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# Dickkopf-related protein 1 and gremlin 1 show different response than frizzled-related protein in human synovial fluid following knee injury and in patients with osteoarthritis

X. Huang † [a], J.N. Post † [a], L. Zhong †, J. Leijten †, S. Larsson ‡, M. Karperien †, A. Struglics ‡ *

† Department of Developmental BioEngineering, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands
‡ Lund University, Faculty of Medicine, Department of Clinical Sciences Lund, Orthopaedics, Lund, Sweden

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SUMMARY

*Objective:* To explore the involvement of the wingless-type MMTV integration site (WNT) and bone morphogenetic protein (BMP) antagonists dickkopf-related protein 1 (DKK1), frizzled-related protein (FRZB) and gremlin 1 (GREM1) in knee injury and osteoarthritis (OA).
*Design:* The antagonists were immunoassayed in synovial fluid from a cross-sectional cohort of nine knee healthy reference subjects, patients with recent (0–77 days, $n = 158$) or old (1–37 years, $n = 50$) knee injuries, and OA ($n = 22$). Cartilage (ARGS-aggrecan, cartilage oligomeric matrix protein and C2C type II collagen) and other biomarkers were assessed in synovial fluid in a subset of samples. Statistical analysis was by Kendall's tau ($\tau$) correlation, Mann–Whitney $U$ test, and linear regression analysis.
*Results:* Compared to references, median concentration of GREM1 (but not DKK1 and FRZB) was elevated 1.5-fold immediately after injury, and FRZB was reduced 1000-folds in OA. All three antagonists decreased with increasing time after injury as well as with increasing age, but the temporal change after injury was less accentuated for FRZB (peaked 8–22 days after injury) compared to that of DKK1 and GREM1 (peaked immediately after injury). In the recent injury group, there was a correlation between GREM1 and DKK1 ($\tau = 0.172$); FRZB concentrations correlated with concentrations of cartilage biomarkers ($\tau$ between 0.257 and 0.369), while DKK1 and GREM1 were inversely correlated ($\tau$ between −0.177 and −0.217) with these markers.
*Conclusions:* Our results indicate separate roles for the antagonists, where DKK1 and GREM1 had similarities in response to injury and in OA, with a different response for FRZB.


## Introduction

The secreted wingless-type mouse mammary tumor virus (MMTV) integration site (WNT) and the bone morphogenetic protein (BMP) signal pathways have been implicated as driving factors in development of osteoarthritis (OA) after joint injury[1]. Numerous studies have revealed a central role of WNT signaling in cartilage homeostasis. In healthy articular cartilage, moderate activity of WNT is essential for chondrocyte proliferation and maintenance of the cell phenotype in the superficial zone of articular cartilage[2]. By contrast, in animal models of OA, aberrant canonical WNT signaling has been implicated in disease development. Indeed, in human OA and in injured cartilage, increased activity of the canonical WNT pathway has been linked to loss of the chondrocyte phenotype and premature hypertrophic differentiation[1,3,4]. Although BMPs have a protective effect in articular cartilage[5,6], they are also implicated in the development of OA[7].

The joint tissues express a number of WNT- and BMP-signaling antagonists that modulate signaling activity. We have identified the WNT antagonists dickkopf-related protein 1 (DKK1) and frizzled-related protein (FRZB) and the BMP antagonist gremlin 1 (GREM1) as critical regulators of cartilage homeostasis[8–10]. These factors are

* Address correspondence and reprint requests to: A. Struglics, Lund University, Faculty of Medicine, Department of Clinical Sciences Lund, Orthopaedics, BMC C12, Klinikgatan 28, SE-221 84 Lund, Sweden. Tel: 46-(0)-46-222-07-62.
*E-mail addresses:* x.huang-1@utwente.nl (X. Huang), j.n.post@utwente.nl (J.N. Post), zhongleilei8@gmail.com (L. Zhong), j.c.h.leijten@utwente.nl (J. Leijten), staffan.larsson@med.lu.se (S. Larsson), h.b.j.karperien@utwente.nl (M. Karperien), andre.struglics@med.lu.se (A. Struglics).
[a] These authors contributed equally.

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secreted soluble proteins able to inhibit WNT and BMP signaling directly or indirectly[11–13].

We have observed that changes in the expression in articular cartilage of WNT antagonists DKK1 and FRZB, and BMP antagonist GREM1 may contribute to the loss of the stable articular cartilage phenotype[8,9,14], and we hypothesize that the changes in expression of these antagonist contribute to the development of OA. In the present report, we therefore study synovial fluid concentrations of DKK1, FRZB and GREM1 in patients with knee injuries and OA, and investigate association with age, time after injury and other biomarkers associated with OA.

## Materials and methods

### *Subjects and samples*

From a cross-sectional convenience cohort, we selected subjects with synovial fluid aspirated once from their index knee from three diagnostic groups. Our main study group was 208 knee-injured patients, of whom 104 did not fulfill the inclusion criteria of a randomized controlled trial[15], and 104 were from previous cross-sectional investigations[16–19] (Table I). The knee-injured subjects were organized by time between injury and synovial aspiration, and were in our primary analysis stratified into a recent injury group (sampling 0–77 days after injury) and an old injury group (sampling 1–37 years after injury). In a secondary analysis, the knee-injured subjects were ordered by time after knee injury, and divided into eight groups of 20–30 subjects each, to increase the resolution of time-dependent changes (Table I). Since the subjects in the old injury group were older than the subjects in the recent injury group (Table I), we selected patients from the recent injury group to generate a subgroup, selected recent injury group ($n = 80$) with similar age and sex distribution as the old injury group ($n = 50$) (Table I).

Samples from one group of 22 subjects with OA (of which seven had been collected during arthroplasty) were used as a proxy for future consequence of knee injury. Another set of samples from nine subjects deemed knee healthy based on absence of knee symptoms or knee injury, or based on normal findings on clinical examination were used as reference (Table I). All samples have been used in previous investigations[15–20]. There was no difference in sex between the four diagnostic groups (*P*-values between 0.066 and 0.842). Synovial fluid was aspirated without lavage, centrifuged at 3000 *g* for 10 min at 4°C and the supernatants were stored at −80°C. All subjects gave informed consent, and the study was approved by the regional (Lund) ethical review board.

### *Analysis of DKK1, FRZB and GREM1 in synovial fluid*

We used enzyme-linked immunosorbent assays (ELISAs) according to the manufacturers' instructions to quantify DKK1 (R&D system, #DY1906) and GREM1 (Bio-Connect Diagnostics, #E01G0253) in synovial fluid. For detection of synovial fluid FRZB levels we used the R&D system ELISA (#DY192) according to the manufacturer, with the following modifications: we added two more standard points (62.5 and 7.77 pg/ml) and used the Reagent Diluent (1% bovine serum albumin in phosphate-buffered saline) as a blank control. The ELISA's technical performance in synovial fluid was conducted as described[21], using three randomly selected synovial fluid control samples to assess dilution linearity and spiking recovery. The lower and upper limits of detections (LLOD and ULOD) for the FRZB, DKK1 and GREM1 ELISAs were between 7.8 and 57 pg/ml and between 8000 and 16,000 pg/ml, respectively (Table II). Within these ranges, good dilution linearity (i.e., 80–120% as described[22]), mean recoveries between 83 and 114%, were observed for synovial fluid control samples diluted 1:2 to 1:8 for FRZB and DKK1, and 1:24 to 1:96 for GREM1; the same dilutions were used for reference and patient synovial fluids for the respective antagonist. Spiking synovial fluid control samples with different amounts of standard showed good recovery (mean recoveries between 84 and 107%) for the FRZB, DKK1 and GREM1 assays; repeated freeze–thaw cycles showed mean recoveries between 81 and 107%. The mean intra coefficient of variations (CV, within plates) for the synovial fluid control samples were between

**Table I**
Characteristics of the study subjects

| Main diagnostic groups | Sub-groups of injury | Time between injury and sampling | *n* (% women) | Age in years, median (range) | Differences in age, *P*-value | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Reference vs | Recent injury vs | Selected recent injury vs | Old injury vs |
| Reference | – | – | 9 (33) | 30 (17–58) | – | | | |
| Knee injury* | – | 0–36.9 years | 208 (25) | 29 (13–70) | 0.856 | | | |
| | Recent injury | 0–77 days | 158 (23) | 25 (13–64) | 0.684 | – | | |
| | Selected recent injury | 0–77 days | 80 (25) | 35 (18–64) | Nd | Nd | – | |
| | Recent injury, sub-stratification | 0 days | 26 (19) | 33 (14–46) | 0.883 | Nd | Nd | |
| | | 1 day | 28 (21) | 24 (13–57) | 0.482 | Nd | Nd | |
| | | 2–3 days | 29 (31) | 26 (16–54) | 0.917 | Nd | Nd | |
| | | 4–7 days | 30 (37) | 25 (14–64) | 0.470 | Nd | Nd | |
| | | 8–22 days | 25 (16) | 22 (15–57) | 0.315 | Nd | Nd | |
| | | 23–77 days | 20 (5) | 29 (17–49) | 0.825 | Nd | Nd | |
| | Old injury† | 1–36.9 years | 50 (30) | 35 (18–70) | 0.108 | **<0.001** | 0.291 | – |
| | Old injury, sub-stratification | 1–2.5 years | 27 (37) | 32 (18–61) | Nd | Nd | Nd | Nd |
| | | 2.8–36.9 years | 23 (22) | 43 (18–70) | Nd | Nd | Nd | Nd |
| OA‡ | – | – | 22 (41) | 64 (39–86) | **<0.001** | **<0.001** | Nd | **<0.001** |

Differences in age between reference vs patient groups, between recent injury vs old injury and OA, between selected recent injury vs old injury, and between old injury vs OA were analyzed (using Student's *t* test); significance ($P < 0.05$) are indicated in boldface. Nd = not determined.

* The knee injury group, with a total $n = 208$, was composed of samples from two convenience cohorts. The clinical diagnoses of the knee-injured subjects were: isolated anterior or posterior cruciate ligament (ACL and PCL, respectively) injuries (ACL $n = 24$ or PCL $n = 4$), ACL injury with meniscus tear ($n = 28$), ACL injury with meniscus tear and other ligament injuries ($n = 31$), ACL injury with other ligament injuries ($n = 32$), isolated meniscus tear ($n = 53$), meniscus tear with other ligament injuries ($n = 6$), patellar dislocation with or without soft tissue injuries ($n = 13$), other types of injuries (medial or lateral collateral ligament tears $n = 5$, PCL tear with meniscus or ligament injuries $n = 1$, give-way $n = 2$), no signs of soft tissue injury ($n = 9$).

† Seven patients in the old injury group had post-traumatic OA (based on OA score ≥ 5)[45], and the group had the following OA score: median = 2, range = 1–8 (information from 36 out of 50 patients).

‡ Symptomatic and/or radiographic diagnosed idiopathic OA with no history of knee trauma. The OA score for the OA group was: median = 7, range = 3–9 (information from 15 out of 24 patients).

**Table II**
Technical performance of the DKK1, FRZB and GREM1 immunoassays using synovial fluid

| Analyte | | | FRZB | DKK1 | GREM1 |
|---|---|---|---|---|---|
| Concentration, pg/ml | | LLOD | 7.77 | 7.77 | 57 |
| | | ULOD | 8000 | 8000 | 16,000 |
| Recovery, mean (range) % | Dilution | Ratio | | | |
| | | 1:2 | 87 (78–96) | 97 (77–115) | Na |
| | | 1:4 | 93 (72–120) | 93 (85–112) | Na |
| | | 1:8 | 91 (71–113) | 87 (76–106) | Na |
| | | 1:24 | Na | Na | 114 (97–131) |
| | | 1:48 | Na | Na | 83 (68–97) |
| | | 1:96 | Na | Na | 98 (82–119) |
| | Spiking | Concentration | | | |
| | | High | 92 (83–110) | 90 (87–94) | 102 (81–124) |
| | | Middle | 90 (84–102) | 85 (80–92) | 107 (94–121) |
| | | Low | 104 (86–122) | 84 (80–90) | 97 (84–115) |
| | Freeze–thaw | Cycle | | | |
| | | Second | 81 (73–89) | 103 (98–104) | 107 (93–121) |
| | | Third | 83 (79–88) | 98 (79–117) | 100 (81–135) |
| CV, mean % | | Assay | | | |
| | | Intra | 5.2 | 6.6 | 8.3 |
| | | Inter | 6.2 | 7.6 | 9.2 |

To calculate dilution linearity randomly selected synovial fluid samples ($n = 3$) were prepared in three different dilutions and analyzed. Results are expressed as % recovery: 100 × [(concentration at a specific dilution) divided by (known concentration divided by the dilution times)]. Spiking recovery was calculated from randomly selected synovial fluid samples ($n = 3$) which were spiked using high (8000 or 16,000 pg/ml), middle (2000 or 8000 pg/ml) or low (500 or 4000 pg/ml) concentrations of standards. Spiking recovery is expressed in % as recovery: 100 × [(spiked sample result) divided by (un-spiked sample result plus the known spike added concentration)]. To analyze freeze-thawing effects on DKK1, FRZB and GREM1 concentrations randomly selected synovial fluid samples ($n = 3$) were freeze/thawed three cycles. Recovery after freeze–thaw cycles is expressed in relation to the amount obtained the first time the sample was thawed. Intra and inter DKK1, FRZB and GREM1 assay CV were calculated using randomly selected synovial fluid samples ($n = 3$) loading five repeats (intra) on a single plate or loading duplicates on four plates (inter). LLOD and ULOD for the DKK1 FRZB and GREM1 assays were estimated. Na = not applicable due to either to low or to high dilution ratio.

5 and 8%, while the inter CV (between plates) were between 6 and 9% (Table II).

*Other biomarkers*

Of the 158 synovial fluids in the recent injury group, between 103 and 152 samples were analyzed in the course of previous investigations for the following biomarkers: sulfated glycosaminoglycan (sGAG) by Alcian Blue precipitation[15]; aggrecan ARGS neoepitope (ARGS-aggrecan) from aggrecanase cleavage at the TEGE$^{392}\downarrow^{393}$ARGS site[15]; osteocalcin, secreted protein acidic and rich in cysteine (SPARC) and osteopontin (Human Bone Marker Panel II, Mesoscale Discovery [MSD] #K11147C)[15]; cartilage oligomeric matrix protein (COMP) (BioVendor R&D #RD194080200)[20]; type II collagen epitope C2C (C2C) (IBEX #60-1001-001)[21]; interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor α (TNF) (Human Pro-inflammatory II 4-Plex Ultra-Sensitive, MSD #K11025C)[15]; markers of complement activation C4d, C3bBbP and soluble terminal complement complex (sTCC)[23].

*Statistics*

Shapiro–Wilk tests indicated skewed distributions of FRZB, DKK1 and GREM1. We therefore used Kruskal–Wallis and Mann–Whitney rank sum tests for comparisons of concentrations between diagnostic groups, and Kendall's tau ($\tau$) to assess correlation between biomarkers. Sex and age differences between groups were assessed by Pearson Chi-square and Student's *t* tests, respectively. The percentage of samples with imputed values was 0% for GREM1, between 0 and 5% for DKK1 and between 3 and 64% for FRZB (supplementary information Table S1). We included imputed values in group comparisons by Mann–Whitney, and these imputations were done as follows: (1) imputed values are equal to half the value of LLOD; and (2) for calculations of extreme cases, one group with imputed values equal to the value of LLOD was compared to another group with imputed values equal to 0. These calculations were also done *vice versa*. Correlation analyses were done taking into consideration values below LLOD[24], and compared to analysis done without values below LLOD; correlation was considered to exist only when both analyses showed statistically significant correlation and the presented data herein ($\tau$ and *P*-values) include imputed values. To explore the relative influence of age and time after injury on biomarker levels in subjects with knee injury, we used hierarchical linear regression models where we entered age followed by log-transformed time after injury into predict concentrations for the three antagonists, reporting results with values below LLOD imputed using maximum likelihood estimation[25], and without imputed value present. For statistical analysis, we used IBM SPSS Statistic (version 21 and 24), SAS (version 9.4) and R (version 3.4.3). All analysis was performed using two-tailed tests and *P*-values less than 0.05 was considered statistically significant. We did not compensate for multiple testing due to the exploratory nature of the study. If not otherwise specified, expressions such as "higher" and "lower" in the text are based on statistically significant differences.

## Results

*Synovial fluid concentration of FRZB, DKK1 and GREM1 in subjects with knee injury*

In the recent injury group (0–77 days after injury) the median synovial fluid concentration of GREM1 was 1.5-fold increased, whereas FRZB and DKK1 concentrations were no different, compared to concentrations in the reference group [Fig. 1(A), Table S1]. In the old injury group (1–37 years after injury) concentrations of the three antagonists were no different from those observed in the reference group [Fig. 1(A), Table S1]. Comparing recent and old injury, the median concentrations of FRZB, DKK1 and GREM1 were 1.7-, 1.3- and 1.2-fold higher in the recent injury group [Fig. 1(A), Table S1].

With injured subjects sub-stratified into eight groups with increasing time between injury and sampling, the response to injury appeared as an immediate increase in GREM1 (2.1-fold

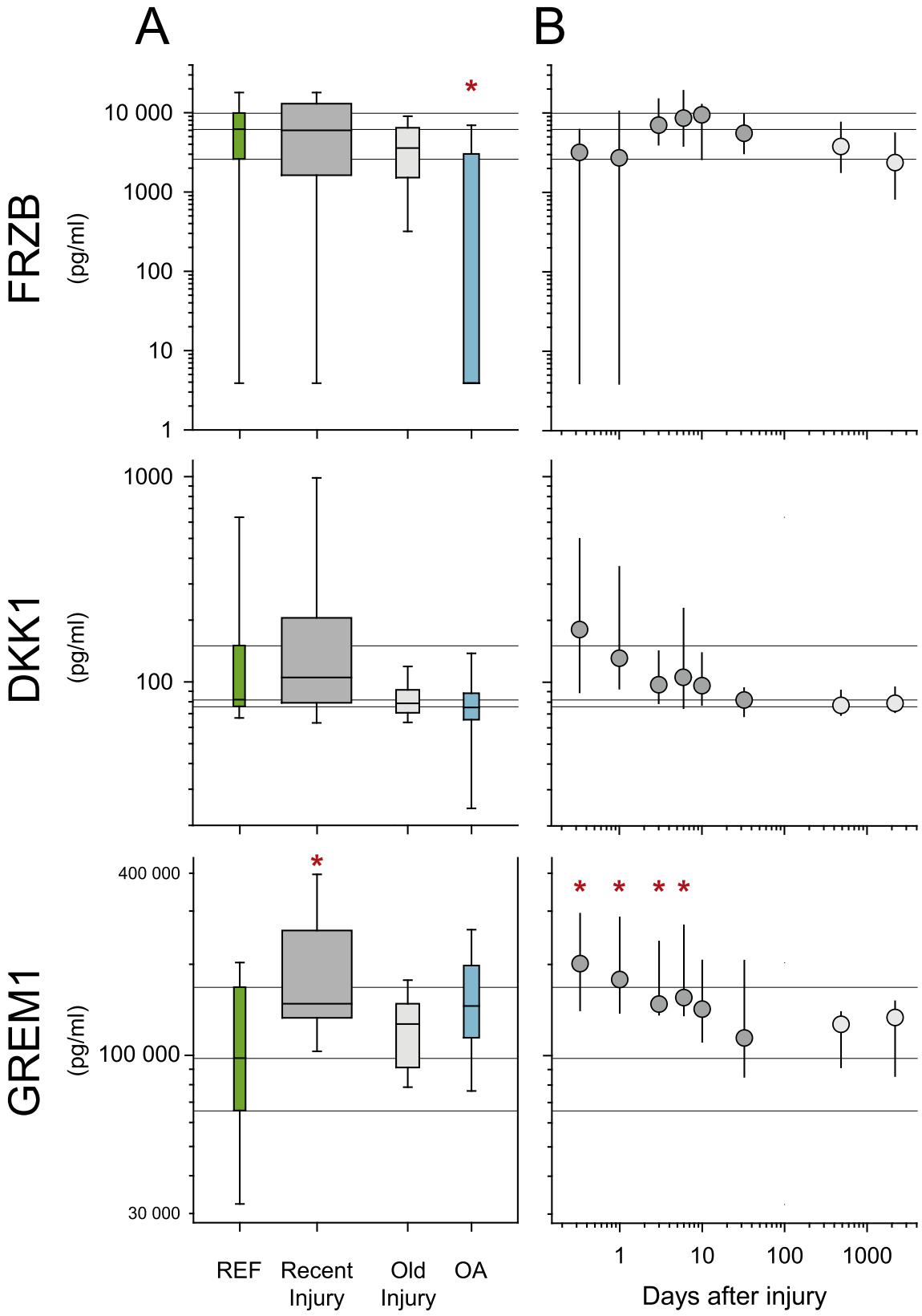



**Fig. 1. Synovial fluid concentrations of FRZB, DKK1 and GREM1.** (A) Box plots with subjects ordered by the diagnostic groups reference (REF; green), recent injury (dark gray), old injury (gray), and OA (blue). Boxes show the quartiles (median, 25th and 75th percentiles) with error bars and whiskers for the 10th and 90th percentiles, with box width indicating the relative group size. The quartiles of the reference group are extended as thin horizontal lines in both panels for comparison. (B) Knee-injured subjects ordered by days after injury in sub-groups of 20–30 subjects (Table I). Quartiles are plotted as filled circles (medians) and error bars (25th and 75th percentiles). Fill color of circles indicate the origin of the sub-group; recent injury (dark gray) or old injury (gray). Statistically significant group differences, determined by Mann–Whitney *U* test, vs the reference group are indicated by asterisks (*). To illustrate the levels of FRZB, samples with concentrations below LLOD were imputed and given a concentration equal to half the LLOD. All group level statistics are presented as supplementary data in Table S1.

higher median concentration at the day of injury compared to the reference group) with subsequently lower concentrations in the sub-groups with increasing time after injury [Fig. 1(B), Table S1]. DKK1 displayed a similar, but less accentuated, pattern after injury as GREM1, with the highest median concentration at the day of injury, with subsequent decreasing concentrations with increasing time after injury [Fig. 1(B), Table S1]. For FRZB the sub-stratification revealed an apparent delayed response to injury, with a peak in concentrations in the sub-group aspirated between 8 and 22 days after injury [Fig. 1(B), Table S1]. Concentrations of DKK1 and FRZB were not statistically significant different from those observed in the reference group at any time interval after injury (Fig. 1, Table S1).

There were no statistically significant differences in concentrations of any of the antagonists between men and women in the reference (*P*-values between 0.167 and 0.905), recent injury (*P*-values between 0.056 and 0.823) and old injury groups (*P*-values between 0.397 and 0.946), and there were no differences in age between the reference and the two injury groups (Table I). This suggests that the increased level of GREM1 in the synovial fluid of recent injured knees compared to reference knees was due to the knee injury.

There was, however, a difference in age between the old and recent injury groups, and all three antagonists were negatively correlated with age in at least one of the knee injury groups (Table III). To rule out that the differences in age were the reason for the observed differences in FRZB, DKK1, and GREM1 concentrations between the recent and old injury groups, we selected patients from the recent injury group generating a new group (selected recent injury group $n = 80$), which had similar age and sex distribution as the old injury group (Table I). In similarity with the comparison between recent and old injury groups, the selected recent injury group had higher median concentrations of FRZB (1.7-fold, *P*-values between 0.005 and 0.008), DKK1 (1.3-fold, $P < 0.001$) and GREM1 (1.1-fold, $P < 0.001$) as compared to levels found in the old injury group, suggesting that the observed differences were not solely due to an age difference. However, for the knee-injured subjects, both time after injury and age influenced the concentrations of these antagonists (Fig. 2, Table III), and age correlated with time after injury ($\tau = 0.155$, $P = 0.001$). To further explore the relative influence of age and time after injury on antagonist levels, we entered age followed by log-transformed time after injury in hierarchical linear regression models to predict concentrations for the three antagonists. This analysis confirmed that DKK1 and GREM1 levels decreased with increasing age as well as with increasing time after injury (based on negative regression coefficients) (Table IV). For FRZB, no association with age (regression coefficient $= -0.001$, $P = 0.947$) or time after injury (regression coefficient $= 0.265$, $P = 0.109$) was noted when imputed values were included ($n = 208$), but when the analysis was done with imputed values removed ($n = 181$), FRZB decreased with both age ($P < 0.001$) and time after injury ($P = 0.035$) (Table IV).

*Synovial fluid concentration of FRZB, DKK1 and GREM1 in patients with OA*

The median concentration of FRZB was 1600 times or more (depending on the two extreme cases used for imputation) decreased in the OA group compared to the reference group, while DKK1 and GREM1 concentrations were not statistically significantly different between these groups [Fig. 1(A), Table S1]. The concentrations of FRZB and DKK1 were 1500- and 1.4-fold lower in the OA group than in the recent injury group, respectively. Also, the concentration of FRZB was 900-fold lower in the OA group than in the old injury group, while the concentration of GREM1 was 1.2-fold higher [Fig. 1(A), Table S1].

The synovial concentrations of antagonists between men and women in the OA group differed for FRZB (*P*-values between 0.016 and 0.342), but not for DKK1 ($P = 0.807$) and GREM1 ($P = 0.431$); but the subjects in the OA group were older than the subjects in the reference, recent injury and old injury groups (Table I). Even though there was no correlation between age and levels of synovial fluid antagonists in the OA group, we cannot exclude that the decreased level of FRZB in the OA group vs reference, as well as decreased levels FRZB and DKK1 in the OA group vs the recent injury group, were partially due to differences in age between OA and these groups.

*Correlation between DKK1, FRZB and GREM1 and other biomarkers in the recent injury group*

In the recent injury group, the synovial fluid concentration of GREM1 correlated positively with the DKK1 level; no other correlations were found between the antagonists (Fig. 3). Concentrations of FRZB showed a positive correlation with concentrations of the cartilage markers sGAG, ARGS-aggrecan, C2C type II collagen and COMP, and with the bone marker osteopontin (Fig. 3). By contrast, weak negative correlations were found between concentrations of DKK1 and ARGS-aggrecan and between DKK1 and C2C type II collagen; a negative correlation was also found between concentrations of GREM1 and C2C type II collagen, and a weak positive correlation between GREM1 and osteocalcin. Both DKK1 and GREM1 showed a weak positive correlation with IL-8 and with the complement markers C4d and C3bBbP. No correlations were found between concentrations of the WNT and BMP antagonists and concentrations of IL-1β, IL-6, TNF and SPARC.

## Discussion

This is the first study to investigate DKK1 and FRZB (both naturally occurring secreted antagonists of the WNT-signaling pathway), and GREM1 (a naturally occurring secreted antagonist of the BMP-signaling pathway) in synovial fluid from subjects with knee injury or OA. Our results indicate different roles for the

**Table III**
Correlations using Kendall's tau (*P*-values) between synovial fluid concentrations of antagonists and age or time after injury

| | Group (*n*) | FRZB | DKK1 | GREM1 |
|---|---|---|---|---|
| Age vs antagonists | Reference (9) | **−0.667 (0.017)** | 0.167 (0.602) | 0.444 (0.118) |
| | Knee injury (208) | −0.033 (0.489) | **−0.155 (<0.001)** | **−0.225 (<0.001)** |
| | Recent injury (158) | 0.092 (0.088) | **−0.148 (0.006)** | **−0.216 (<0.001)** |
| | Old injury (50) | **−0.435 (<0.001)** | 0.065 (0.514) | −0.037 (0.713) |
| | OA (22) | −0.167 (0.340) | −0.109 (0.498) | 0.052 (0.756) |
| Time after injury vs antagonists | Knee injury (208) | −0.019 (0.701) | **−0.279 (<0.001)** | **−0.274 (<0.001)** |
| | Recent injury (158) | 0.092 (0.088) | **−0.148 (0.006)** | **−0.216 (<0.001)** |
| | Old injury (50) | **−0.435 (<0.001)** | 0.065 (0.514) | −0.037 (0.713) |

Age distributions and concentrations of FRZB, DKK1 and GREM1 in synovial fluid are presented for the different subject groups in Tables I and S1. Statistically significant correlations ($P < 0.05$) are indicated in boldface.

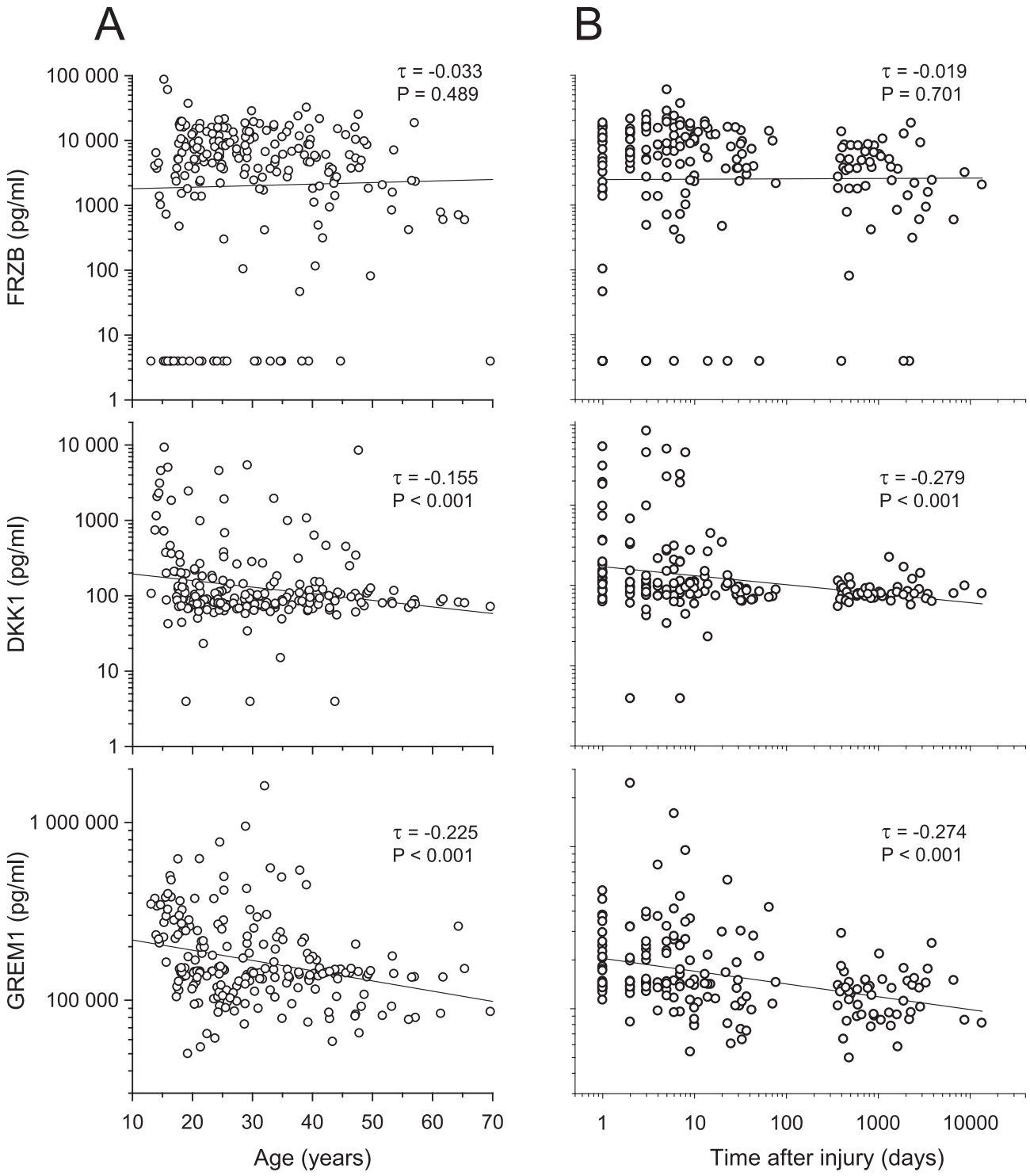



**Fig. 2. Bi-variate scatter plots of FRZB, DKK1 and GREM1 vs age and time after injury in knee-injured subjects**. Regression lines and correlations, using Kendall's tau ($\tau$) and *P*-values, with age (A) or with time after injury (B) are indicated for each antagonist. Knee-injured subjects (0–37 years from injury), $n = 208$.

antagonists, where DKK1 and GREM1 have high similarities in response to injury and in OA, whereas FRZB appears to have a separate response. This is suggested by differences in temporal change after injury as well as by differences in correlation with other biomarkers that are affected by injury and OA. In response to injury, both DKK1 and GREM1 appeared to be increased immediately after injury (although this was statistically significant only for GREM1). For FRZB we did not see the same immediate increase after injury, but instead a delayed response with a peak several days after injury. Also, in these recent knee-injured patients, there was no correlation between the synovial fluid levels of FRZB and DKK1, while DKK1 correlated positively with GREM1. Further, FRZB correlated positively with the cartilage markers (sGAG, ARGS-aggrecan, C2C type II collagen and COMP), while DKK1 and GREM1 correlated positively with IL-8 and complement markers (i.e., C4d and C3bBbP).

**Table IV**
Model summary obtained by hierarchical multiple regression for prediction of antagonist concentrations for the knee injury group with predictors age entered in the first model, and age and time after injury in the second model

| Antagonists* | Model | Predictors† | Effect | *P*-value |
|---|---|---|---|---|
| FRZB ($n = 208$) | 1 | Age | 0.007 | 0.662 |
| | 2 | Age | −0.001 | 0.947 |
| | | Time after injury | 0.265 | 0.109 |
| FRZB ($n = 181$)‡ | 1 | Age | **−0.030** | **<0.001** |
| | 2 | Age | **−0.026** | **0.001** |
| | | Time after injury | **−0.153** | **0.035** |
| DKK1 ($n = 208$) | 1 | Age | **−0.020** | **0.002** |
| | 2 | Age | **−0.013** | **0.046** |
| | | Time after injury | **−0.228** | **<0.001** |
| DKK1 ($n = 205$)‡ | 1 | Age | **−0.020** | **0.001** |
| | 2 | Age | −0.012 | 0.054 |
| | | Time after injury | **−0.259** | **<0.001** |
| GREM1 ($n = 208$) | 1 | Age | **−0.013** | **<0.001** |
| | 2 | Age | **−0.009** | **0.007** |
| | | Time after injury | **−0.151** | **<0.001** |

Effect (regression coefficient): the estimate of the average change in natural log-transformed antagonist concentration that corresponds to a 1-unit change in the predictor (1 year for age, 1 log 10-unit for the transformed time [days] after injury). Statistically significant effects and *P*-values ($P < 0.05$) are indicated in boldface.

* Natural log-transformed concentrations (ng/ml) of antagonists in synovial fluid.

† Age in years and log 10 transformed time after injury (days).

‡ Modeled with imputed values removed.

While there is no existing report of synovial fluid concentrations of FRZB in knee healthy subjects, the synovial fluid concentrations of DKK1 of reference subjects found in this study were in the same range as previously reported[26]. Likewise, the synovial fluid concentrations of GREM1 in OA patients were in the same range as reported[27]. DKK1 has received quite some attention as a possible biomarker for assessing the joint's condition[28]. However, based on the many fold lower concentration of FRZB in synovial fluid of OA patients compared to the reference group, and compared to the much smaller (and not statistically significant) difference in DKK1 between OA and reference groups, we suggest that FRZB might prove to be a more sensitive alternative to DKK1 as a OA biomarker.

FRZB is structurally similar to the cysteine rich WNT binding domain of frizzled (FZD) receptors. When FRZB binds WNT in the extracellular space it prevents WNTs to bind to the FZD receptors[29] and by that FRZB is blocking both the canonical and the non-canonical pathways[30]. By contrast, DKK1 binds low-density lipoprotein receptor-related protein (LRP)5/6, the membrane-bound frizzled co-receptor[31], and by that DKK1 specifically inhibits only the canonical pathway. The more specialized role of DKK1 and its localization on the cell surface may require lower concentrations of this antagonist, as compared to concentrations of FRZB.

The difference in concentration of DKK1 and FRZB observed after injury (median concentrations 93 and 5170 pg/ml, respectively) may be due to a difference in binding affinity of the factors for their respective targets. The binding of a protein with its substrate is dependent both on the concentrations of the protein and its substrate, as well as the affinity of the protein-substrate interaction. The affinity, or rather, the dissociation constant $K_D$, of both FRZB and DKK1 was reported to be 20- to 200-fold lower for FRZB to WNT ($K_D$ = 0.1–10 nM) than for DKK1 to LRP5/6 ($K_D$ = 50 pM)[32–36]. These differences in dissociation constants of FRZB and DKK1 are in the same range as the differences in median concentration in the reference and injury groups, which indicates that the high affinity of DKK1 to LRP5/6 may compensate for the relative low concentration in synovial fluid, and that the antagonistic capacity of FRZB and DKK1 may be similar.

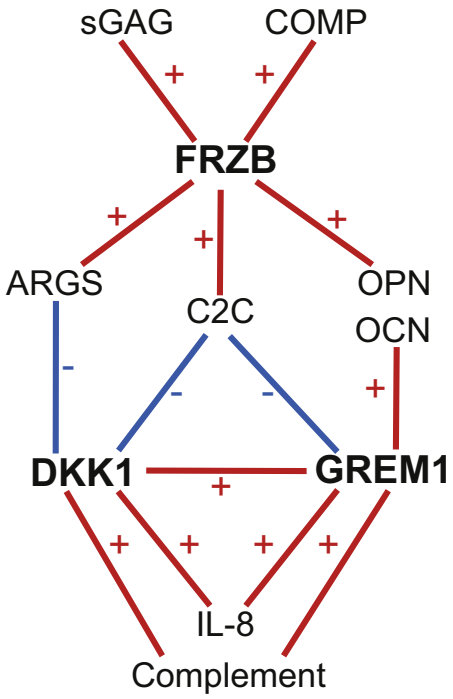
Sub-stratification of the recent injury group indicated that the DKK1 concentration in the synovial fluid increased immediately after injury. This mimics the increases in synovial fluid concentrations of cartilage proteins, complement factor and cytokines which has been observed directly after knee injury[18,21,23,37–39]. In the current report, the expression of the pro-inflammatory cytokine IL-8 correlated with DKK1 expression in the recent injury patient group, which is in accordance with previous observations[40,41].

In OA and rheumatoid arthritis, GREM1 is expressed by the chondrocytes and synovial fibroblasts[8,9,14,42]. We have previously shown that GREM1 co-localizes with chondrocytes in healthy cartilage, and that the mRNA and protein expression increase significantly in OA cartilage[14]. Here, we found GREM1 to be elevated in synovial fluid immediately following injury, with subsequent decreasing levels with increasing time after injury, with concentrations in the OA group no different from those observed in the reference group. Although we did not discriminate between different grades of OA, Yi and co-workers reported that synovial fluid and serum concentrations of GREM1 associated with the severity of knee OA[27].

It has been reported that joint defects in young people (10–26 years) tend to develop less often into OA compared to similar defects in older people[43,44]. Interestingly, in this study we found for the knee injury groups that the measured concentrations of DKK1 and GREM1 in synovial fluid decreased with increasing age. We speculate that there is a gradual increase in WNT and BMP signaling in the joint with increasing age as a direct result of the age-related decrease in WNT and BMP antagonist expression, and that this may contribute to the increased incidence of OA development in relatively older patients with a joint defect.

We found positive correlations in synovial fluid between FRZB concentrations and concentrations of cartilage makers of aggrecan (sGAG and ARGS-aggrecan), COMP and type II collagen (epitope C2C). On the other hand, synovial fluid DKK1 and GREM1 concentrations correlated negatively with concentrations of the C2C epitope of type II collagen, and DKK1 correlated negatively with ARGS-aggrecan. This suggests that directly after knee injuries DKK1 and GREM1 work co-operatively to block and/or balance the catabolic signaling, and this process is partly separated from the action of FRZB.

This study was limited by its cross-sectional design, which hampered the ability to draw firm conclusions on temporal change since no repeated sampling was made within individual patients. Second, group sizes were small, especially the reference group, which limits the power to detect differences between groups. Third, we used imputed data for FRZB and DKK1 due to limited sensitivity of the assays, which may have concealed or exaggerated the true differences, temporal variations, or associations too small to be detected with the assays. Fourth, there were differences in age between groups, and in the injury groups both time after injury and age influenced the concentrations of GREM1 and DKK1. However, in the age and sex matched selected recent injury and old injury groups, we observed that there were statistically significant differences in concentrations of DKK1, FRZB and GREM1, indicating that the differences were at least in part due to trauma, and not solely on increasing age. The linear regression analysis revealed that time after injury accounted for a slightly larger proportion of the change in concentrations of GREM1 and DKK1 than age in the injured subjects. The linear regression analysis further revealed that the strategy of imputation of FRZB concentrations below the limit of detection likely concealed that also FRZB decreased with increasing time after injury as well as with increasing age, and that age appeared to account for a larger proportion of the change in FRZB compared to time after injury. This suggests that the concentrations of all three antagonists are different in young and old patients, and that age




| | n | FRZB, τ (p value) | DKK1, τ (p value) | GREM1, τ (p value) |
|---|---|---|---|---|
| FRZB | 158 | - | | |
| DKK1 | 158 | 0.022 (0.685) | - | |
| GREM1 | 158 | Nd[1] | **0.172 (0.001)** | - |
| IL-1β | 104 | -0.113 (0.095) | Nd[1] | 0.048 (0.474) |
| IL-6 | 104 | -0.111 (0.099) | 0.105 (0.114) | 0.011 (0.871) |
| IL-8 | 104 | -0.122 (0.070) | **0.182 (0.006)** | **0.151 (0.023)** |
| TNF | 104 | -0.006 (0.937) | 0.099 (0.138) | 0.107 (0.111) |
| C4d | 149 | -0.001 (0.995) | **0.194 (<0.001)** | **0.174 (0.002)** |
| C3bBbP | 149 | 0.026 (0.642) | **0.267 (<0.001)** | **0.163 (0.003)** |
| sTCC | 149 | -0.004 (0.949) | Nd[2] | **0.133 (0.016)** |
| sGAG | 136 | **0.345 (<0.001)** | -0.031 (0.593) | -0.000 (0.999) |
| ARGS | 103 | **0.369 (<0.001)** | **-0.186 (0.005)** | -0.084 (0.208) |
| COMP | 104 | **0.257 (<0.001)** | -0.110 (0.099) | -0.012 (0.862) |
| C2C | 123 | **0.273 (<0.001)** | **-0.177 (0.004)** | **-0.214 (<0.001)** |
| Osteocalcin | 104 | 0.034 (0.622) | 0.101 (0.131) | **0.133 (0.046)** |
| SPARC | 104 | -0.002 (0.980) | 0.086 (0.196) | 0.113 (0.091) |
| Osteopontin | 104 | **0.270 (<0.001)** | -0.097 (0.148) | -0.046 (0.494) |

**Fig. 3. Correlation between DKK1, FRZB, GREM1 and other biomarkers in knee-injured subjects**. Correlations, using Kendall's tau ($\tau$), between synovial fluid biomarkers were analyzed in samples from the recent injury group (0–77 days after injury). Red = positive correlation, blue = negative correlation. Significant correlations ($P < 0.05$) are indicated in boldface. The percentage of samples above the LLOD in the correlation analyses were as follows: FRZB, between 76 and 85%; DKK1, between 88 and 98%; GREM1, between 88 and 100%. ARGS, ARGS-aggrecan; OPN, osteopontin; OCN, osteocalcin. Markers of complement activation: C4d, C3bBbP and sTCC. (1) Not determined (Nd); results using imputed values gave positive (IL-1) or negative (GREM1) statistically significant correlations while using data without imputed values gave no correlation. (2) Not determined; results using imputed values gave no correlation while using data without imputed values gave a statistically significant positive correlation.

needs to be accounted for when studying the effect of time after injury on levels of these antagonists.

In conclusion, we found elevated synovial fluid concentrations of GREM1 (but not DKK1 and FRZB) immediately after injury, and decreased concentrations of FRZB in OA, compared to knee healthy references. We further found that in knee injured all three antagonists decreased with increasing time after injury as well as with increasing age, but that the temporal change after injury was less accentuated for FRZB compared to that of DKK1 and GREM1. The positive correlation between FRZB and cartilage biomarkers, compared to inverse correlations between DKK1 and GREM with the same cartilage biomarkers, further indicated that the role of FRZB after knee injury and in OA is different compared to the roles of DKK1 and GREM1.

## Authors' contributions

Design of the study: LZ, JNP, MK and AS. Performed the experiments: LZ, JL and XH. Analyzed the results: LZ, XH, JL, SL, JNP and AS. Drafting of manuscript: all authors. All authors approved the final version of manuscript for submission.

## Conflict of interests

The authors declare that they have no competing interests.

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**Supplementary data**

Supplementary data related to this article can be found at https://doi.org/10.1016/j.joca.2018.02.904.

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