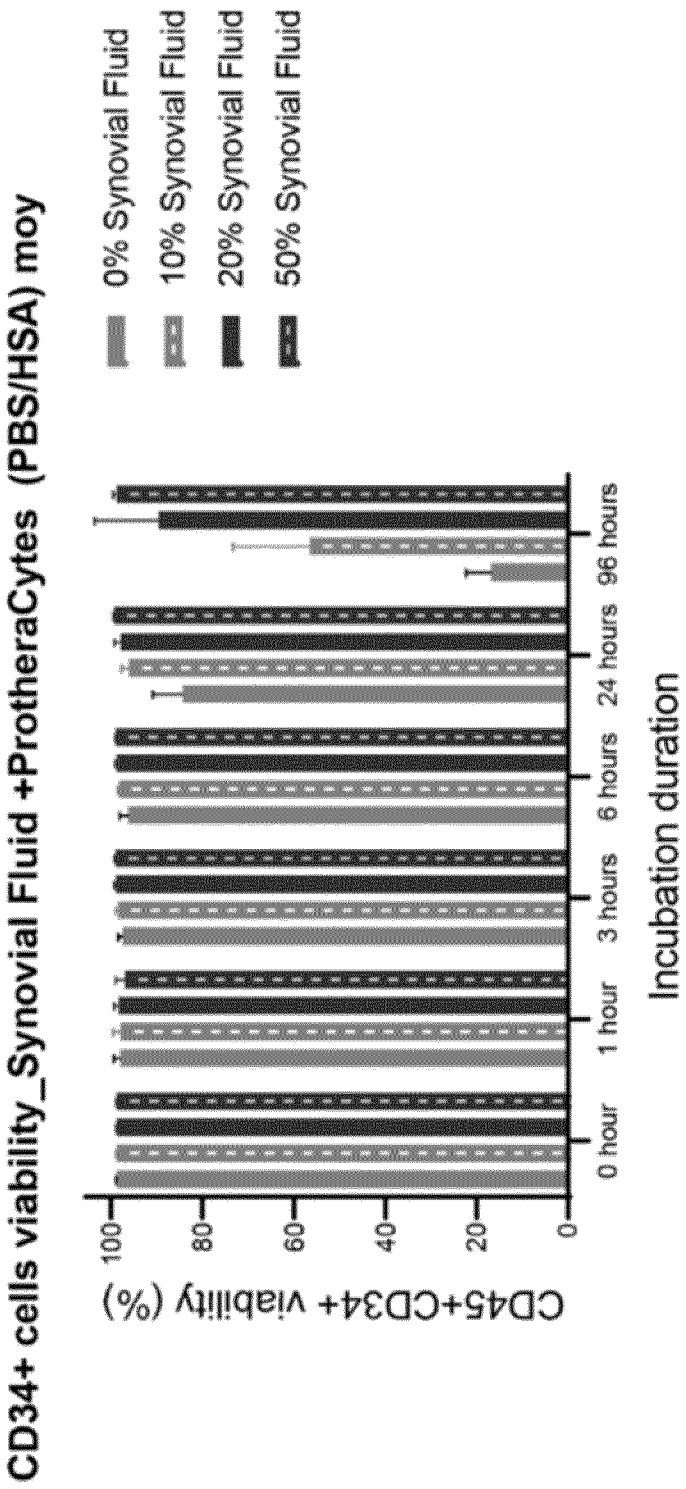
12. The population of CD34+ cells for use of any preceding claim, wherein the method further comprises the steps of:

i. administering a hematopoietic growth factor to a subject,
ii. collecting a peripheral blood sample from the subject,
iii. isolating and/or expanding CD34+ cells from the blood sample, to obtain a population of CD34+ cells, and/or
iv. administering the population of CD34+ cells to the subject, optionally wherein one dose of cells is administered.

13. The population of CD34+ cells for use of any preceding claim, wherein the method comprises administering at least $8x10^6$ CD34+ cells, and/or wherein the volume administered per dose is about 15 ml.

14. The population of CD34+ cells for use of any preceding claim, wherein the CD34+ cells increase the expression of collagen II, aggrecan, and/or Sox9, and/or wherein the CD34+ cells decrease the expression of IL1β, IL8, TNF, ADAMTS-4, MMP-1, and/or MMP-13.

15. The population of CD34+ cells for use of any preceding claim, wherein the CD34+ cells are for use in treating a patient with grade 2 or grade 3 osteoarthritis according to the Kellgren-Lawrence scale.


CD34+ cells viability_Synovial Fluid +ProtheraCytes (PBS/HSA) moy
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10% Synovial Fluid
20% Synovial Fluid
50% Synovial Fluid
CD45+CD34+ viability (%)
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0 hour
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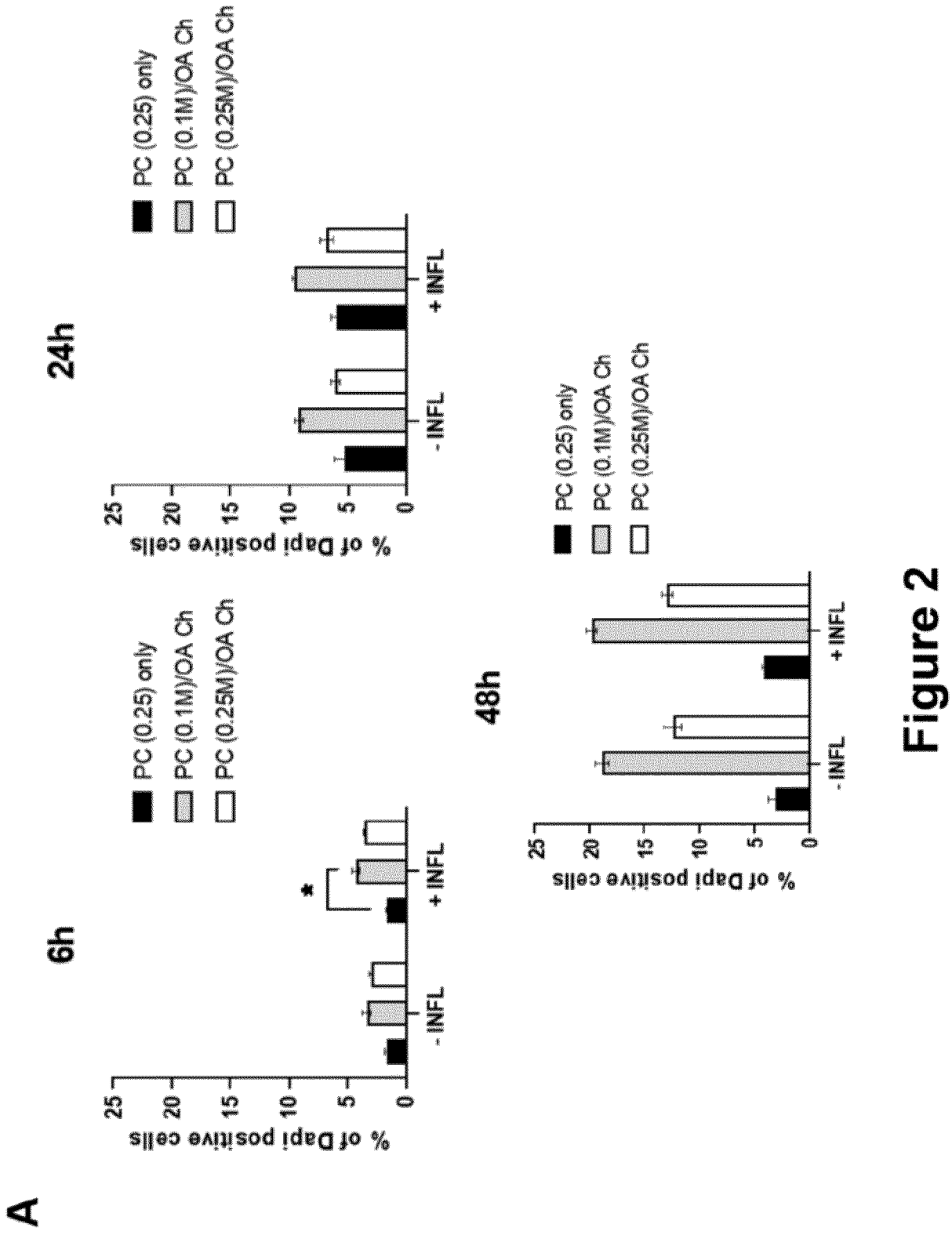
3 hours
6 hours
24 hours
96 hours
Incubation duration

Figure 1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% of Dapi positive cells
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25
- INFL
+ INFL
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Figure 2

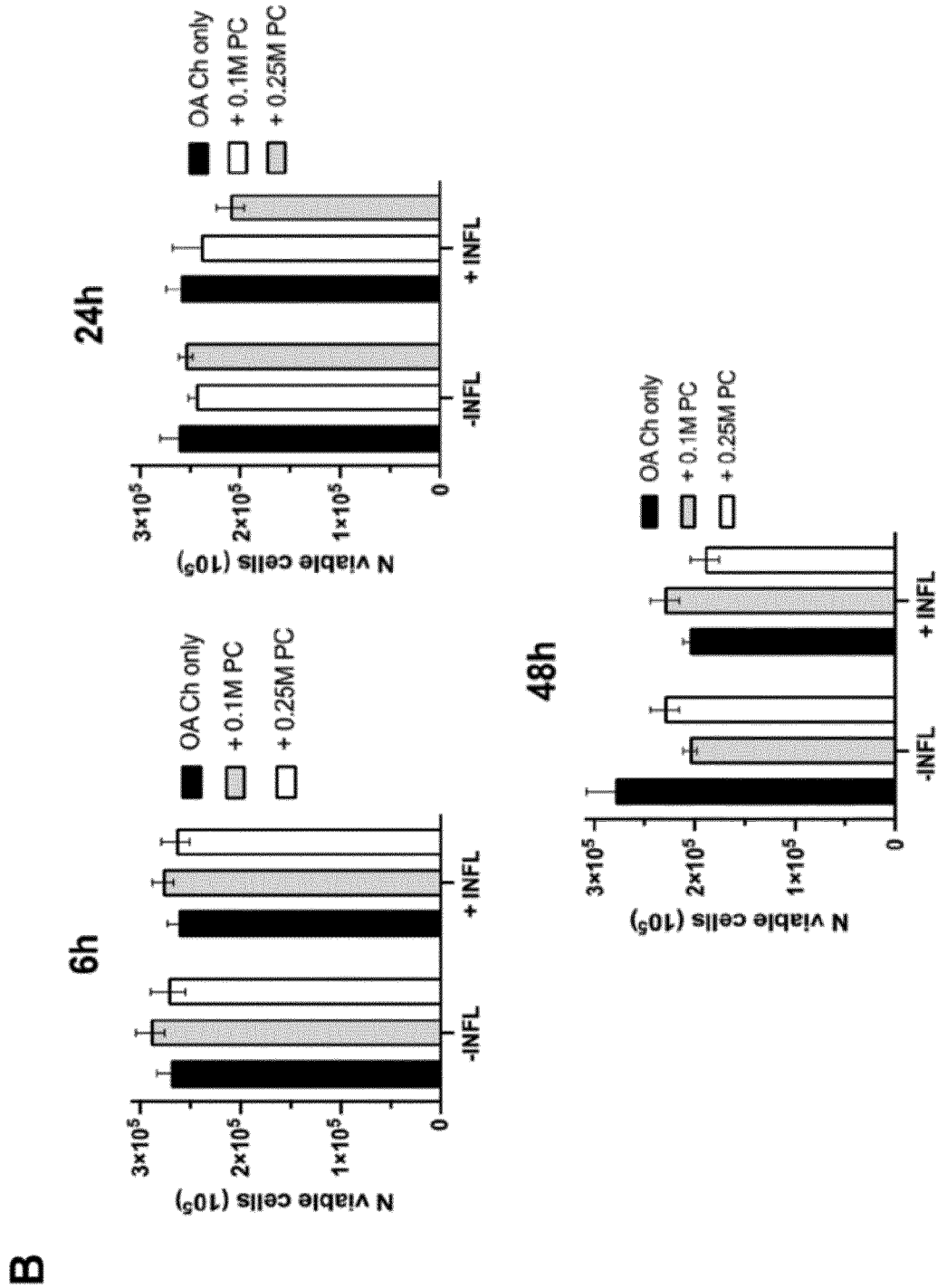
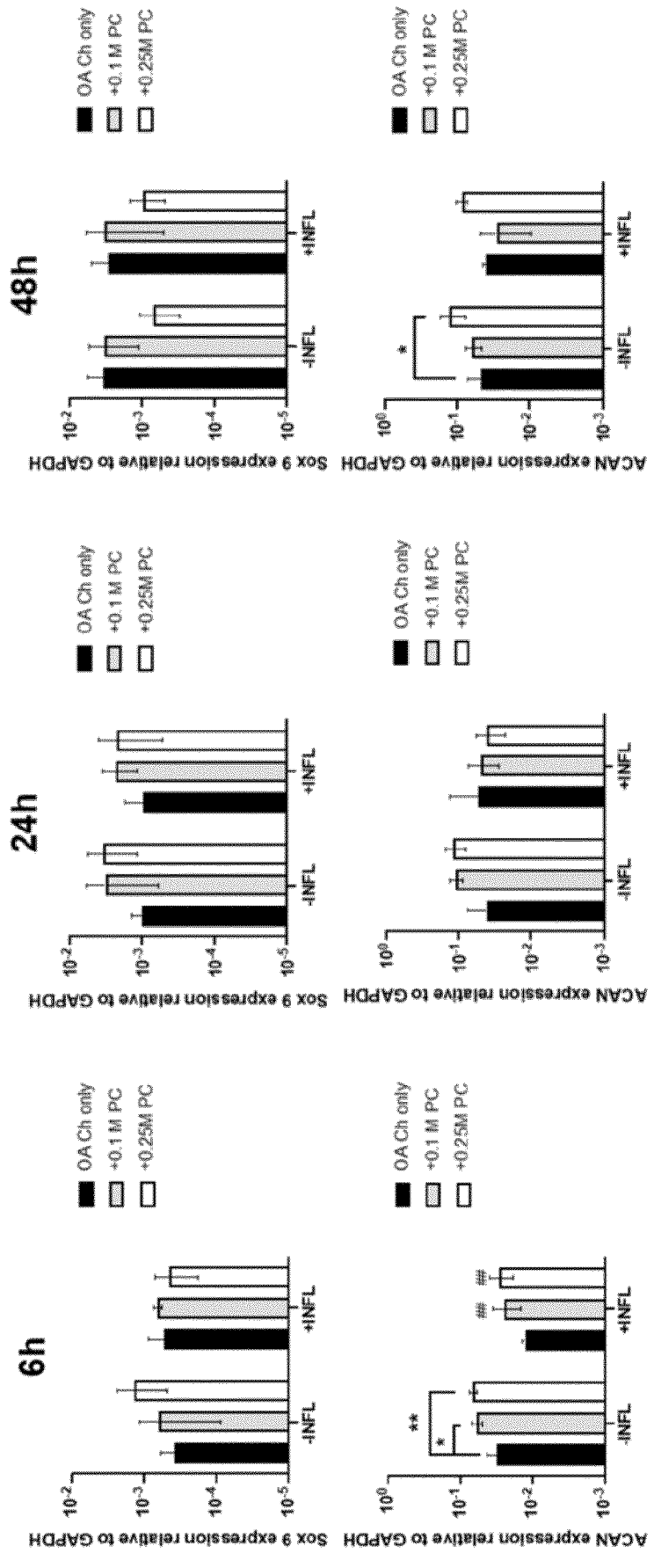


Figure 2 cont.


6h
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Sox 9 expression relative to GAPDH
ACAN expression relative to GAPDH
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Figure 3

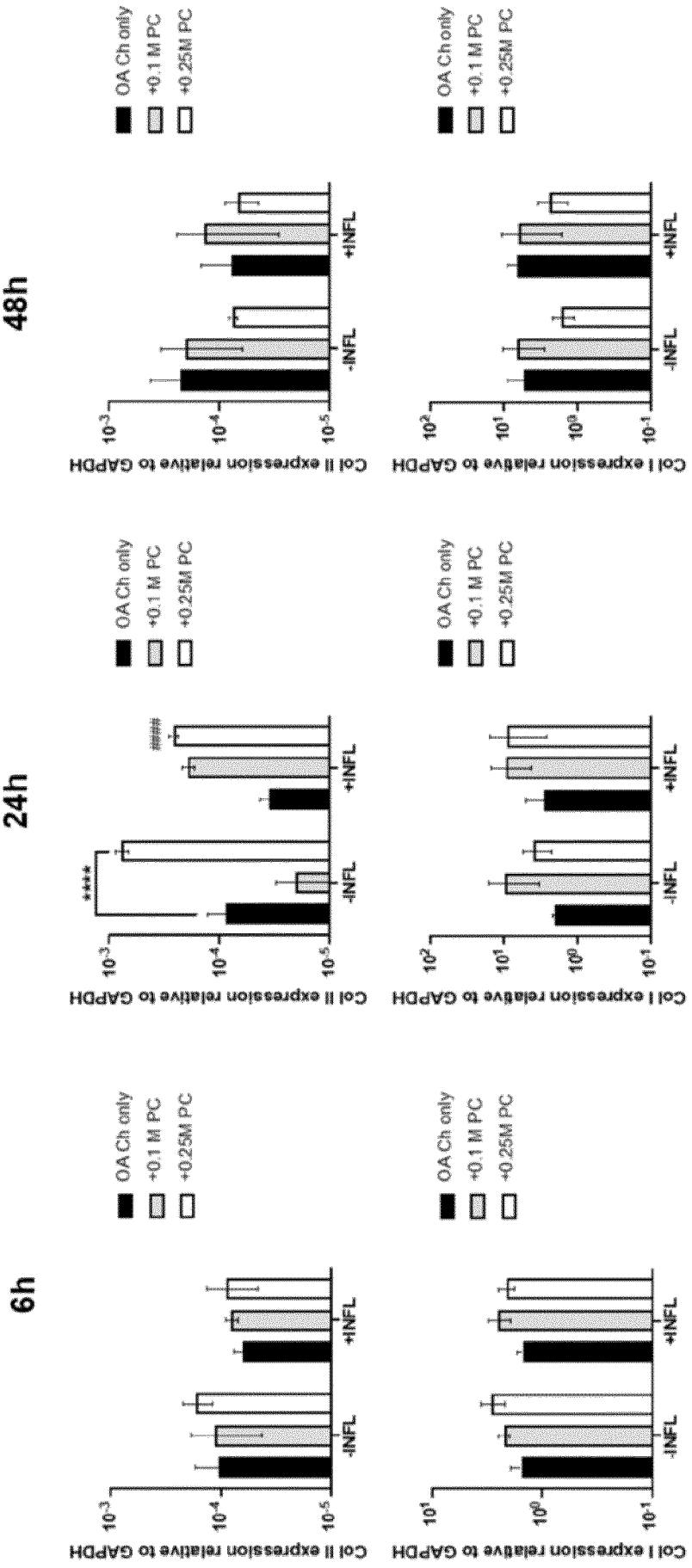


Figure 3 cont.

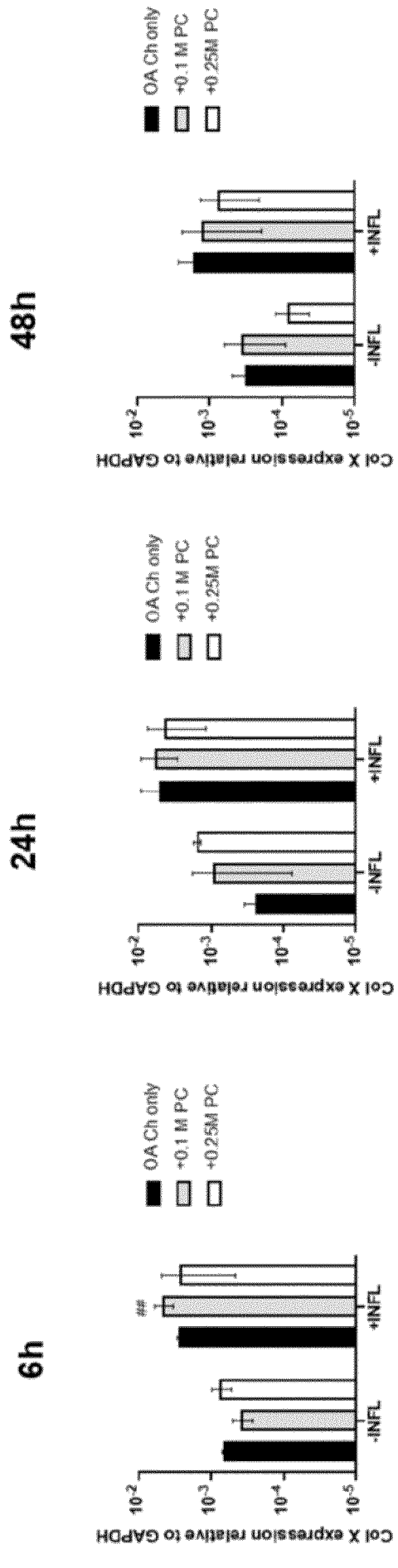
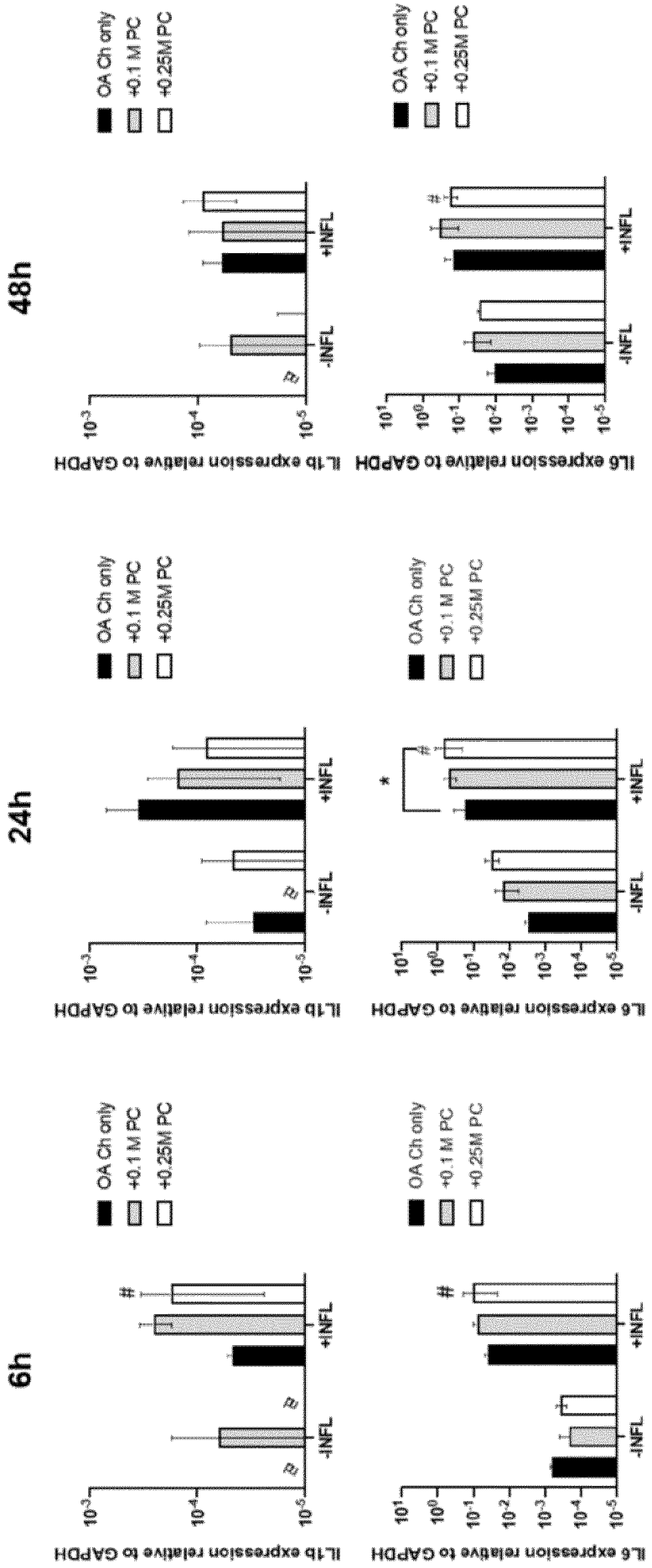


Figure 3 cont.


6h
24h
48h
OA Ch only
+0.1 M PC
+0.25M PC
IL1b expression relative to GAPDH
IL6 expression relative to GAPDH
-INFL
+INFL
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*

Figure 4

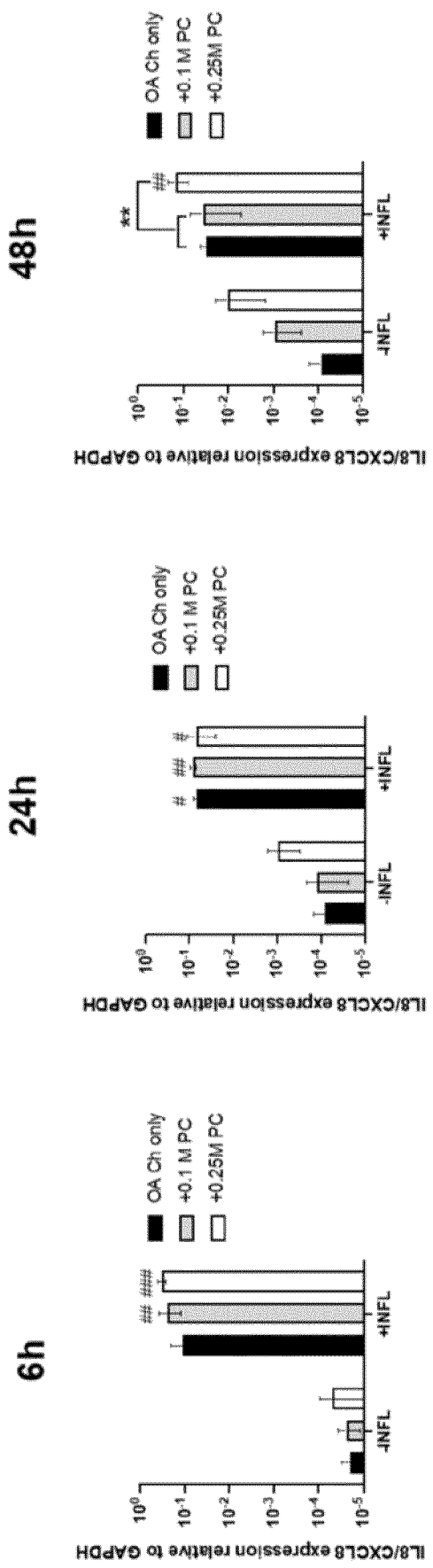

Figure 4 cont.

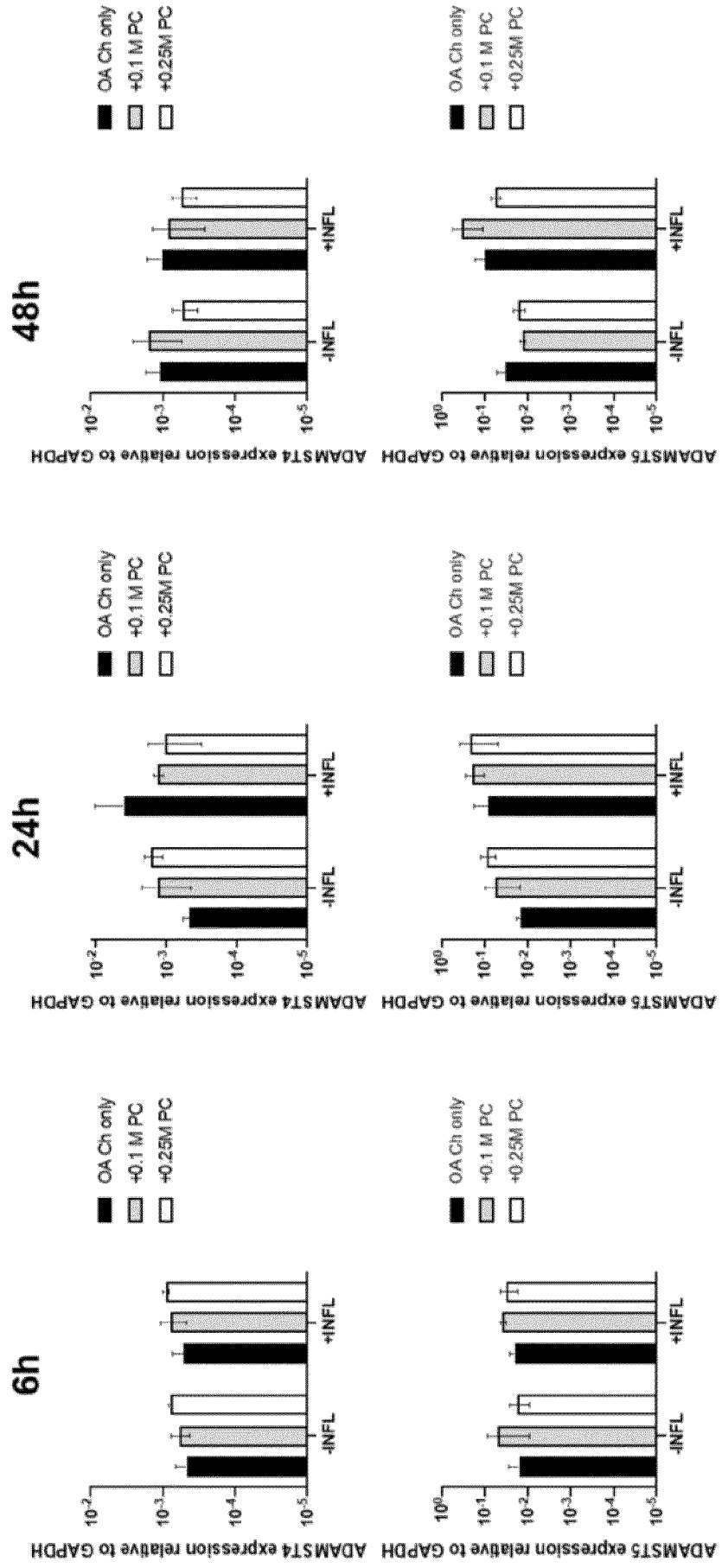
48h
24h
6h
OA Ch only
+0.1 M PC
+0.25M PC
-INFL
+INFL
ADAMST4 expression relative to GAPDH
ADAMST5 expression relative to GAPDH

Figure 5

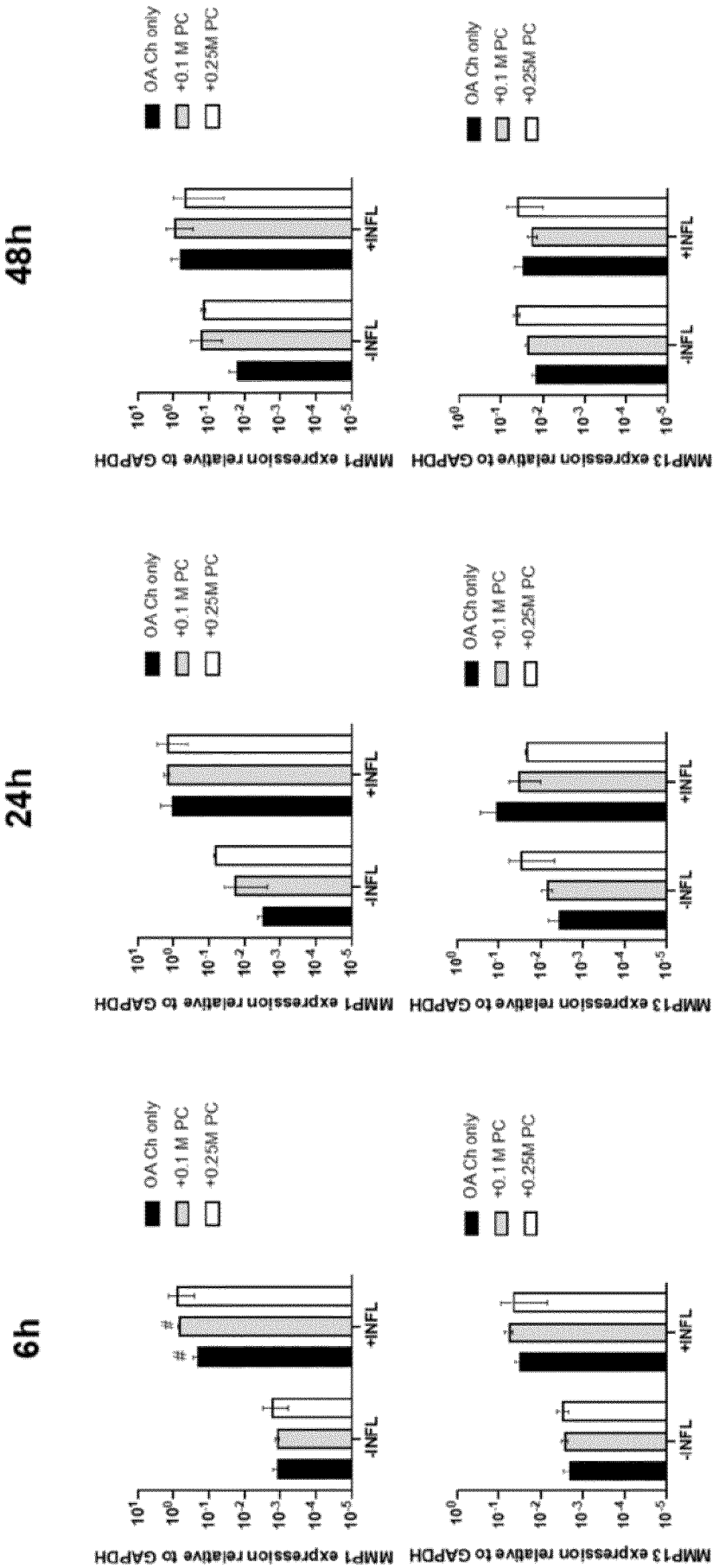

Figure 5 cont.

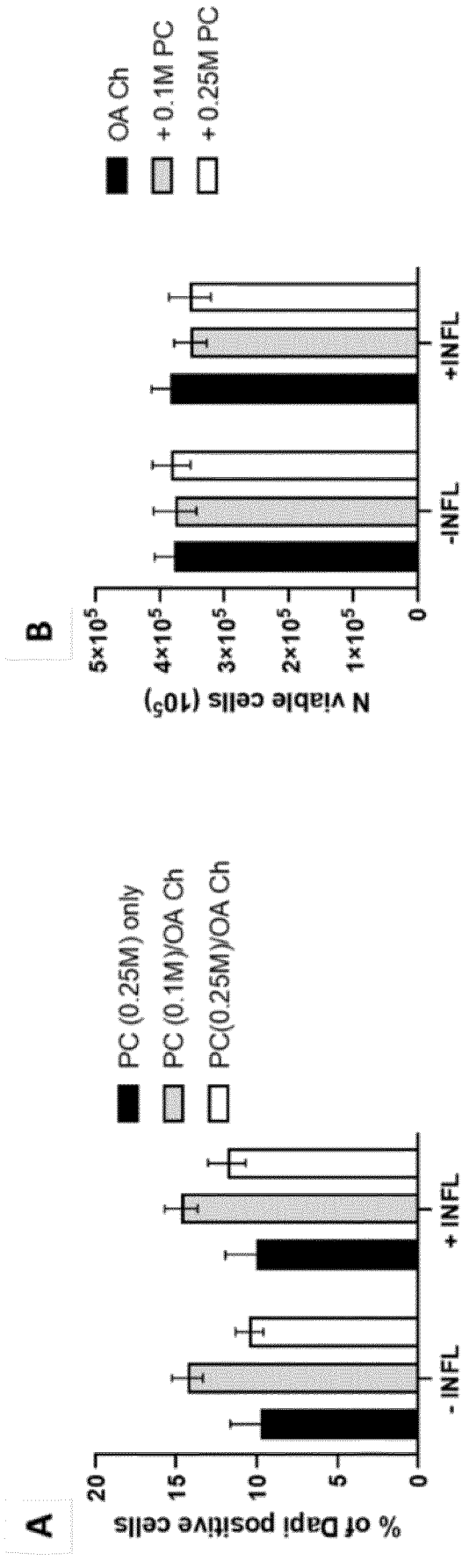
A
% of Dapi positive cells
0
5
10
15
20
- INFL
+ INFL
PC (0.25M) only
PC (0.1M)/OA Ch
PC(0.25M)/OA Ch
B
N viable cells (10^5)
0
1×10^5
2×10^5
3×10^5
4×10^5
5×10^5
-INFL
+INFL
OA Ch
+ 0.1M PC
+ 0.25M PC

Figure 6

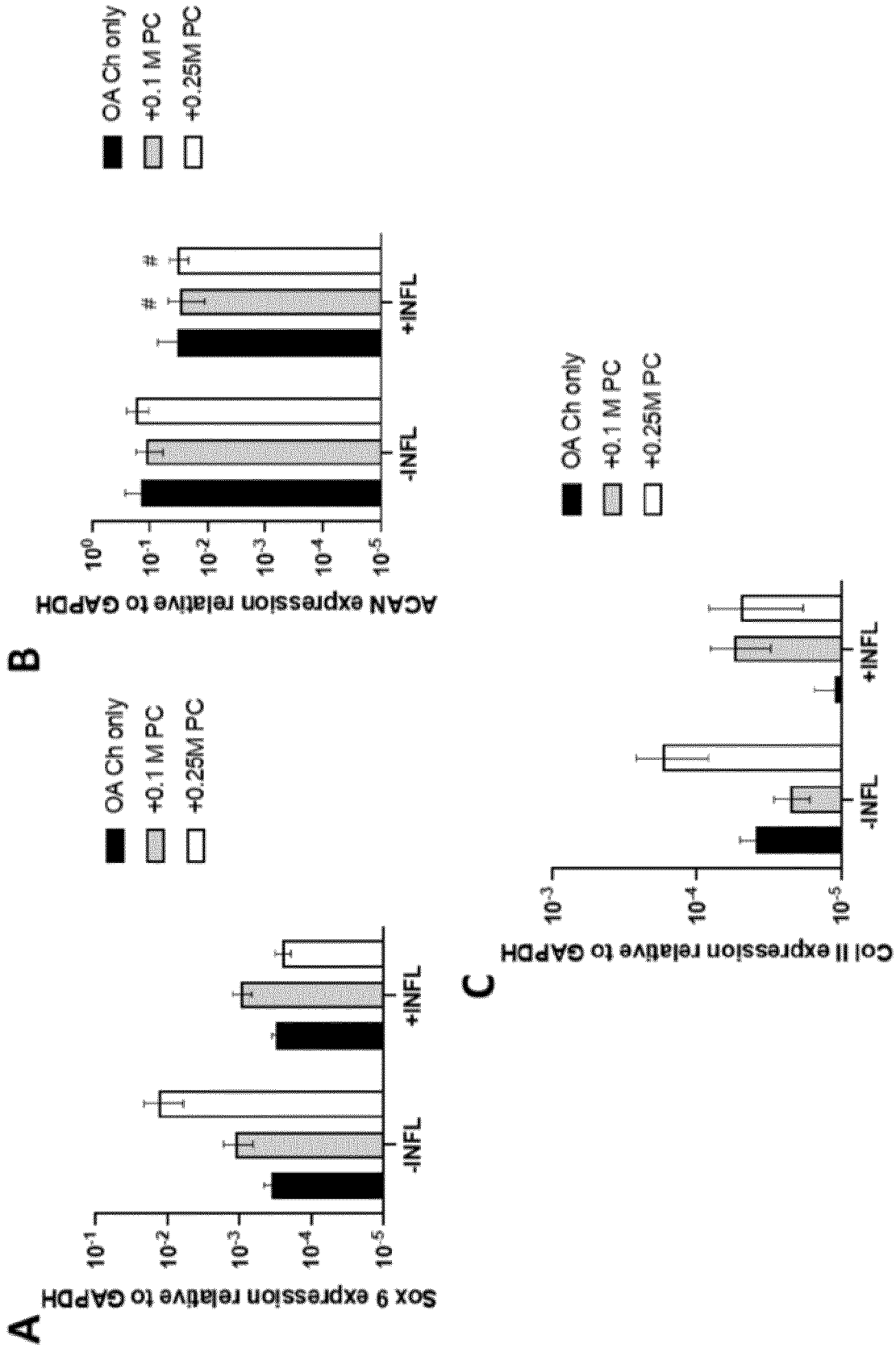
A
Sox 9 expression relative to GAPDH
OA Ch only
+0.1 M PC
+0.25M PC
-INFL
+INFL
B
ACAN expression relative to GAPDH
OA Ch only
+0.1 M PC
+0.25M PC
-INFL
+INFL
C
Col II expression relative to GAPDH
OA Ch only
+0.1 M PC
+0.25M PC
-INFL
+INFL

Figure 7

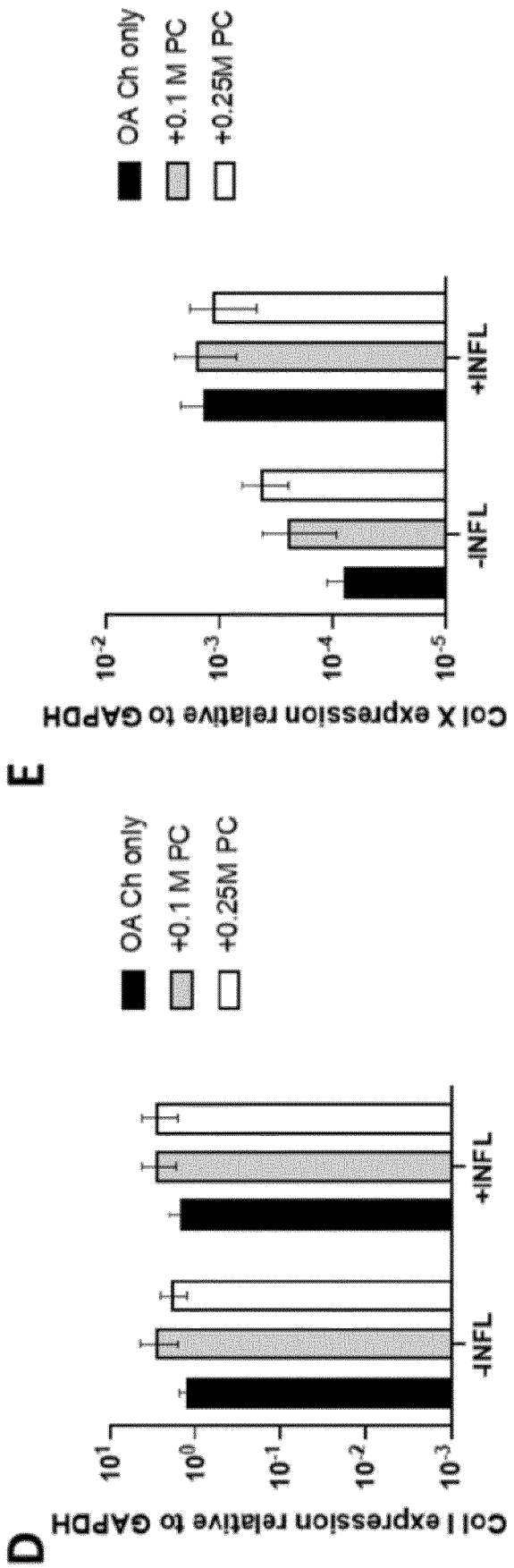

Figure 7 cont.

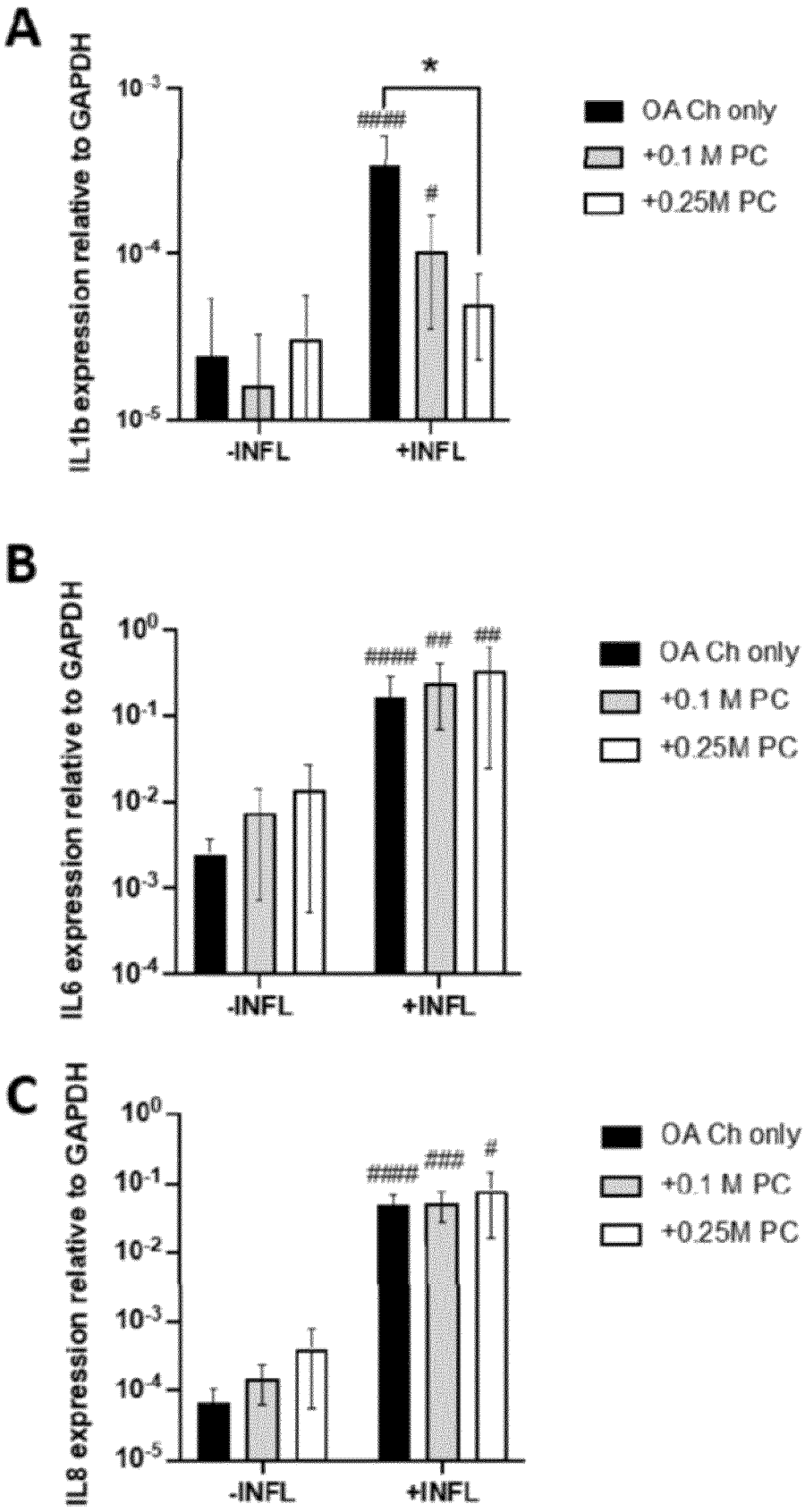
A
IL1b expression relative to GAPDH
10-3
10-4
10-5
*
####
#
-INFL
+INFL
OA Ch only
+0.1 M PC
+0.25M PC
B
IL6 expression relative to GAPDH
100
10-1
10-2
10-3
10-4
####
##
##
-INFL
+INFL
OA Ch only
+0.1 M PC
+0.25M PC
C
IL8 expression relative to GAPDH
100
10-1
10-2
10-3
10-4
10-5
####
###
#
-INFL
+INFL
OA Ch only
+0.1 M PC
+0.25M PC

Figure 8

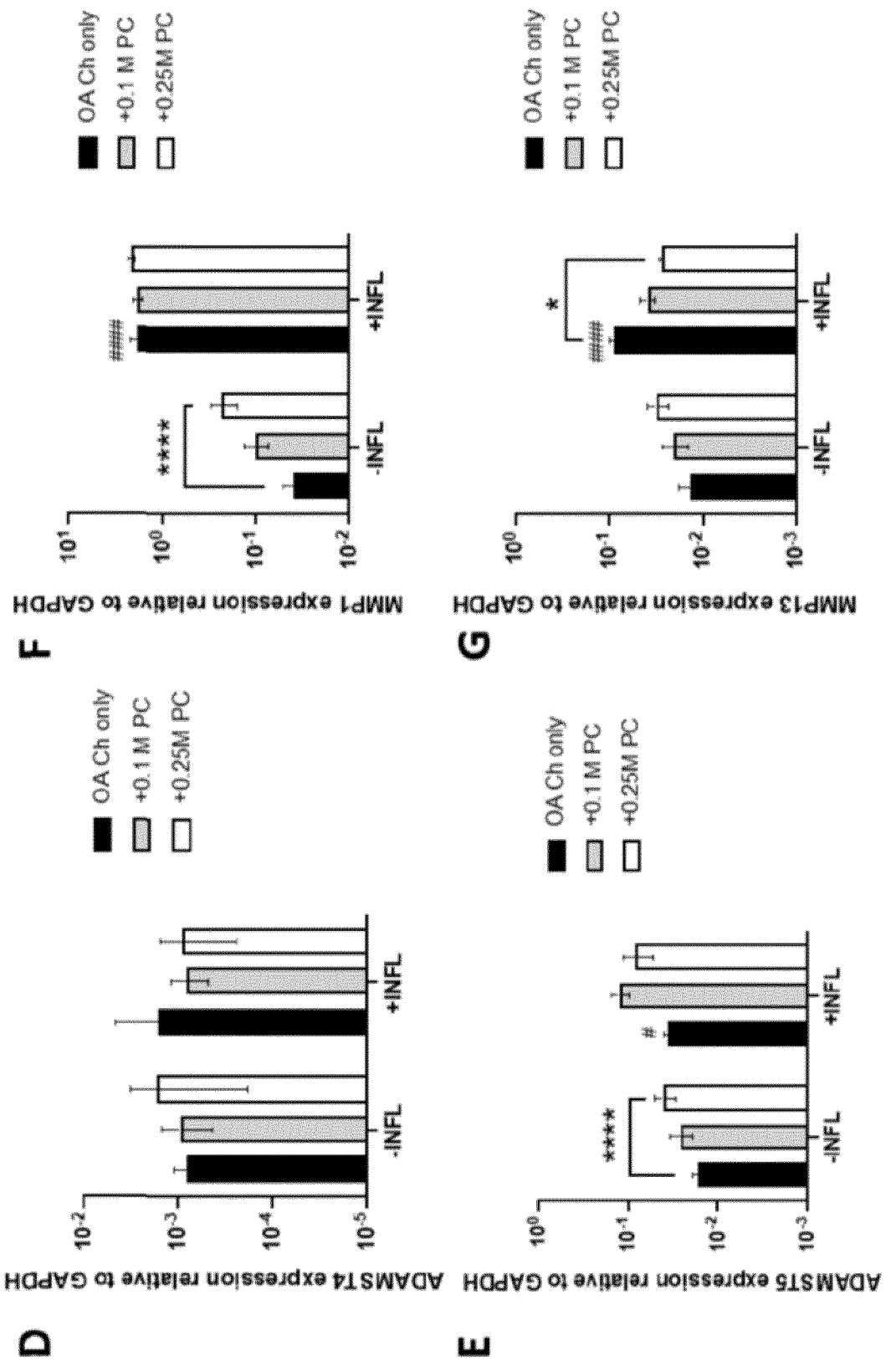

Figure 8 cont.

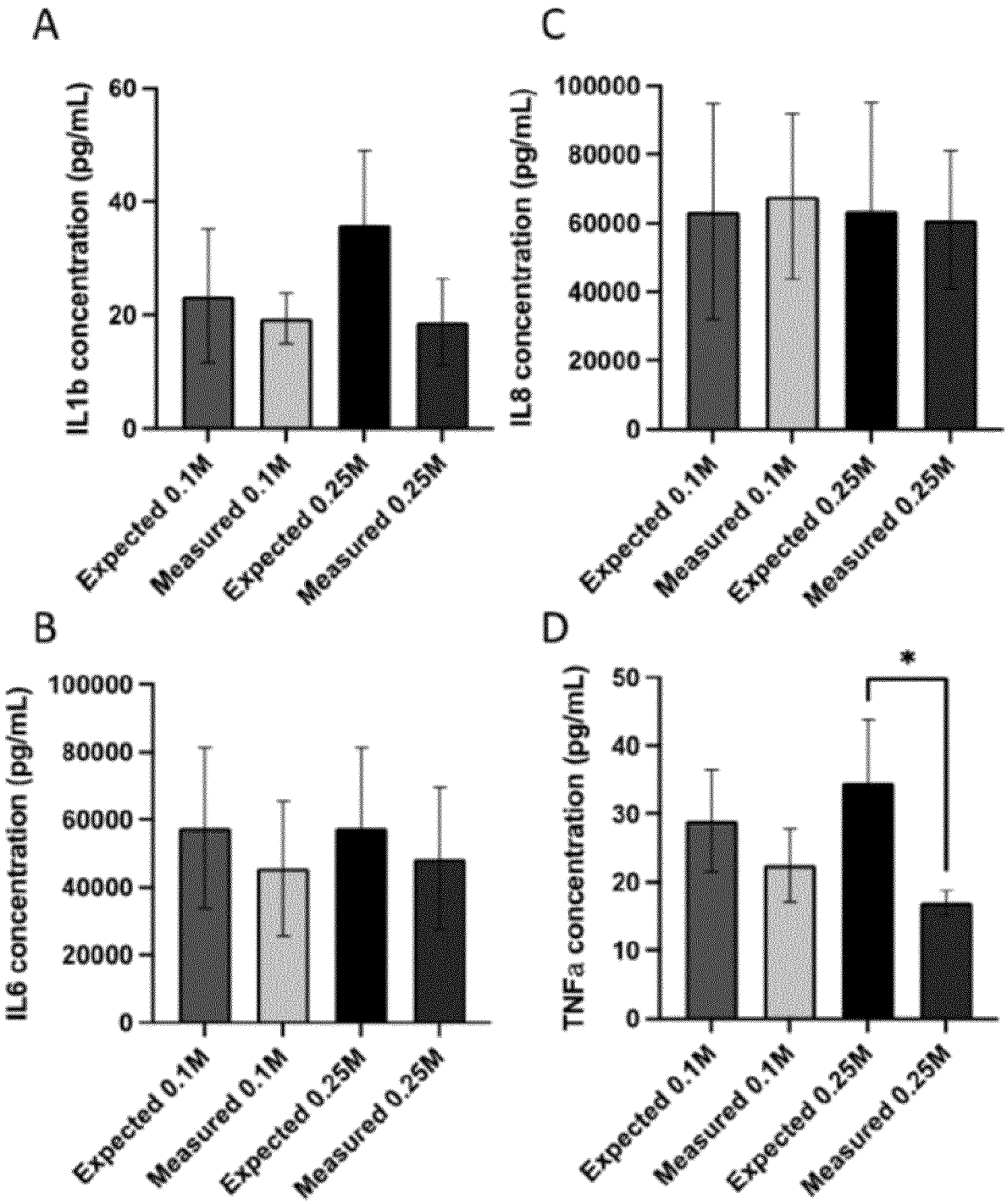
A
IL1b concentration (pg/mL)
60
40
20
0
Expected 0.1M
Measured 0.1M
Expected 0.25M
Measured 0.25M
C
IL8 concentration (pg/mL)
100000
80000
60000
40000
20000
0
Expected 0.1M
Measured 0.1M
Expected 0.25M
Measured 0.25M
B
IL6 concentration (pg/mL)
100000
80000
60000
40000
20000
0
Expected 0.1M
Measured 0.1M
Expected 0.25M
Measured 0.25M
D
TNFa concentration (pg/mL)
50
40
30
20
10
0
*
Expected 0.1M
Measured 0.1M
Expected 0.25M
Measured 0.25M

Figure 9

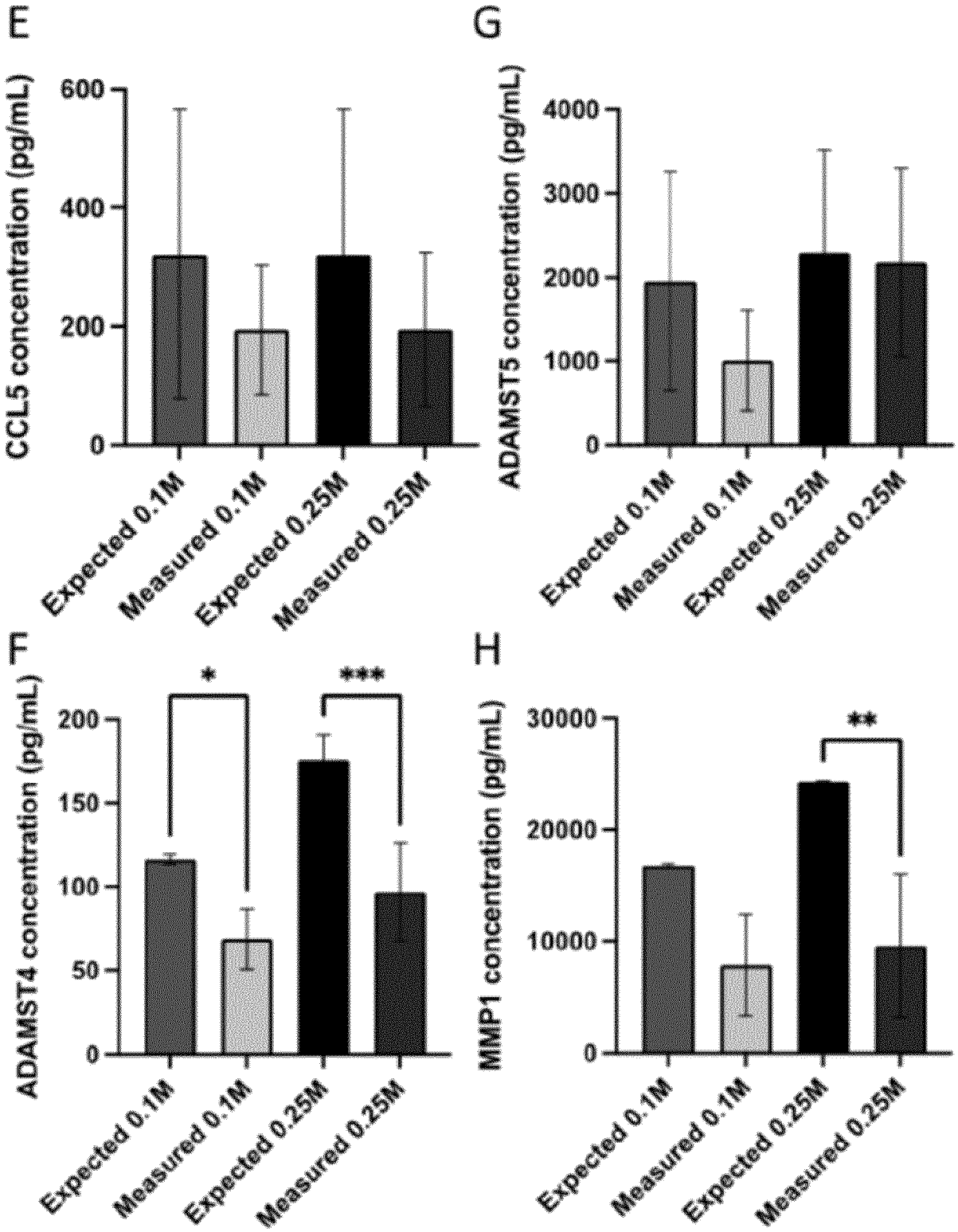

Figure 9 cont.

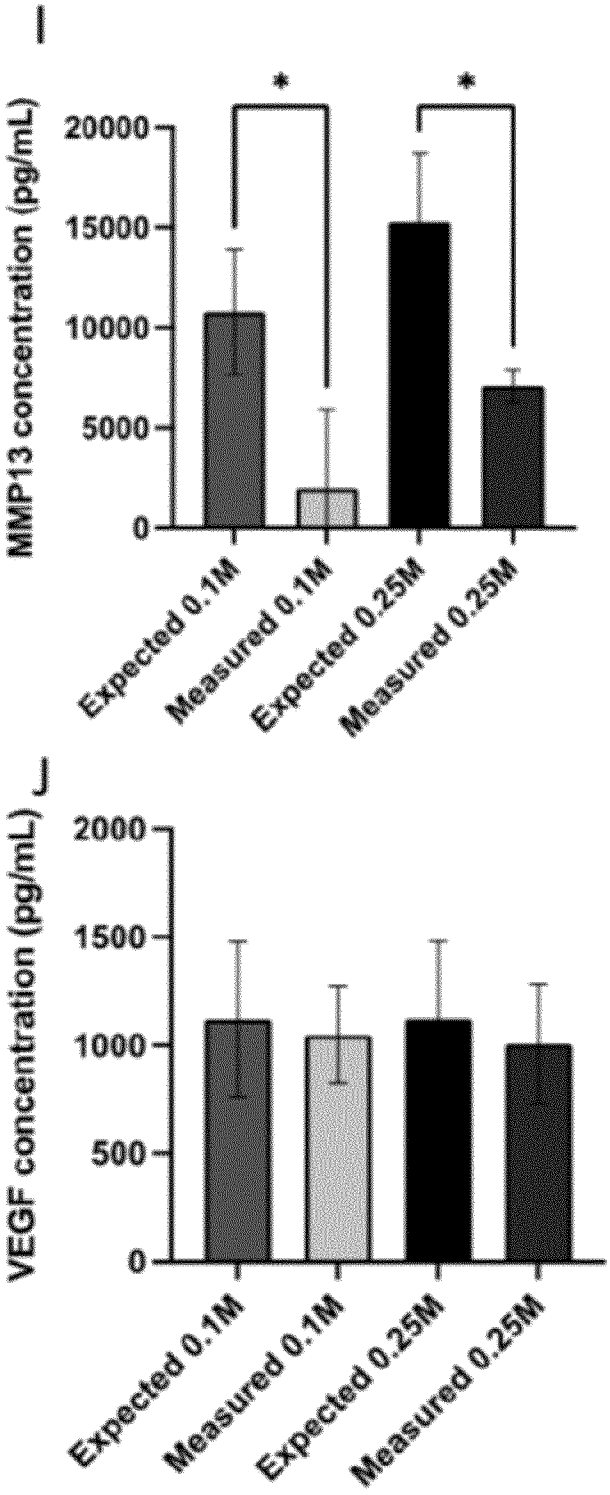

Figure 9 cont.

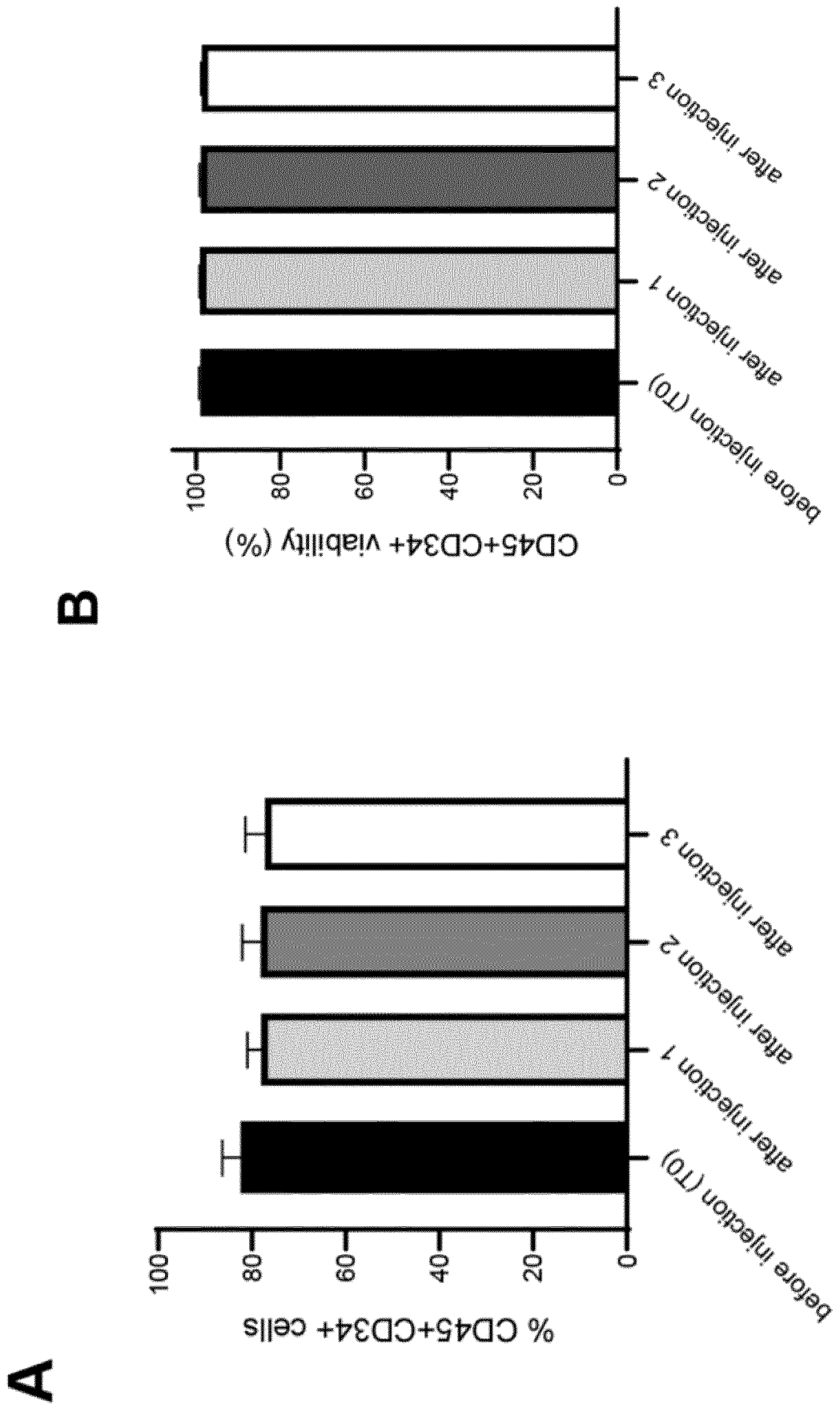
A
% CD45+CD34+ cells
100
80
60
40
20
0
before injection (T0)
after injection 1
after injection 2
after injection 3
B
CD45+CD34+ viability (%)
100
80
60
40
20
0
before injection (T0)
after injection 1
after injection 2
after injection 3

Figure 10

C

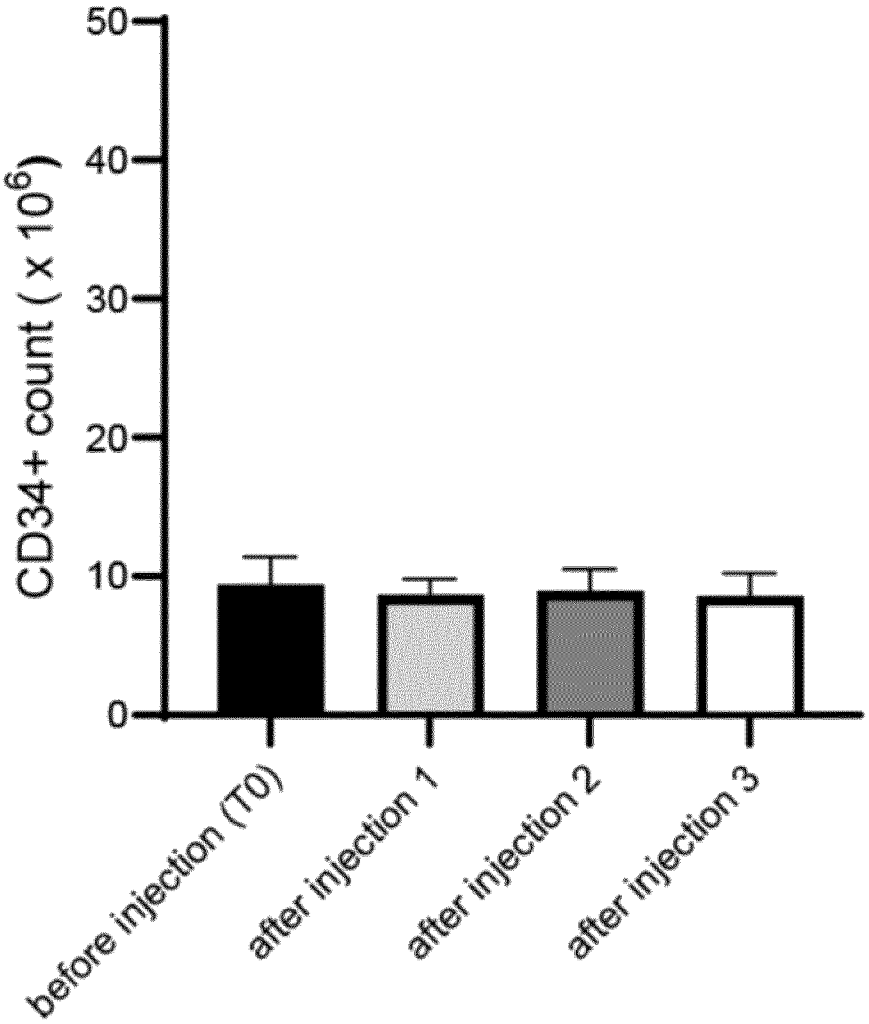

Figure 10 cont.

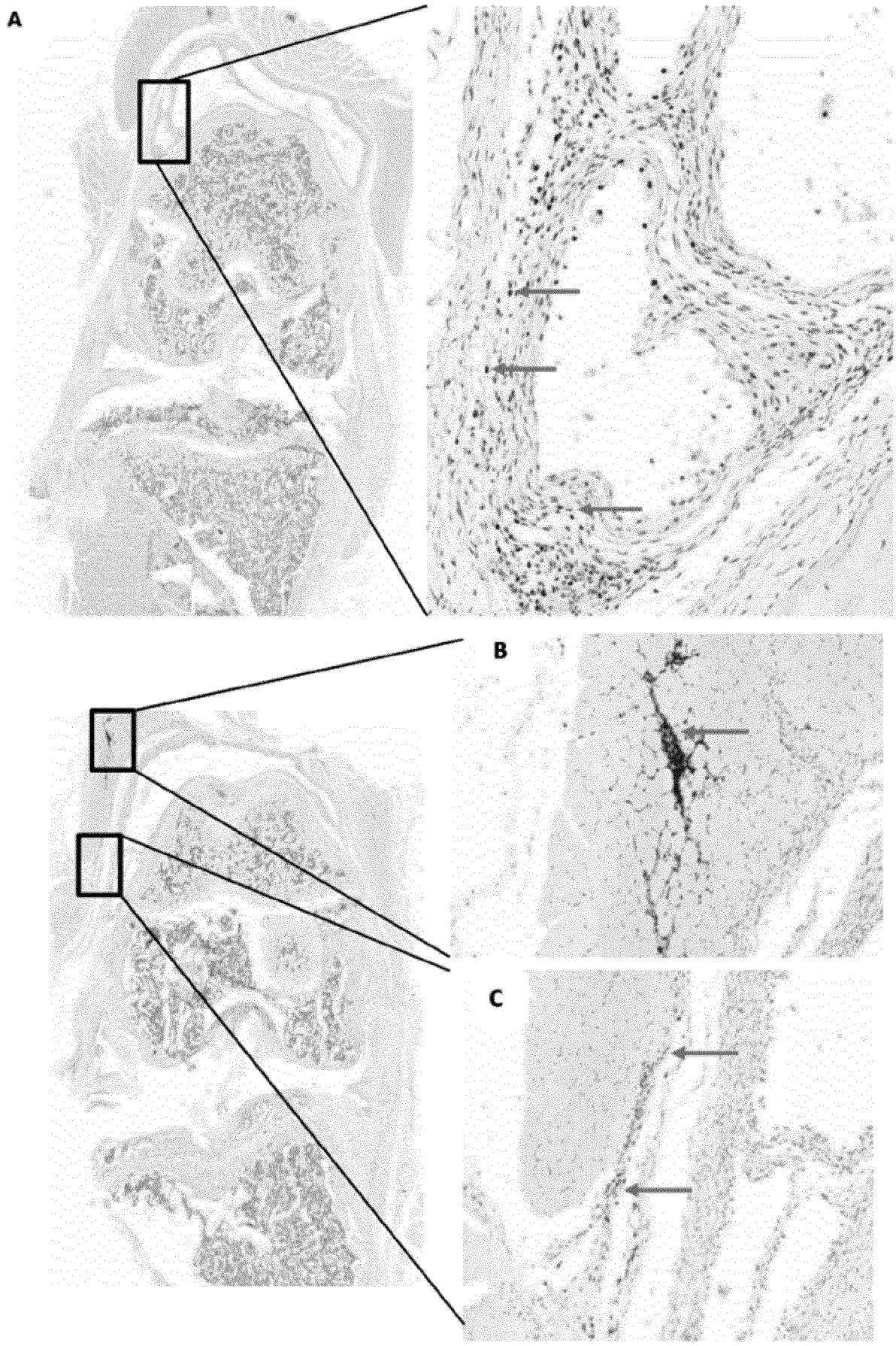

Figure 11

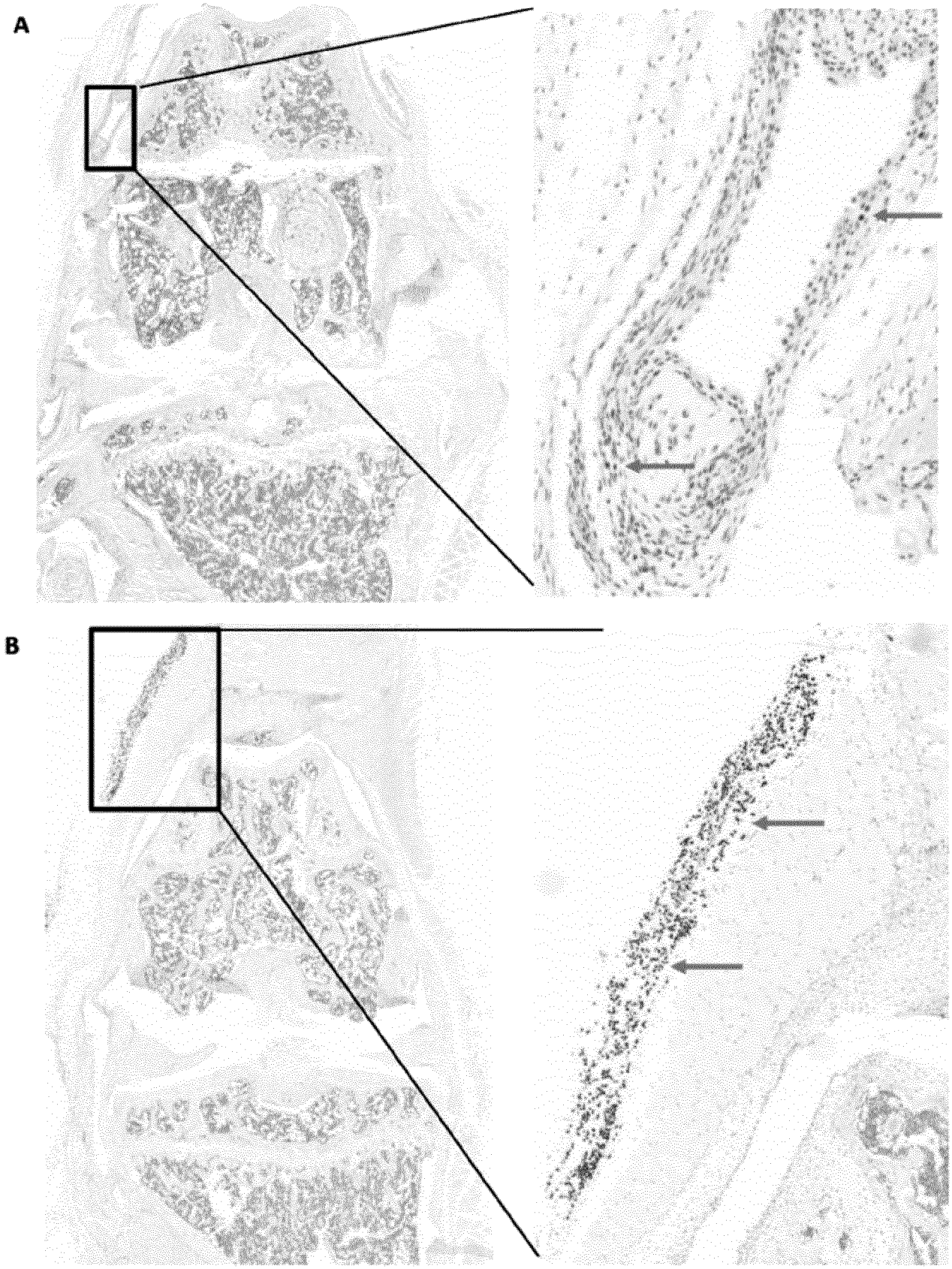

Figure 12

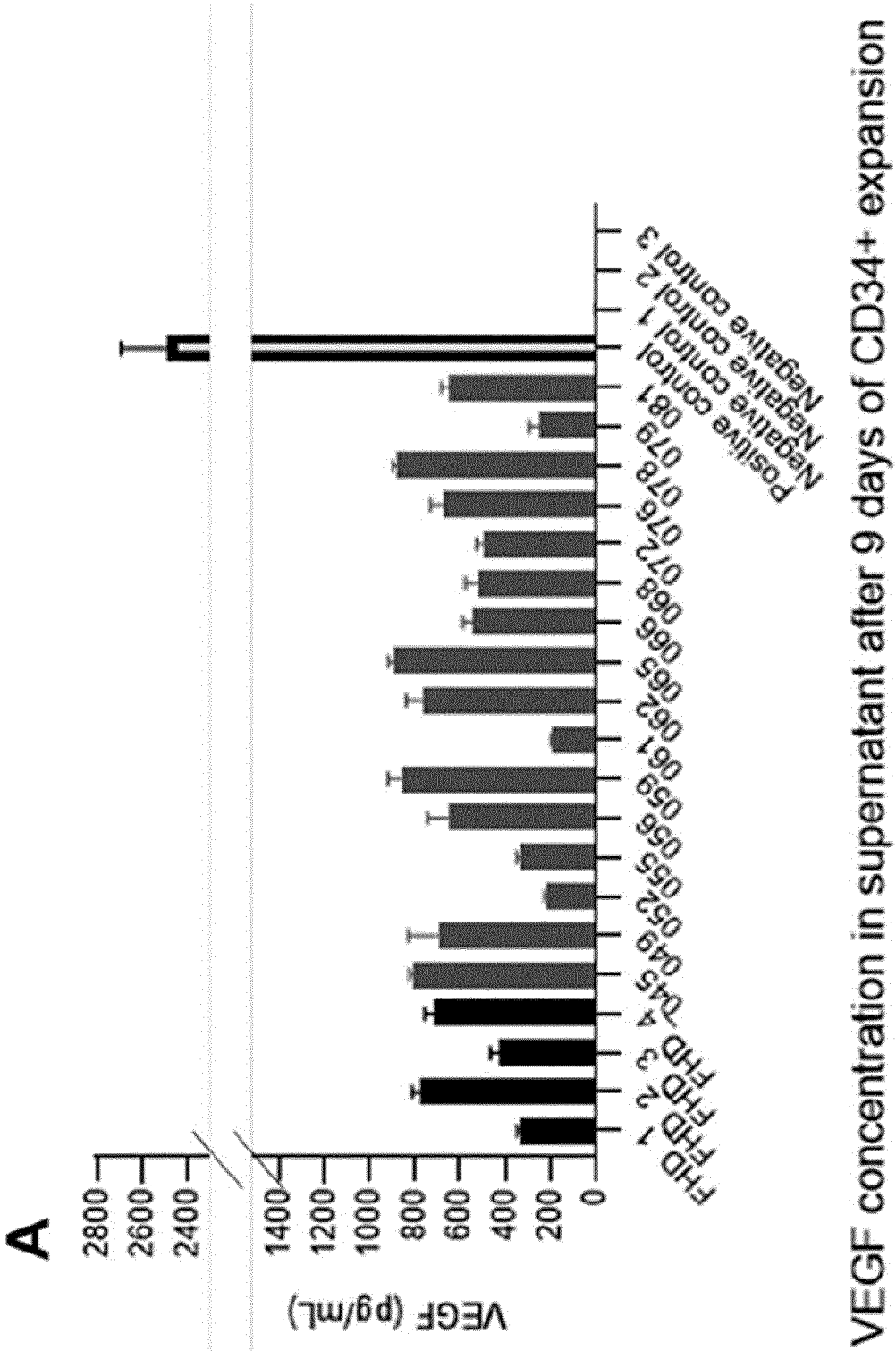
A
VEGF (pg/mL)
2800
2600
2400
1400
1200
1000
800
600
400
200
0
FHD 1
FHD 2
FHD 3
FHD 4
FHD 045
049
052
055
056
059
061
062
065
066
068
072
076
078
079
081
Negative control 1
Negative control 2
Negative control 3
Positive control
VEGF concentration in supernatant after 9 days of CD34+ expansion

Figure 13

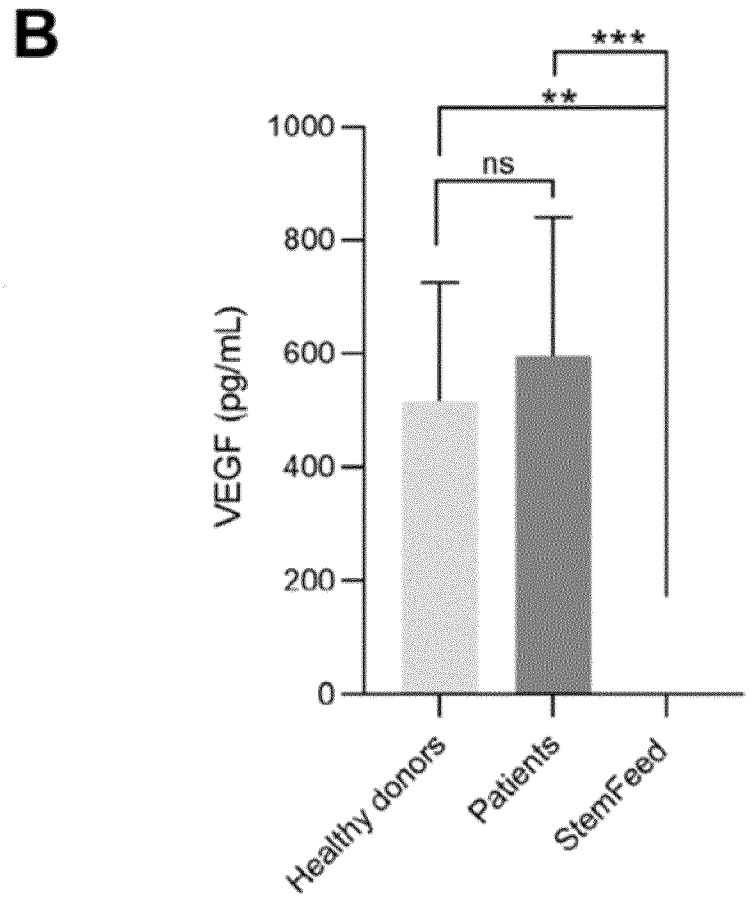

Figure 13 cont.

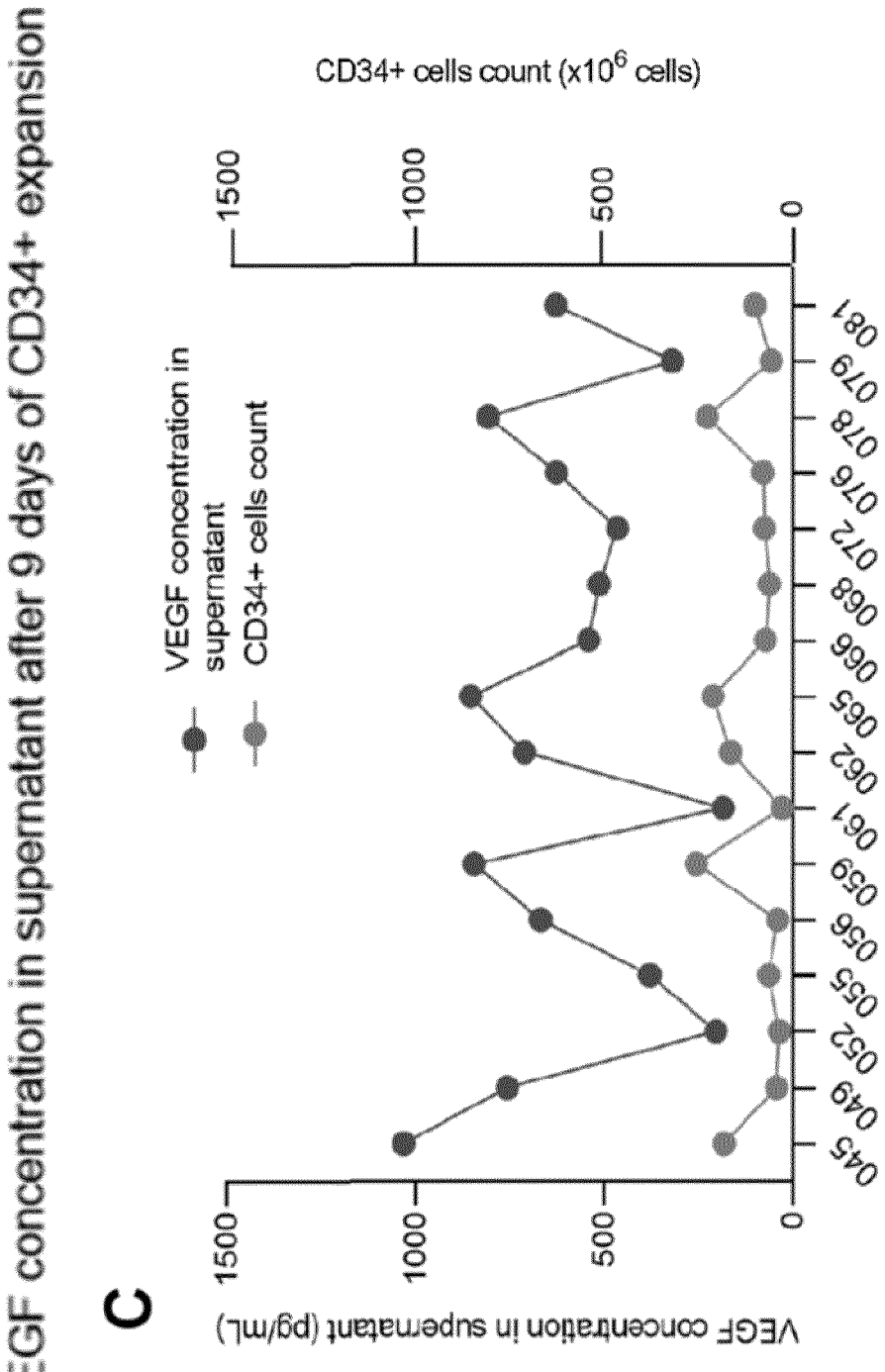

Figure 13 cont.

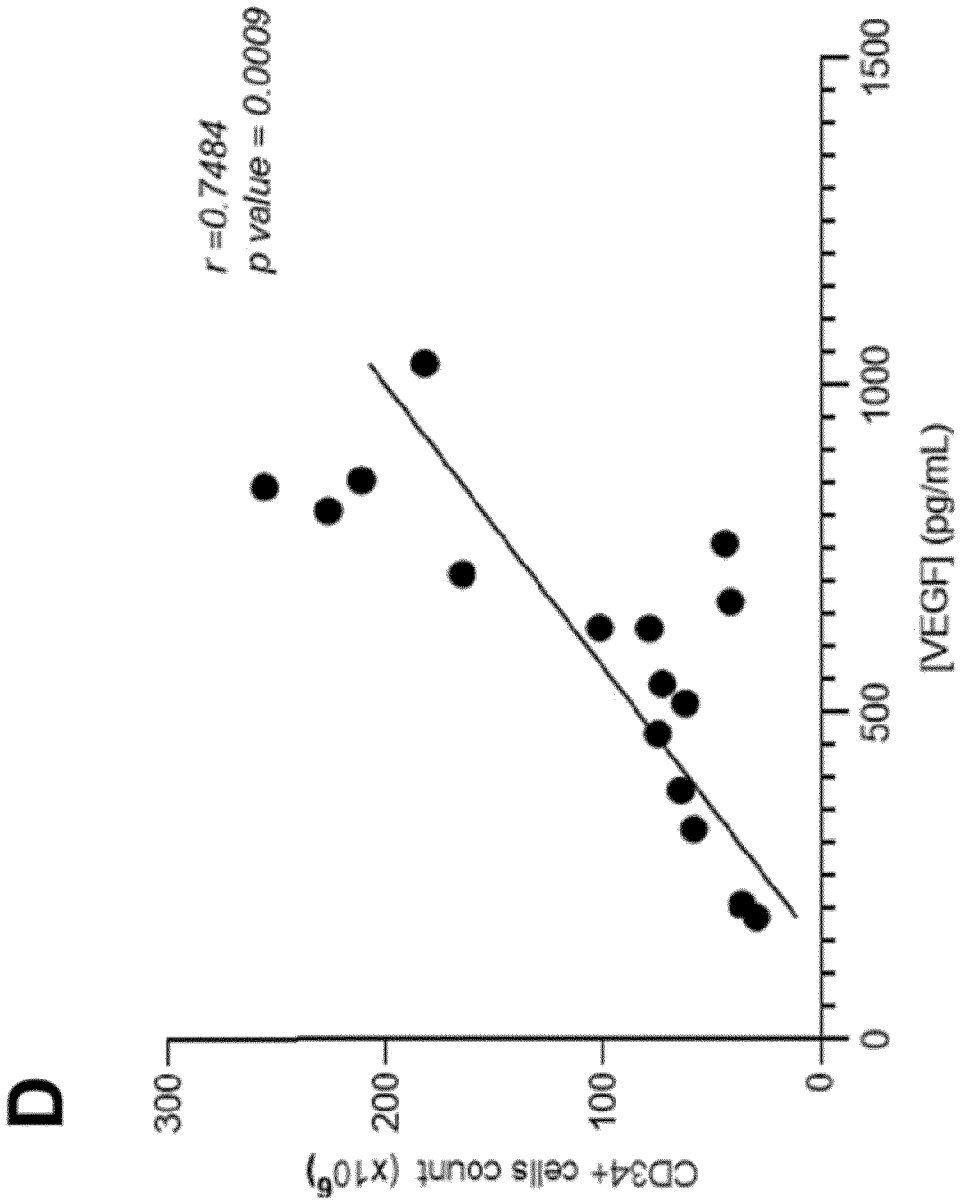

Figure 13 cont.

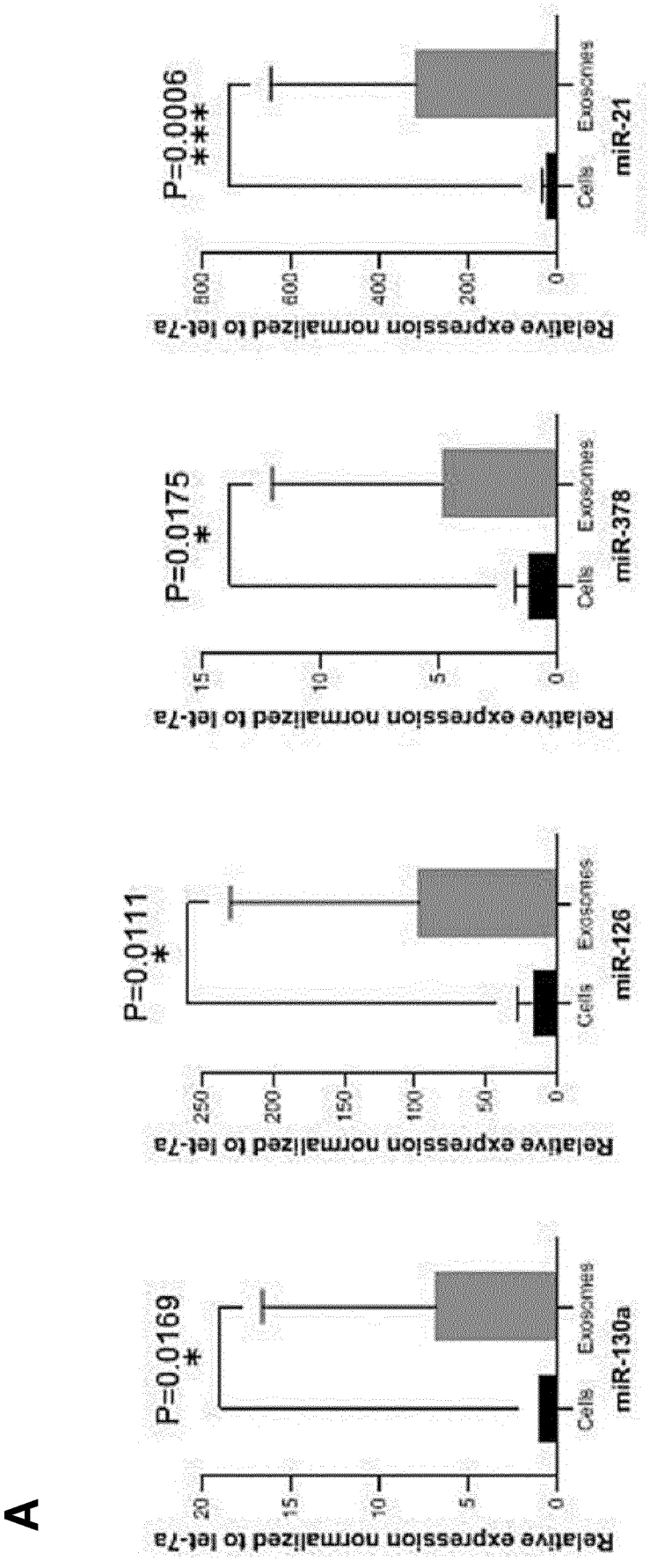
A
Relative expression normalized to let-7a
0
5
10
15
20
P=0.0169
*
Cells
Exosomes
miR-130a
Relative expression normalized to let-7a
0
50
100
150
200
250
P=0.0111
*
Cells
Exosomes
miR-126
Relative expression normalized to let-7a
0
5
10
15
P=0.0175
*
Cells
Exosomes
miR-378
Relative expression normalized to let-7a
0
200
400
600
800
P=0.0006
***
Cells
Exosomes
miR-21

Figure 14

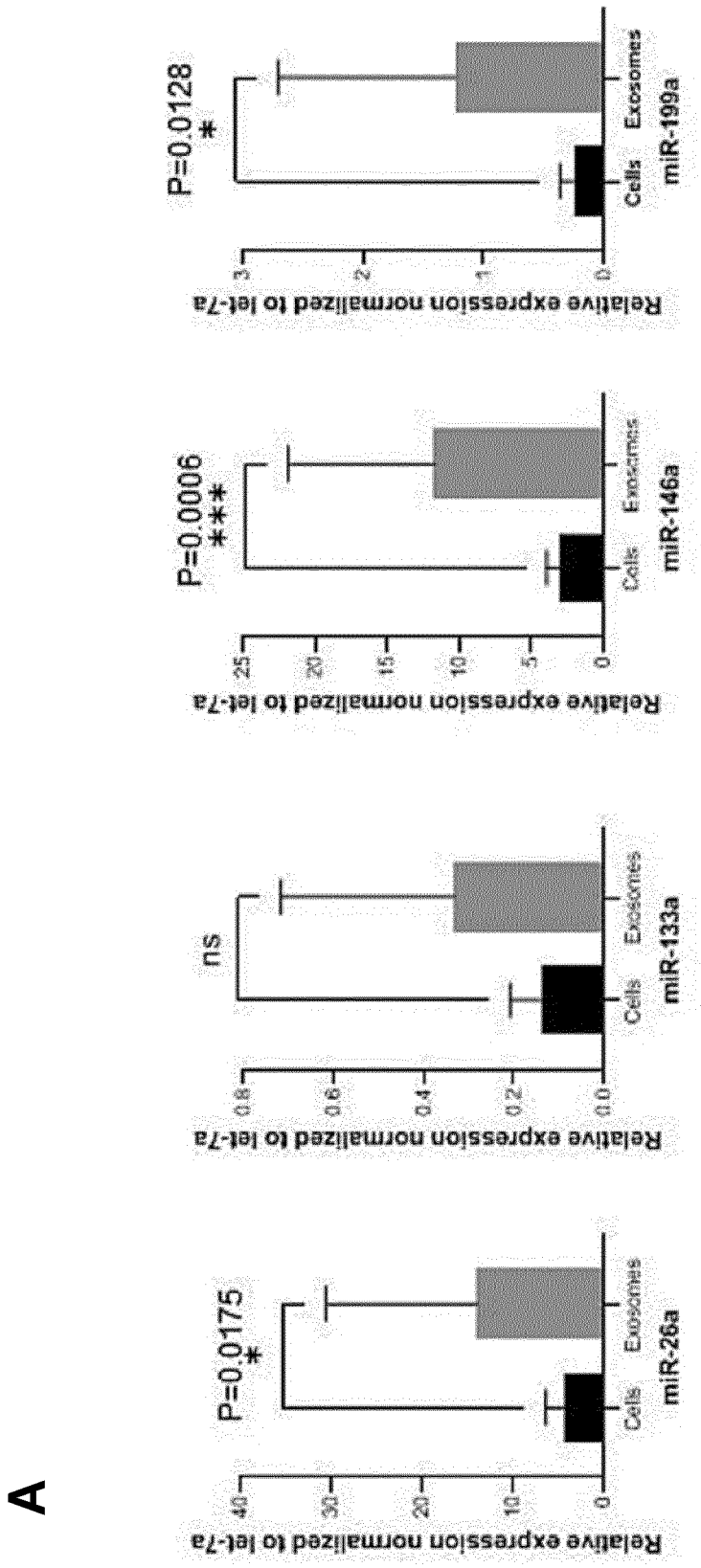

Figure 14 cont.

A

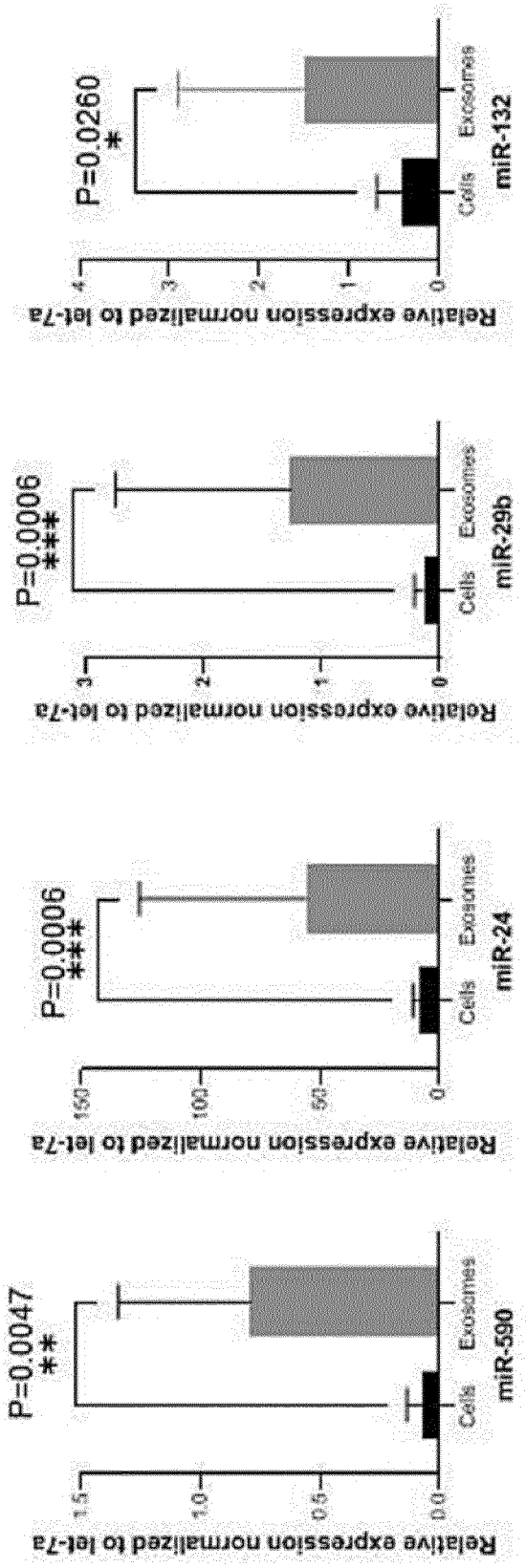

Figure 14 cont.

B

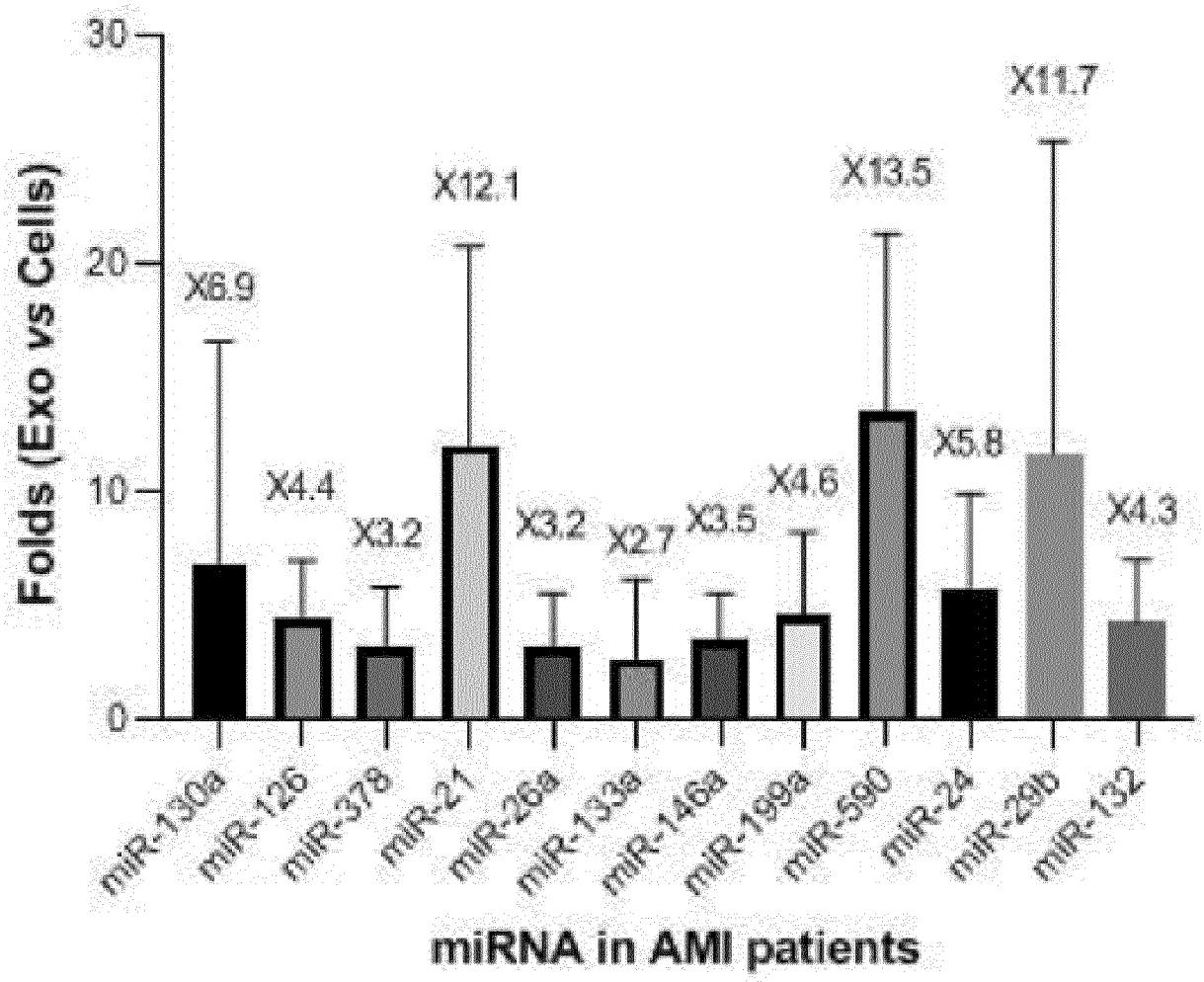

Figure 14 cont.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2024/056983

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/056983

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/28 A61K35/51 A61P19/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IBON GARITAONANDIA: "383. Autologous Expanded Peripheral Blood Derived CD34+ Stem Cells for the Treatment of Moderate Knee Osteoarthritis", 25TH ANNU MEET AM SOC GEN CELL THER (ASGCT) WASHINGTON, D.C., 1 May 2022 (2022-05-01), pages 182-182, XP093160670, Retrieved from the Internet: URL:chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://www.cell.com/molecular-therapy-family/molecular-therapy/pdf/S1525-0016(24)00237-5.pdf> abstract ----- -/--	1-15

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 May 2024	21/05/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Vandenbogaerde, Ann

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/056983

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAUCOURT CLAIRE ET AL: "Design and Validation of an Automated Process for the Expansion of Peripheral Blood-Derived CD34+ Cells for Clinical Use After Myocardial Infarction", STEM CELLS TRANSLATIONAL MEDICINE, vol. 8, no. 8, 1 August 2019 (2019-08-01), pages 822-832, XP093043806, US ISSN: 2157-6564, DOI: 10.1002/sctm.17-0277 cited in the application the whole document	1-15
X	US 2007/264238 A1 (SHAW ROBERT A [US] ET AL) 15 November 2007 (2007-11-15) claims; examples	1-3,9, 11-13
X	A KUBSIK-GIDLEWSKA: "CD34+ stem cell treatment for knee osteoarthritis: a treatment and rehabilitation algorithm", JOURNAL OF REHABILITATION MEDICINE - CLINICAL COMMUNICATIONS, vol. 1, no. 1, 1 January 2018 (2018-01-01) , page 1000012, XP093160705, ISSN: 2003-0711, DOI: 10.2340/20030711-1000012 the whole document	1-3,9, 11-13
X	ZHANG X ET AL: "Evaluation of CD34+ hematopoietic stem cell-associated extracellular vesicles as a potential personalized therapy for osteoarthritis", OSTEOARTHRITIS AND CARTILAGE, ELSEVIER, AMSTERDAM, NL, vol. 28, 1 April 2020 (2020-04-01), XP086140891, ISSN: 1063-4584, DOI: 10.1016/J.JOCA.2020.02.512 [retrieved on 2020-04-20] the whole document	1-3,9, 11-13

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2024/056983

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2007264238 A1	15-11-2007	NONE	