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Stimulation of Fibrotic Processes by the Infrapatellar Fat Pad in Cultured Synoviocytes From Patients With Osteoarthritis

A Possible Role for Prostaglandin $F_{2\alpha}$

Yvonne M. Bastiaansen-Jenniskens,[1] Wu Wei,[1] Carola Feijt,[1] Jan H. Waarsing,[1] Jan A. N. Verhaar,[1] Anne-Marie Zuurmond,[2] Roeland Hanemaaijer,[2] Reinout Stoop,[2] and Gerjo J. V. M. van Osch[1]

Objective. **Stiffening of the joint is a feature of knee osteoarthritis (OA) that can be caused by fibrosis of the synovium. The infrapatellar fat pad (IPFP) present in the knee joint produces immune-modulatory and angiogenic factors. The goal of the present study was to investigate whether the IPFP can influence fibrotic processes in synovial fibroblasts, and to determine the role of transforming growth factor β (TGFβ) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in these processes.**

Methods. **Batches of fat-conditioned medium (FCM) were made by culturing pieces of IPFP obtained from the knees of 13 patients with OA. Human OA fibroblast-like synoviocytes (FLS) (from passage 3) were cultured in FCM with or without inhibitors of TGFβ/activin receptor–like kinase 5 or $PGF_{2\alpha}$ for 4 days. The FLS were analyzed for production of collagen and expression of the gene for procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*; encoding lysyl hydroxylase 2b, an enzyme involved in collagen cross-linking) as well as the genes encoding α-smooth muscle actin and type I collagen α1 chain. In parallel, proliferation and migration of the synoviocytes were analyzed.**

Results. **Collagen production and *PLOD2* gene expression by the FLS were increased 1.8-fold ($P < 0.05$) and 6.0-fold ($P < 0.01$), respectively, in the presence of FCM, relative to control cultures without FCM. Moreover, the migration and proliferation of synoviocytes were stimulated by FCM. Collagen production was positively associated with $PGF_{2\alpha}$ levels in the FCM ($R = 0.89$, $P < 0.05$), and inhibition of $PGF_{2\alpha}$ levels reduced the extent of FCM-induced collagen production and *PLOD2* expression. Inhibition of TGFβ signaling had no effect on the profibrotic changes.**

Conclusion. **These results indicate that the IPFP can contribute to the development of synovial fibrosis in the knee joint by increasing collagen production, *PLOD2* expression, cell proliferation, and cell migration. In addition, whereas the findings showed that TGFβ is not involved, the more recently discovered profibrotic factor $PGF_{2\alpha}$ appears to be partially involved in the regulation of profibrotic changes.**

Osteoarthritis (OA) is a multifactorial disease of the articular joints, with an incidence that is higher in women, obese subjects, and older individuals (1,2). The role of the synovium in OA pathology is becoming more evident. In conjunction with cartilage damage and bone alterations, the pathologic features of inflammation, hyperplasia, and extensive fibrosis are also often observed in the synovium of OA joints (3–5).

Fibrosis can be seen as an abnormal healing process that is characterized by excessive deposition of

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[1]Yvonne M. Bastiaansen-Jenniskens, PhD, Wu Wei, MD, Carola Feijt, Jan H. Waarsing, PhD, Jan A. N. Verhaar, MD, PhD, Gerjo J. V. M. van Osch, PhD: Erasmus MC University Medical Center, Rotterdam, The Netherlands; [2]Anne-Marie Zuurmond, PhD, Roeland Hanemaaijer, PhD, Reinout Stoop, PhD: The Netherlands Organization for Applied Scientific Research (TNO), Leiden, The Netherlands.

Address correspondence to Yvonne M. Bastiaansen-Jenniskens, PhD, Erasmus MC University Medical Center, Department of Orthopaedics, Ee1614, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands. E-mail: y.bastiaansen@erasmusmc.nl.

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extracellular matrix proteins, in particular collagen, which, in turn, results in alteration of the structure of the tissue and, finally, even loss of function of this tissue. Fibrotic processes are a response to a variety of insults, such as infection, trauma, autoimmunity, or inflammation, resulting in an inflammatory reaction with rapid recruitment of monocytes from the circulation or macrophages resident in the tissue. These cells are important sources of fibrotic cytokines, such as transforming growth factor β (TGFβ) and platelet-derived growth factor, that, in turn, recruit fibroblasts to the site of injury and stimulate them to proliferate (6).

Fibroblasts can also differentiate toward myofibroblasts. Myofibroblasts, which are characterized by the presence of α-smooth muscle actin, are present during normal tissue repair, but when the wound is closed, they disappear from the site. In the case of fibrosis, myofibroblasts persist in the damaged tissue (7–9). An additional hallmark of fibrosis is the increased level of hydroxyallysine collagen crosslinks, which is regulated by lysyl hydroxylase 2b (LH2b), an enzyme encoded by the gene for procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) (10). These hydroxyallysine collagen crosslinks are found in fibrotic tissues and are associated with irreversible accumulation of collagen (11). Recently, it was demonstrated that *PLOD2* expression and the presence of LH2b are up-regulated in the fibrotic synovium of mice with OA (12).

TGFβ is a potent inducer of *PLOD2* expression and LH2b activity (12,13), making TGFβ a very potent fibrotic growth factor involved in all of the processes seen in fibrosis. In addition to TGFβ, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was recently discovered as a cytokine that facilitates pulmonary fibrosis independent of TGFβ. Mice lacking the $PGF_{2\alpha}$ receptor did not develop pulmonary fibrosis in response to bleomycin, and cells stimulated with $PGF_{2\alpha}$ produced more collagen (14,15).

In addition to cartilage, menisci, ligaments, and synovium, the knee joint contains fat pads. One of the largest of the fat pads is the infrapatellar fat pad (IPFP). The main role of the IPFP is to facilitate distribution of synovial fluid and distribute mechanical forces through the knee joint. Several adipokines and cytokines, such as tumor necrosis factor α, interleukin-6 (IL-6), IL-10, leptin, and vascular endothelial growth factor, are known to be produced by the IPFP (16–20). The IPFP is located in close proximity to the synovial layers and cartilage surfaces, making the IPFP able to influence inflammatory processes in the knee (21). Many secreted proteins are derived from the non-adipocyte fraction of adipose tissue (macrophages, T cells, and B cells) (18,22), and it is suggested that most of the cytokines produced by the adipose tissue are macrophage-derived (23,24).

Since synovial fibrosis is often seen in end-stage OA, the goal of the present study was to investigate whether the IPFP and its secreted factors influence fibrotic processes in synovial fibroblasts, and to determine the role of 2 potent fibrotic inducers, TGFβ and $PGF_{2\alpha}$, in the relationship between the IPFP and synovial fibrosis.

MATERIALS AND METHODS

Preparation and analysis of fat-conditioned medium (FCM). Samples of IPFP were derived from anonymized leftover knee tissue material obtained from patients with OA who had undergone total knee arthroplasty. The IPFP samples were used to produce FCM. The patients implicitly consented to the use of these tissues for scientific research, in accordance with the guidelines of the Federation of Biomedical Scientific Societies (http://www.federa.org), with approval from the local ethics committee in Rotterdam, The Netherlands (approval no. MEC 2008-181). The mean age of the donors was 67.9 years (range 54–81 years) and the mean body mass index (BMI) of the donors was 30.52 kg/m^2 (range 19.6–44.5 kg/m^2).

The inner parts of the fat pads, where no synovium is present, were cut into small pieces of ~10 mg and cultured in suspension for 24 hours in a concentration of 50 mg tissue/ml in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Gibco BRL), containing insulin, transferrin, selenic acid, and albumin (ITS+) (dilution 1:100; BD Biosciences) as well as 50 μg/ml gentamicin and 1.5 μg/ml Fungizone (both from Gibco BRL). As a control medium, we used identically composed culture medium that did not contain pieces of IPFP, cultured in parallel. After 24 hours, the medium was harvested, centrifuged at 300*g* for 8 minutes to remove (immune) cells, and frozen at −80°C in aliquots of 1.5 ml, resulting in 29 different batches of FCM. This incubation time was chosen arbitrarily, since each mediator has its own optimum regarding release kinetics (22,25–27).

Isolation and culture of fibroblast-like synoviocytes (FLS). Samples of human synovium were also obtained as anonymized leftover material from patients with OA who had undergone total knee arthroplasty (approval no. MEC 2004-322). On the basis of its specific structure, the synovium could be distinguished and removed from the adjacent tissue. The synovium samples were then digested in Pronase (2 mg/ml; Sigma) for 2 hours and in collagenase B (1.5 mg/ml; Roche Diagnostics) overnight. Digested cells were plated out as 3,500 cells per cm^2 and expanded in Iscove's modified Dulbecco's medium with 10% fetal calf serum (FCS), 50 μg/ml gentamicin, and 1.5 μg/ml Fungizone (all from Gibco BRL). Cells from passage 3 were allowed to adhere at a density of 50,000 cells per cm^2 in DMEM with Glutamax, 10% FCS, and antibiotics (all from Gibco BRL). After overnight attachment, the culture medium was removed and the cells were washed carefully 3 times with saline. FCM was mixed 1:1 with fresh DMEM with Glutamax, supplemented with 50 μg/ml gentamicin, 1.5 μg/ml

Fungizone, and ITS+ (dilution 1:100), and applied to the attached FLS. Thereafter, the FLS were cultured for 4 days. The mean age of the FLS donors was 67 years (range 60–81 years) and the mean BMI was 32.4 kg/m^2 (range 24.2–42.5 kg/m^2).

To investigate the involvement of TGFβ or PGF$_{2\alpha}$, 1 μM SB505124 (Sigma), an inhibitor of TGFβ signaling, or 10 μM AL8810 (Cayman Chemical), an inhibitor of PGF$_{2\alpha}$ signaling, was added 1 hour prior to adding the FCM; as positive control for the inhibition, 1 ng/ml TGFβ or 1 μM PGF$_{2\alpha}$ was added. Concentrations and timing were based on those previously described in a study by Oga et al (14).

Analysis of collagen deposition. Collagen was examined in the tissue samples using a QuickZyme soluble collagen assay, according to the manufacturer's guidelines (QuickZyme Biosciences). Briefly, culture medium was removed and the cell/matrix fraction was solubilized by overnight incubation in 0.5M acetic acid at 4°C. The QuickZyme assay is based on binding of collagen with sirius red.

Analysis of gene expression. After culture, monolayers of synoviocytes were suspended in 350 μl RLT buffer (Qiagen) supplemented with 1% β-mercaptoethanol. RNA was extracted, and complementary DNA was analyzed for gene expression using previously described methods (28). The primer sequences for the genes were as follows: for the *GAPDH* reference gene, forward GTCAACGGATTTGGTCGTATTGGG, reverse TGCCATGGGTGGAATCATATTGG, and probe FAM-CGCCCAATACGACCAAATCCGTTGAC-TAMRA; for the type I collagen α1 chain gene (*COL1A1*), forward CAGCCGCTTCACCTACAGC, reverse TTTTGTATTCAATCACTGTCTTGCC, and probe FAM-CCGGTGTGACTCGTGCAGCCATC-TAMRA; for *PLOD2*, forward CCCTCCGATCAGAGATGATT and reverse AATGTTTCCGGAGTAGGGGAGTCTTTTT; and for the gene encoding α-smooth muscle actin (*ASMA*), forward CGTTGCCCCTGAAGAGCAT and reverse CCGCCTGGATAGCCACATACA. Primers for the type III collagen gene (*COL3*) were purchased from Qiagen Assays-on-Demand (QT00058233). For analysis of *GAPDH* and *COL1A1*, TaqMan 2× Universal Polymerase Chain Reaction (PCR) Master Mix (Applied Biosystems) was used in the reaction. For analysis of *PLOD2* and *ASMA*, quantitative PCR Master Mix Plus SYBR Green I (Eurogentec) was used in the reaction. In determining the optimal housekeeping gene, we compared *GAPDH*, 18S RNA, β_2-microglobulin, and hypoxanthine guanine phosphoribosyltransferase, and observed that *GAPDH* was the most stable housekeeping gene in our experiments.

Migration assay. To investigate the migration of the synoviocytes in response to soluble factors, a scratch wound assay was performed. Synoviocytes from passage 3 were seeded at 100,000 cells per cm^2 in 12-well plates and allowed to adhere overnight in DMEM containing 10% FCS. A 20-μl pipette tip was used to make a scratch in the confluent monolayer of the synoviocytes, after marking the scratch location on the bottom of the well. When applicable, SB505124 or AL8810 was added 1 hour prior to making the scratch. Cell debris was removed by washing with saline, and the cell culture was continued in DMEM–1% ITS+ mixed with FCM (1:1) with or without SB505124 or AL8810.

Photographic images were obtained directly after scratching and at 14, 16, and 19 hours after scratching. These time points were chosen on the basis of pilot experiments showing that, from 14 hours onward, migration of the synoviocytes is best visualized (results not shown) without interference from proliferation of the cells. Closure was measured using TScratch software (Computational Science & Engineering Laboratory). The extent of migration is presented as the percentage of closure after wounding.

Proliferation assay. To analyze proliferation, synoviocytes from passage 3 were seeded at a density of 10,000 cells per cm^2 in 12-well plates and allowed to adhere overnight in DMEM containing 10% FCS. To arrest cells in the S_1 phase (which was necessary in order to reduce variation), cells were cultured in DMEM containing 0.1% FCS for 24 hours after adherence. Following 24 hours of starvation, the culture was continued in DMEM–1% ITS+ mixed with FCM (1:1) with or without the addition of SB505124 or AL8810. When applicable, SB505124 or AL8810 was added 1 hour prior to adding the FCM. Samples for DNA measurement were obtained after 1, 2, 3, and 4 days by suspending the monolayer in phosphate buffered saline with 0.1% Triton. The amount of DNA in each sample was determined using ethidium bromide, with calf thymus DNA (Sigma) as standard.

Enzyme-linked immunosorbent assay (ELISA) for TGFβ1. To determine the activity of TGFβ1 in the FCM, a human TGFβ1 Quantikine ELISA kit (R&D Systems) was used according to the manufacturer's guidelines. To activate latent TGFβ1 to the immunoreactive form, acid activation with 1N HCl and neutralization with 1.2N NaOH/0.5M HEPES was performed.

PGF$_{2\alpha}$ measurements using mass spectrometry. To determine the levels of PGF$_{2\alpha}$ in the FCM samples that remained (n = 7), samples were analyzed with liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously (29). Briefly, to prepare the samples for measurement, FCM samples, with deuterated PGF$_{2\alpha}$-d4 (Cayman Chemical) added as internal standard, were extracted with LC-MS–grade methanol (Riedel-de-Häen). The methanol extract was loaded on an HLB SPE column (Oasis), after which the samples were reconstituted in 100 μl ethanol containing CUDA (Cayman Chemical) as a second internal standard, and immediately used for LC-MS/MS analysis on an Acquity C18 BEH Ultra Performance liquid chromatography column coupled to a Xevo TQ-S mass spectrometer (Waters). Cone voltage and collision energy were optimized for each compound individually. Parent and product mass/charge (m/z) values of PGF$_{2\alpha}$ were 353.1 and 193.0. Parent and product m/z values of PGF$_{2\alpha}$-d4 were 357.1 and 313.4. Parent and product m/z values of CUDA were 339.1 and 214.1. Identification and quantification of peak values were performed using MassLynx software version 4.1.

Statistical analysis. Experiments examining the effect of FCM were performed with samples from 3 different FLS donors and 13 different FCM batches representing 13 different IPFP donors. Experiments examining the effect of inhibition of TGFβ or PGF$_{2\alpha}$ together with FCM stimulation were performed with samples from 2 different FLS donors and 8 different FCM batches representing 8 different IPFP donors. All experiments were performed with triplicate samples per condition, which was taken into account in the statistical analysis. A mixed linear model, followed by a Bonferroni post

hoc test, was used to analyze gene expression, collagen deposition, and cell migration. A univariate general linear model was used to analyze the results from the proliferation assays. Since not every FCM batch was tested on FLS from every donor, we allowed for this in the statistical analysis by adding a subject variable indicating the FLS donor. Spearman's rho correlations were determined to examine associations between TGFβ or $PGF_{2\alpha}$ levels and other parameters. Data were analyzed with IBM SPSS statistical software (version 20.0).

RESULTS

Induction of fibrotic processes with medium conditioned by IPFP. In culture conditions with 8 of the 13 FCM batches (derived from 13 different OA IPFP donors), collagen production by FLS was increased 1.8-fold after 4 days of culture, from a mean 1.9 μg/monolayer in control conditions without FCM to a mean 3.4 μg/monolayer in cultures with FCM (Figure 1A [all 13 included in the figure]). In addition, gene expression of the enzyme involved in the formation of pyridinoline-based collagen crosslinks, *PLOD2*, was increased 6.0-fold in the presence of FCM. Surprisingly, *COL1A1* expression on day 4 was 2.5-fold lower than that in control conditions without FCM, and *ASMA* expression was 1.7-fold lower (Figure 1B). *COL3* expression was unaltered by the addition of FCM (results not shown). The observed increase in collagen production but decrease in *COL1A1* expression might be explained by the hypothesis that altered processing of the collagen would lead to more efficient translation, but would not alter expression of *COL3*. Also, the collagen deposition represents accumulation in the total culture for 4 days, whereas the collagen gene expression is the specific expression at the moment of harvest.

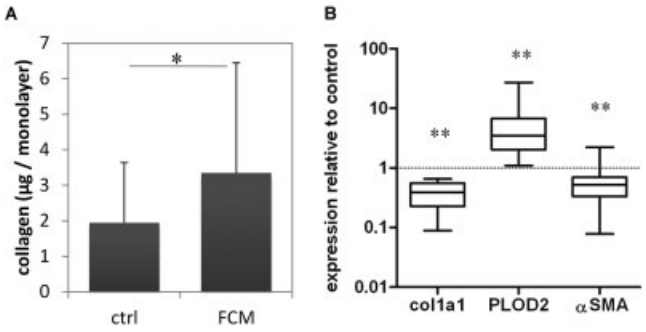

Figure 1. Effects of fat-conditioned medium (FCM) on total collagen production by fibroblast-like synoviocytes (FLS) in monolayer after 4 days of culture **(A)** and gene expression by FLS after 4 days of culture, relative to control conditions without FCM (set at 1; broken horizontal line) **(B)**. Genes describing the fibrotic process were genes encoding type I collagen α1 chain (*COL1A1*), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*), and α-smooth muscle actin (α-SMA). In total, 13 different FCM batches were tested on FLS from 3 different donors. In **A,** bars show the mean ± SD. In **B,** data are shown as box plots, in which each box represents the 25th to 75th percentiles, lines inside the boxes represent the median, and lines outside the boxes represent the minimum and maximum values. * = $P < 0.05$; ** = $P < 0.01$ versus control.

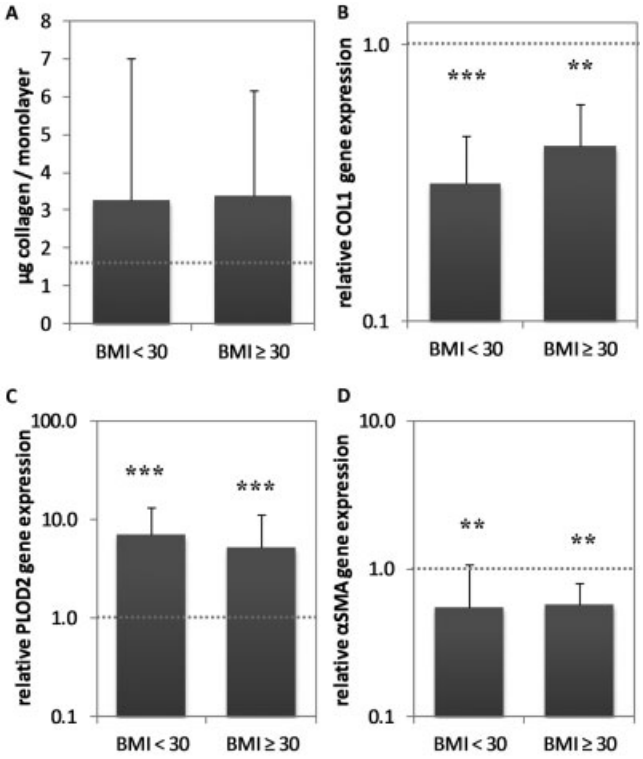

Figure 2. Effects of fat-conditioned medium (FCM) from subgroups of nonobese human donors (body mass index [BMI] <30 kg/m^2; n = 5, 1 male and 4 female) and obese human donors (BMI ≥30 kg/m^2; n = 8, 1 male and 7 female) on collagen deposition **(A)** and expression of genes for type I collagen α1 chain (*COL1A1*) **(B)**, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) **(C)**, and α-smooth muscle actin (α-SMA) **(D)** by fibroblast-like synoviocytes, as compared to control conditions without FCM (set at 1.0; broken horizontal line). Bars show the mean ± SD. ** = $P < 0.01$; *** = $P < 0.005$ versus control.

Since TGFβ is known to be a potent inducer of collagen deposition and of *COL1A1*, *PLOD2*, and *ASMA* expression, we also included, as a positive control a culture condition in which TGFβ was added in all of the experiments. Indeed, irrespective of which FLS donor was used, TGFβ induced the same fibrotic processes as seen in FLS cultures with FCM (results not shown).

To investigate whether the BMI of the IPFP donor had any influence, we performed a post hoc

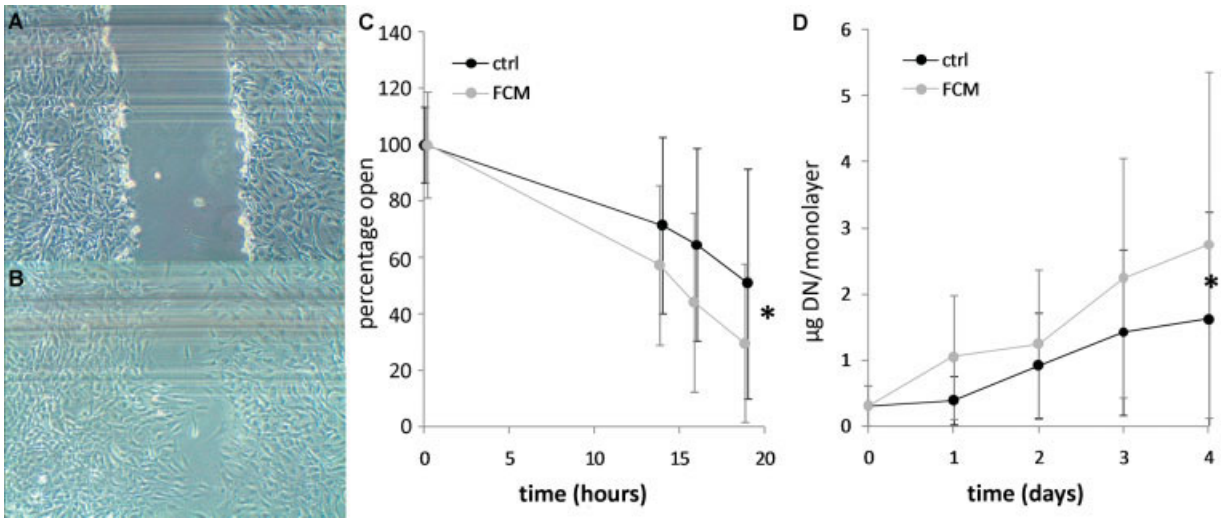

Figure 3. Effects of fat-conditioned medium (FCM) on migration and proliferation of fibroblast-like synoviocytes (FLS), as determined by scratch wound assay. **A** and **B,** Images of the scratch were obtained directly after making the scratch **(A)** and after migration for 19 hours **(B)**. The lines at the top of **A** and **B** mark the bottom of the well as the reference point for taking pictures during migration. **C** and **D,** The effects of FCM on the migration **(C)** and proliferation **(D)** of the FLS were tested. In total, 9 different FCM batches were tested on FLS from 3 different donors, and results were compared to control conditions without FCM. Results are shown as the mean ± SD. $* = P < 0.05$ versus control at all time points.

subgroup analysis comparing the FCM batches made from IPFPs of donors who had a BMI lower than 30 kg/m^2 (n = 5) with FCM batches made from IPFPs of donors who had a BMI equal to or higher than 30 kg/m^2 (n = 8). No differences in collagen deposition or expression of *COL1A1*, *PLOD2*, or *ASMA* were observed between the 2 BMI subgroups. Both groups still showed significant differences in gene expression when compared with the control condition without FCM (Figure 2).

To determine the effects on FLS migration, we performed a scratch wound assay (typical examples right after scratching and 19 hours after scratching are shown in Figures 3A and B). Migration of FLS ($P = 0.005$) and proliferation of FLS ($P = 0.002$) were stimulated in the presence of FCM (Figures 3C and D).

Association of FCM effects with the presence of TGFβ1 or PGF$_{2\alpha}$. TGFβ and PGF$_{2\alpha}$ are both known to be potent profibrotic mediators and candidate factors for involvement in the processes seen in the FLS in

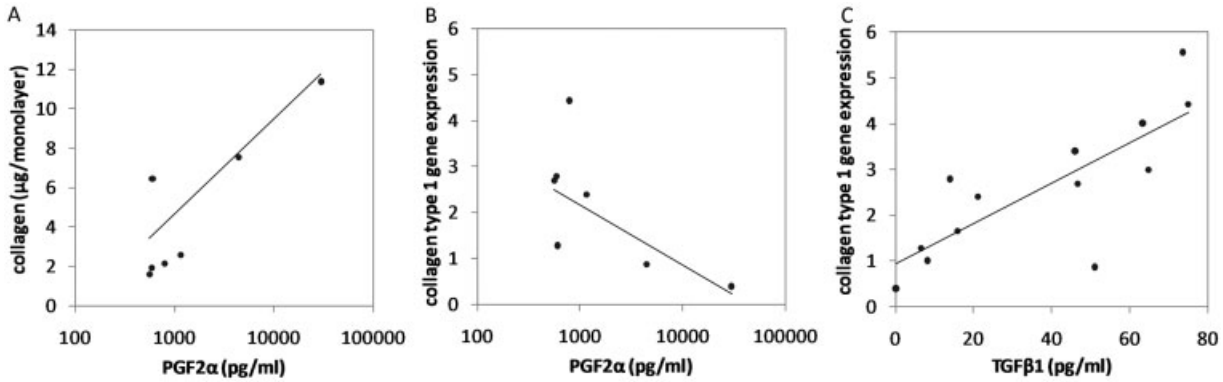

Figure 4. Associations between the concentration of transforming growth factor β1 (TGFβ1) and prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) in the fat-conditioned medium (FCM) and the effect of FCM on fibroblast-like synoviocytes (FLS) in culture. Positive associations were observed between the PGF$_{2\alpha}$ level in the FCM and collagen deposition by the FLS (R = 0.89, $P = 0.028$) **(A)** and between the TGFβ1 level in the FCM and gene expression of type I collagen α1 chain (*COL1A1*) (R = 0.77, $P = 0.012$) **(C)**, while a negative association was observed between the PGF$_{2\alpha}$ level in the FCM and *COL1A1* expression (R = −0.82, $P = 0.023$) **(B)**. *P* values were corrected for multiple comparisons.

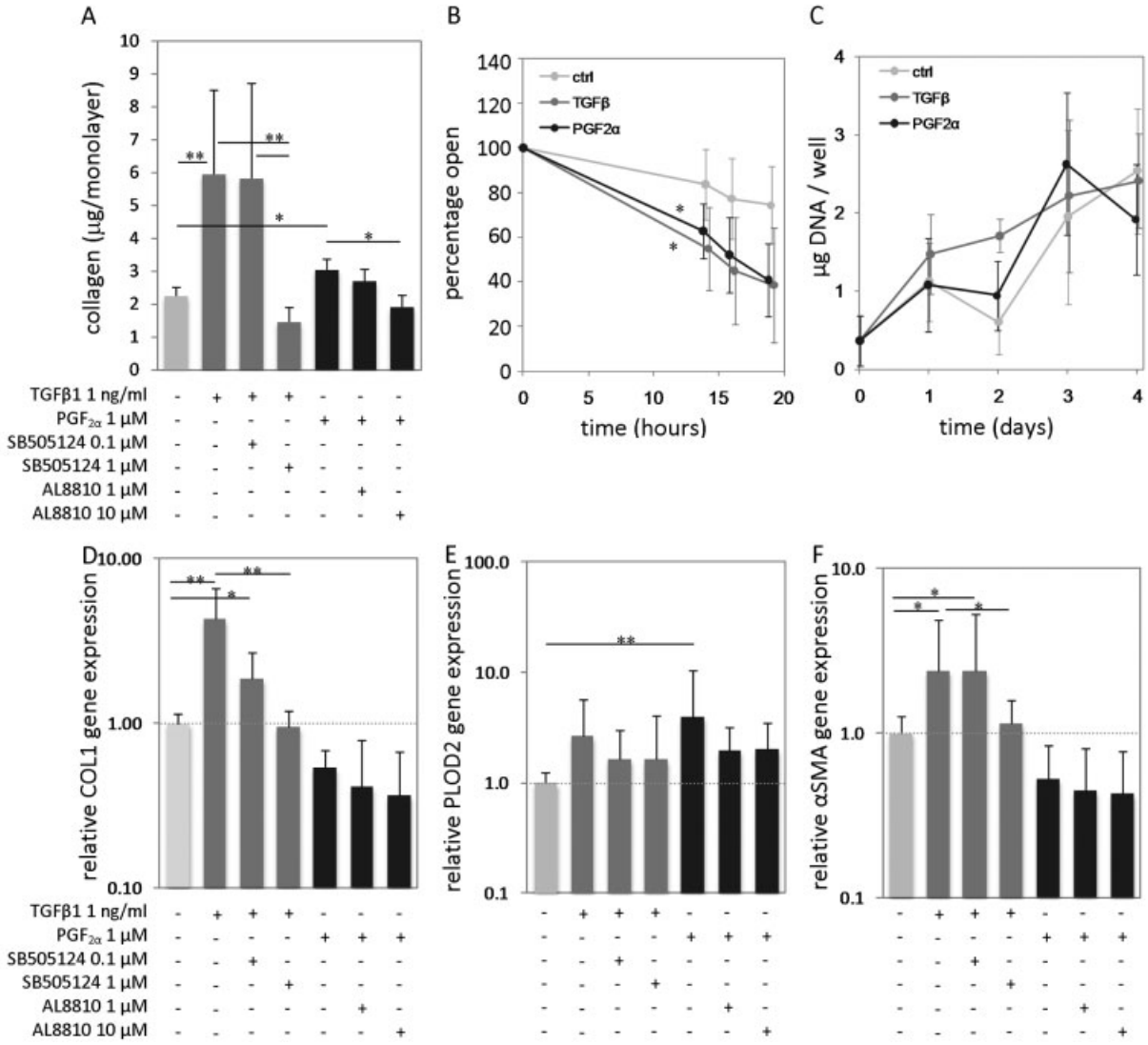

Figure 5. Effects of 1 ng/ml transforming growth factor β1 (TGFβ1) and 1 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and their inhibitors, SB505124 and AL8810, respectively, in the absence of fat-conditioned medium on collagen production by fibroblast-like synoviocytes (FLS) **(A)**, migration of FLS **(B)**, and proliferation of FLS **(C)** as well as gene expression of type I collagen α1 chain (*COL1A1*) **(D)**, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) **(E)**, and α-smooth muscle actin (α-SMA) **(F)** by FLS, relative to control conditions without these additions (set at 1.0; broken horizontal line). Results are shown as the mean ± SD in samples from 3 FLS donors, in experiments performed in triplicate. $* = P < 0.05$; $** = P < 0.01$.

response to FCM. The mean TGFβ1 content of the FCM was 37.3 pg/ml, ranging from 0.1 pg/ml to 74.9 pg/ml. The mean $PGF_{2\alpha}$ content in the FCM was 6,204 pg/ml, ranging from 560 pg/ml to 29,718 pg/ml. The level of $PGF_{2\alpha}$ was positively correlated with the extent of collagen deposition by the FLS (Figure 4A) and negatively associated with *COL1A1* expression (Figure 4B). The level of TGFβ1 was positively associated with *COL1A1* expression (Figure 4C). No other associations were seen.

To evaluate the effect of TGFβ1 and $PGF_{2\alpha}$ on FLS in our culture system, we added these compounds to FLS cultures without the presence of FCM. The addition of 1 ng/ml TGFβ1 increased total collagen deposition, *COL1A1* expression, and *ASMA* expression. The addition of 1 μM $PGF_{2\alpha}$ increased *PLOD2* expres-

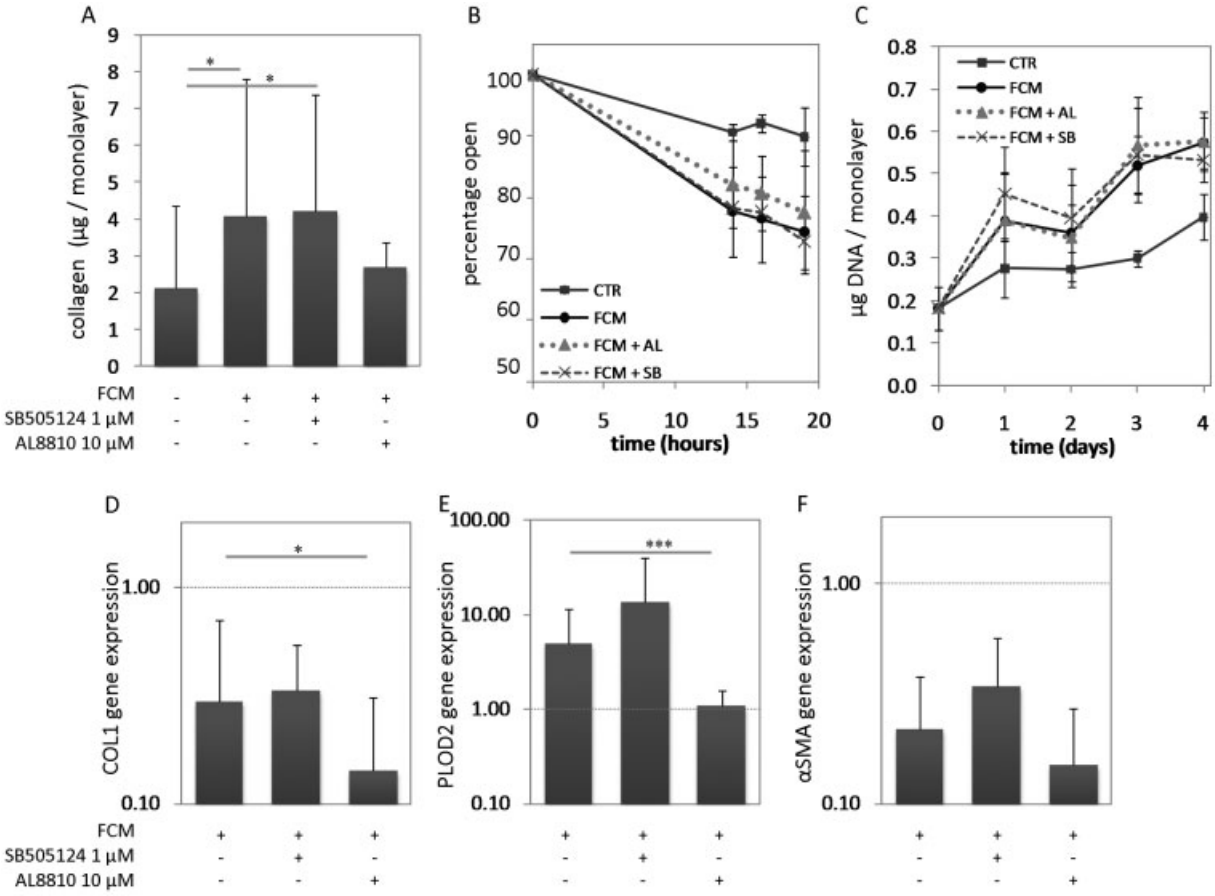

Figure 6. Effects of inhibition of transforming growth factor β (TGFβ)/activin receptor–like kinase 5 signaling (with the inhibitor SB505124) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) signaling (with the inhibitor AL8810) on the changes induced by fat-conditioned medium (FCM) in fibroblast-like synoviocyte (FLS) cultures, in terms of the effects on collagen production by FLS (**A**), migration of FLS (**B**), and proliferation of FLS (**C**) as well as gene expression of type I collagen α1 chain (*COL1A1*) (**D**), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) (**E**), and α-smooth muscle actin (α-SMA) (**F**) by FLS. Control cultures were performed in the absence of FCM (set at 1.00 for the gene expression analyses; broken horizontal line). Results are shown as the mean ± SD of 8 different FCM batches using FLS from 2 different donors. $* = P < 0.05$; $*** = P < 0.005$.

sion and total collagen deposition (Figures 5A and D–F). TGFβ1 and $PGF_{2\alpha}$ both increased the migration of the FLS, but had no effect on proliferation of the FLS (Figures 5B and C). The effects of $PGF_{2\alpha}$ best simulated the effects of FCM as seen in our experiments (as described in Figures 1 and 2).

Counteraction of the profibrotic effect of FCM via inhibition of $PGF_{2\alpha}$ signaling. To examine the involvement of TGFβ and $PGF_{2\alpha}$ in the profibrotic effect of FCM, the FLS were cultured with FCM with or without a TGFβ receptor type I kinase inhibitor, SB505124, or a selective PGF receptor antagonist, AL8810. First, we verified whether SB505124 could indeed inhibit the effect of 1 ng/ml TGFβ1, and whether AL8810 could indeed inhibit the effect of 1 μM $PGF_{2\alpha}$. SB505124 blocked the effect of TGFβ1 on collagen production, *COL1A1* expression, and *ASMA* expression, confirming the efficacy of the inhibitor. AL8810 inhibited the effect of $PGF_{2\alpha}$ on collagen production and there was a trend toward normalization of *PLOD2* expression ($P = 0.08$) (Figures 5A and D–F). The presence of 1 μM SB505124 or 10 μM AL8810 alone seemed to have no influence on collagen deposition or *COL1A1*, *PLOD2*, and *ASMA* expression (results available from the corresponding author upon request).

Inhibition of TGFβ/activin receptor–like kinase 5 signaling with SB505124 did not alter the FCM-induced effects on FLS, indicating that the effect of FCM was not caused by TGFβ. Blocking the PGF receptor with AL8810, on the other hand, inhibited the increase in

collagen deposition that had been induced by FCM, bringing the collagen deposition back to the levels seen in control conditions without FCM (Figure 6A). The increase in *PLOD2* expression induced by FCM was similarly abrogated, with a return to the levels seen in control conditions without FCM, when the FLS were coincubated with FCM and AL8810 (Figure 6E).

The effects of FCM on the migration and proliferation of synoviocytes and on the level of *ASMA* expression were not counteracted by the addition of the PGF receptor inhibitor AL8810 (Figures 6B, C, and F). *COL1A1* expression, which had been decreased in FLS cultures with FCM, was decreased even more in cultures with AL8810 (Figure 6D). This is consistent with the minimal decrease in *COL1A1* expression that was observed when only AL8810 was added to the FLS cultures (Figure 5D).

DISCUSSION

OA is a disease of the articular joints in which synovial fibrosis is often seen (3,4,12). Accumulating data have been presented to suggest that OA is an inflammatory disease in which cytokines and immune cells play a role (30). Adipose tissue can, in general, be considered to be an endocrine organ that secretes cytokines and growth factors and that exhibits significant infiltration of immune cells, including macrophages (31–33). In earlier studies conducted by our group and other investigators, it was shown that the IPFP is able to produce cytokines, adipokines, and growth factors, and thereby contributes to their levels in the synovial fluid (18–20,34). In the current study, we demonstrate that medium conditioned by samples of IPFP obtained from the joints of patients with end-stage OA stimulates fibrotic processes in FLS.

Culturing the FLS with FCM increased collagen production, the expression of *PLOD2* encoding for the enzyme LH2b (involved in pyridinoline-based collagen crosslinks), and the migration and proliferation of FLS, which are hallmarks of a fibrotic process (11,13,35). These effects were independent of the BMI of the IPFP donor (BMI <30 kg/m^2 versus BMI ≥ 30 kg/m^2).

TGFβ1, and more recently, $PGF_{2\alpha}$ (14), have been suggested to act as profibrotic factors in the joints. We found that both TGFβ1 and $PGF_{2\alpha}$ were present in the FCM batches used for culture with the FLS; this finding is in addition to the previously described presence of many other cytokines, adipokines, and growth factors (17). When we compared the levels of TGFβ1 and $PGF_{2\alpha}$ in the FCM with our functional parameters, we found a positive association between $PGF_{2\alpha}$ levels and collagen deposition, a negative association between $PGF_{2\alpha}$ levels and *COL1A1* expression, and a positive association between TGFβ1 levels and *COL1A1* expression. These associations indicate that $PGF_{2\alpha}$ was responsible for some of the effects of the FCM. The absence of associations between $PGF_{2\alpha}$ levels and *PLOD2* expression and between TGFβ1 levels and *ASMA* expression might be explained by the fact that the FCM contains, in addition to $PGF_{2\alpha}$ and TGFβ1, many other unknown factors that could also have influenced the fibrotic processes in FLS.

Furthermore, our experiments indicate that the effects of FCM on FLS are comparable to the effects of adding $PGF_{2\alpha}$ to FLS cultures without FCM, again indicating that the presence of $PGF_{2\alpha}$ contributes to the FCM effect. The profibrotic effect of FCM may be attributable not only to the $PGF_{2\alpha}$ present in the FCM, but also to the $PGF_{2\alpha}$ that is produced by FLS in response to FCM. Fibroblasts, in general, are known to produce $PGF_{2\alpha}$ (36,37). This may explain the discrepancy between our findings of $PGF_{2\alpha}$ increasing *PLOD2* expression and AL8810 bringing *PLOD2* expression back to control levels and our findings of the absence of a correlation between $PGF_{2\alpha}$ levels in the FCM and *PLOD2* expression.

Messenger RNA (mRNA) and protein levels are, in general, associated with each other. This was true for the *COL1A1* mRNA and protein levels in this study, when we cultured the FLS with TGFβ. However, collagen deposition is regulated on many levels, and its regulation through variation in the amount of mRNA is only the beginning. For example, after synthesis of the different collagen chains, posttranslational modification through enzymes such as the lysyl and prolyl hydroxylases and lysyl oxidases can occur, while correct folding of the collagen molecules requires the involvement of chaperones such as Hsp47. These changes not only are directly involved in collagen synthesis but also can indirectly regulate collagen content. The level of collagen crosslinking, for example, can have an effect on the sensitivity of collagen to degradation by matrix metalloproteinases (38). Of course, degradation of collagen can have a major role in determining to what extent collagen content increases over time. In this respect, it is very exciting to see that despite a reduction in type I collagen mRNA, the presence of FCM or $PGF_{2\alpha}$ does result in increased collagen deposition, and that there are differences between stimulation with $PGF_{2\alpha}$ and stimulation with TGFβ. Unfortunately, we were not able to quantify deposition of specific types of collagen.

To further examine the involvement of TGFβ1 and $PGF_{2\alpha}$ present in the FCM in the different fibrotic processes, we used a TGFβ receptor type I kinase inhibitor, SB505124, and a selective PGF receptor antagonist, AL8810, together with the FCM incubation. Blockade of $PGF_{2\alpha}$ with AL8810 brought collagen deposition and *PLOD2* expression back to the levels in control conditions without FCM, whereas the presence of the TGFβ inhibitor SB505124 did not alter the FCM effect on FLS. Since the addition of AL8810 decreased *PLOD2* expression, our results indicate indirectly that $PGF_{2\alpha}$ levels are associated with *PLOD2* expression, in addition to the already-shown association between $PGF_{2\alpha}$ and collagen production. The latter is confirmed by the fact that inhibition of the PGF receptor with AL8810 normalized collagen deposition in FCM-treated FLS. Inhibition of TGFβ signaling with SB505124 in combination with FCM did not normalize collagen deposition or *PLOD2* expression. From these results, we conclude that $PGF_{2\alpha}$ might be a more important factor than TGFβ in the FCM-induced fibrotic processes in FLS.

No effect of the inhibitors was seen on the FCM-induced migration and proliferation of synoviocytes, and coincubation of FCM with AL8810 decreased *COL1A1* expression even more than that with FCM alone. Thus, next to $PGF_{2\alpha}$, other factors also influenced the parameters of fibrosis, since not all processes induced by FCM were counteracted by AL8810. In addition, the extra inhibition of *COL1A1* expression that occurred when the FLS were cultured with FCM and AL8810 could be explained by the fact that, in our culture system, there is no direct effect of $PGF_{2\alpha}$ on *COL1A1* expression and that other factors are involved in this relationship.

Like other organs, adipose tissue contains a resident population of cells of the innate immune system, in particular macrophages and T lymphocytes. In our earlier study, we demonstrated that macrophages were present in the IPFP, many of which have an M2 phenotype (39). Alternatively activated M2 macrophages have an antiinflammatory or repair phenotype and produce, predominantly, IL-10 but also growth factors such as TGFβ and insulin-like growth factor 1, and almost no IL-12 or IL-23 (40). The presence of M2 macrophages in the IPFP of patients with end-stage OA might contribute to the profibrotic effect of the FCM on FLS described in the present study. Earlier studies also found that macrophages are able to produce $PGF_{2\alpha}$ (41,42). In addition to macrophages, adipocytes, T lymphocytes, or other cells from the stromal vascular fraction might also contribute to $PGF_{2\alpha}$ production in the IPFP (43).

Prostaglandins, including PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2, are produced when phospholipids are cleaved in response to stimuli, resulting in the release of arachidonic acid, which is then metabolized by cyclooxygenase to produce prostaglandins. $PGF_{2\alpha}$ is considered to be a major and stable metabolite of PGE_2 (44). Reduction in PGE_2 production is the classic mode of action of antiinflammatory agents such as nonsteroidal antiinflammatory drugs (NSAIDs), which are commonly used in medical management of OA, and numerous studies have demonstrated a lower PGE_2 concentration in synovial fluid following NSAID treatment (45,46). More recently, $PGF_{2\alpha}$ was also found in the synovial fluid of horses, the levels of which increased after stimulation of acute inflammation and which were shown to be decreased after the treatment of inflamed knees with an NSAID (47). NSAIDs might, therefore, also be useful in the prevention of the synovial fibrosis seen in OA.

To our knowledge, this is the first study to examine the effect of adipose tissue on the synovium and to assess the potential involvement of the IPFP on the development of the synovial fibrosis often seen in OA (3). The results of this study indicate that the IPFP in the knees of patients with end-stage OA not only inhibits catabolic mediators in cartilage (39) but also exerts profibrotic effects on the synovium, and these profibrotic effects can be partially explained by the presence of $PGF_{2\alpha}$. However, since not all of the fibrotic effects can be explained by the presence of $PGF_{2\alpha}$, other factors may also play a role. Additional experiments are required to examine the effect of FCM on the entire OA fibrotic process, and to investigate whether the effect that we found is specific to the IPFP in end-stage OA or whether the IPFP from an earlier stage of OA would have the same profibrotic effect. Future studies should also investigate whether FLS from patients with OA in an earlier stage would respond in a manner comparable to that of FLS from patients with end-stage OA. In addition, it should be examined whether OA patients have increased levels of $PGF_{2\alpha}$ in their synovial fluid and whether this is associated with severe changes in their synovium. The continuing expansion of this knowledge might eventually contribute to more optimal treatment or even the prevention of OA.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Bastiaansen-Jenniskens had full

access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study conception and design. Bastiaansen-Jenniskens, Verhaar, Hanemaaijer, Stoop, van Osch.
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Analysis and interpretation of data. Bastiaansen-Jenniskens, Wei, Waarsing, Zuurmond, Stoop, van Osch.

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