International Orthopaedics (SICOT) (2014) 38:1885–1892
DOI 10.1007/s00264-014-2406-y

ORIGINAL PAPER

Angiogenic cytokine expression profiles in plasma and synovial fluid of primary knee osteoarthritis

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Received: 9 May 2014 / Accepted: 2 June 2014 / Published online: 26 June 2014

Abstract
Purpose The aim of this study was to compare angiogenic cytokine levels in knee osteoarthritis (OA) patients and healthy controls and to investigate the relationships between angiogenic cytokines and the OA severity.
Methods Thirty-one knee OA patients and 15 healthy controls were recruited. Nine angiogenic cytokines (angiopoietin-2, follistatin, granulocyte-colony stimulating factor (G-CSF), hepatocyte growth factor (HGF), interleukin (IL)-8, leptin, platelet-derived growth factor-BB (PDGF-BB), platelet endothelial cell adhesion molecule (PECAM)-1, and vascular endothelial growth factor (VEGF)) in plasma and synovial fluid were measured using a multiplex immunoassay.
Results PECAM-1, HGF, VEGF, angiopoietin-2, follistatin, G-CSF, and IL-8 concentrations in plasma were significantly higher in OA patients than those in controls. Plasma angiopoietin-2 was significantly greater in advanced OA than in early OA. Synovial fluid VEGF was positively correlated with the severity (r=0.367, P=0.04). Plasma follistatin was significantly lower in advanced knee OA than in early OA and was negatively correlated with the severity (r=−0.374, P<0.05).
Conclusions Angiogenic cytokine concentrations in plasma can distinguish between controls and OA patients. Local and circulating levels of angiogenic cytokines could give an insight into the pathophysiology of OA. Follistatin, angiopoietin-2, and VEGF may have potential as biochemical markers for the assessment of OA severity.

Keywords Angiogenesis · Cytokine · Osteoarthritis · Plasma · Synovial fluid

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Introduction

Osteoarthritis (OA) is a progressive degenerative joint disease characterised by articular cartilage loss. Whilst the exact aetiology of OA remains elusive, a number of risk factors are associated with knee osteoarthritis including age, obesity, gender, genetics and previous joint trauma [1]. OA is one of the most prevalent joint diseases and is expected to become more common with ageing and overweight populations.

Inflammation of the synovium, destruction of the extracellular matrix leading to articular cartilage degradation, and remodeling of the subchondral bone tissue are characteristics of OA. Additionally, angiogenesis plays an important role in the pathophysiology of tissue repair and OA.

In OA, neovascularisation occurs in the synovium, pannus, menisci, and the osteochondral junction [2]. At the subchondral junction, new blood vessels extend from the pre-existing vascular networks in the subchondral bone plate into the normally avascular non-calcified cartilage. Vascularisation is facilitated by the up-regulation of osteoclast activity and the formation of channels in the bone through to the articular cartilage above [3, 4]. Osteophyte formation, which is characteristic of OA and used in radiographic

diagnosis and assessment, is also influenced by angiogenesis [5]. Furthermore, angiogenesis occurs in the synovium where it is highly linked to inflammation and macrophage infiltration, and enhances with increasing inflammation [6]. The development and growth of blood vessels from a pre-existing vascular network is also closely associated with nerve growth [7]. As a result, angiogenesis is thought to be associated with increased pain in OA patients. Whilst the understanding of the mechanism of angiogenesis in OA remains unclear, a number of angiogenic factors have been proposed to play substantial roles in the pathogenesis of OA.

Previous studies have investigated cytokines in synovial fluid of knee OA patients [8–10]; however, to our knowledge this study is the first to focus on angiogenic cytokines in plasma and synovial fluid of primary knee OA patients. Therefore, the objectives of the present study were to compare angiogenic cytokine levels in knee OA patients and healthy controls and to investigate the relationships between angiogenic cytokines and the radiographic severity of knee OA.

Materials and methods

Study population

Thirty-one patients diagnosed with knee OA (53–84 years of age) were recruited in the present study. They were diagnosed in accordance with the criteria of the American College of Rheumatology. All patients were scheduled for total knee replacement surgery or therapeutic knee arthroscopy. Fifteen healthy controls (50–70 years of age) were also enrolled in this study. The controls had no pre-existing medical conditions and no personal or familial history of OA. None of the participants had other forms of arthritis, histories of corticosteroid medication, or underlying diseases such as diabetes, advanced liver or renal diseases, cancer, or other chronic inflammatory diseases. All participants gave written informed consent prior to their participation. This study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University, and was conducted in agreement with the guidelines of the Declaration of Helsinki.

OA severity was assessed using the Kellgren-Lawrence (KL) grading system [5]. Radiographs of the knee were taken with each participant standing on both legs with fully extended knees and the X-ray beam centred at the level of the joint. Depending on changes observed in conventional weight-bearing anteroposterior radiographs of the affected knee in extension, OA patients were divided into five grades (0–4): grade 0 (normal findings), no X-ray changes; grade 1 (questionable), doubtful narrowing of joint space and possible osteophytic lipping; grade 2 (mild), definite osteophytes and possible joint space narrowing; grade 3 (moderate), multiple moderate osteophytes, definite narrowing of joint space, bone sclerosis and possible deformity of bone contour; grade 4 (severe), large osteophytes, marked joint space narrowing, severe sclerosis and deformity of bone contour. OA patients were defined as having radiographic knee OA of KL grade ≥2 in at least one knee. The grading of the worst affected knee in each patient was used for data analysis. Controls were defined as having neither radiographic hip OA or knee OA, as indicated by KL grades of 0 for both hips and both knees.

Sample collections

Venous blood samples were drawn from all participants after 12 hours over-night fasting into ethylenediamine tetraacetic acid (EDTA) tubes and immediately centrifuged. Plasma was separated and then stored at −80 °C until further analysis. OA patient plasma samples were collected on the day of surgery. Synovial fluid was aspirated prior to surgery by sterile puncture in the knee with the most severe KL grade. The samples were centrifuged to remove debris and stored at −80 °C.

Laboratory procedures

A magnetic-bead based multiplex angiogenesis assay kit (Bio-Plex Pro, Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to analyse nine angiogenic proteins simultaneously on 96-well plates. Briefly, beads were pre-coated in a ratio of two fluorophores to create differing spectral addresses, one for each target protein. These beads were then coupled with target-specific antibodies. All nine bead types were added to the same well to facilitate simultaneous assessment of the targets. A total of 50 μl of samples and standards were added to the wells and incubated for 30 minutes. Biotinylated detection antibodies specific for secondary epitopes of each target were added to each well. Fluorescent-labeled streptavidin was added and bound to the detection antibodies; excess was washed out of each well leaving only bound bead-target-streptavidin complexes. A Bio Plex-200 array reader was used to analyse the samples. In a flow cytometry style system, a reporter laser was used to scan individual beads and identify its spectral address. Simultaneously, a laser with a different wavelength was used to determine the streptavidin fluorescence and therefore the level of target protein bound to the bead. The concentration of each protein investigated was found from the mean fluorescences intensity. The proteins investigated and their detection levels were: angiopoietin-2 (≤500 pg/ml), follistatin (≤100 pg/ml), granulocyte-colony stimulating factor (G-CSF) (≤2 pg/ml), hepatocyte growth factor (HGF) (≤100 pg/ml), interleukin (IL)-8 (≤2 pg/ml), leptin (≤100 pg/ml), platelet-derived growth factor-BB (PDGF-BB) (≤100 pg/ml), platelet endothelial cell adhesion molecule (PECAM)-1 (≤1,000 pg/ml), and vascular

endothelial growth factor (VEGF) (≤2 pg/ml). The procedure was performed in accordance with the manufacture's protocol.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). The baseline characteristics of the controls and patients were analysed by Student's unpaired *t*-tests and chi-square tests where appropriate. Angiogenic cytokine levels were analysed using the Mann–Whitney tests and Spearman's rank correlation coefficient. $P \leq 0.05$ was considered statistically significant for differences and correlations. Values are presented throughout as median (lower quartile — upper quartile).

Results

Thirty-one knee osteoarthritis patients and 15 healthy controls were age and gender matched ($P=0.08$ and $P=0.2$, respectively) as shown in Table 1. In addition, the male/female ratio was in nine/22 OA patients and seven/eight in controls ($P=0.2$). The descriptive data of angiogenic cytokines in controls and OA patients are presented in Table 2.

Non-parametric analysis showed seven of the nine cytokine concentrations, angiopoietin-2, follistatin, VEGF, G-CSF, HGF, PECAM-1, and IL-8, were significantly higher in OA plasma than control plasma samples ($P<0.001$) (Fig. 1). There were no significant difference in plasma leptin and PDGF-BB levels between controls and OA patients ($P=0.07$ and $P=0.5$, respectively).

When cytokine concentrations were compared in OA plasma and OA synovial fluid, five exhibited significant differences. As illustrated in Fig. 1, angiopoietin-2, follistatin, G-CSF, and HGF had significantly higher values in plasma with respect to paired synovial fluid, whereas VEGF levels were significantly lower in plasma than in paired synovial fluid. PECAM-1, IL-8, and leptin showed no significant difference between plasma and synovial fluid ($P=0.6$, $P=0.8$ and $P=0.9$, respectively). PDGF-BB was not detected in any synovial fluid samples.

Subsequent analysis revealed that there was a positive correlation between synovial fluid VEGF levels and the severity of OA ($r=0.367$, $P=0.04$). Conversely, plasma follistatin levels were negatively correlated with the OA severity ($r=-0.374$, $P<0.05$). Moreover, there was a strong positive correlation between plasma and synovial fluid leptin levels ($r=0.876$, $P<0.001$).

Table 1 Baseline clinical characteristics data of study participants

Characteristic	Knee OA	Control	*P*
No.	31	15	–
Age (mean±SD, years)	66.1±7.1	62.4±5.0	0.08
Gender (male/female)	9/22	7/8	0.2

In order to compare the angiogenic cytokine values among OA patients, they were divided into two groups: early knee OA (KL grade 2) and advanced knee OA (KL grade 3 and 4). The angiogenic cytokine values in plasma of controls and OA patients are displayed in Table 3. The median plasma concentrations of G-CSF, HGF, angiopoietin-2, follistatin and IL-8 were significantly higher in early OA patients than controls ($P<0.001$) (Fig. 2). Plasma PECAM-1 and VEGF levels were also significantly greater in early OA compared with controls ($P=0.01$ and $P=0.002$, respectively). In comparison with controls, plasma PECAM-1, HGF, VEGF, angiopoietin-2, follistatin, and IL-8 were significantly increased in advanced OA patients ($P<0.001$). When compared with early OA patients, plasma angiopoietin-2 was significantly higher in advanced OA patients ($P=0.02$); however, plasma follistatin was significantly lower in advanced OA patients ($P<0.05$).

Discussion

This cross-sectional study used a multiplex immunoassay system to analyse angiogenic cytokine levels in plasma and synovial fluid from knee OA patients compared with plasma of healthy controls. This is the first study to determine angiogenic cytokine expression profiles in both plasma and synovial fluid in knee OA.

In the present study, angiopoietin-2, follistatin, VEGF, G-CSF, HGF, PECAM-1, and IL-8 all presented higher levels in OA plasma samples than control plasma samples. Furthermore, these cytokines were also detected in significantly higher levels in plasma samples of early OA patients compared with those of controls. However, plasma levels of follistatin in advanced knee OA decreased significantly. Plasma levels of VEGF, G-CSF, HGF, PECAM-1, IL-8, leptin, and PDGF were not different between early and advanced knee OA. Only the plasma levels of angiopoietin-2 continued to increase in advanced knee OA patients.

VEGF and angiopoietin-2

VEGF is a potent angiogenic factor secreted by endothelial cells, synoviocytes, and chondrocytes located within the articular cartilage of OA affected joints [2, 6, 11–13], and VEGF expression is upregulated in inflamed and hypoxic states [14]. In line with our recent study, synovial fluid VEGF levels in OA patients were significantly higher than paired plasma and were positively correlated with the radiographic severity of knee OA patients. [15]. In addition, Walsh et al. demonstrated a

Table 2 Descriptive data of angiogenic cytokines in healthy controls and OA patients

Cytokines		Mean	SD	Median	IQR (Q1–Q3)	Range (min–max)
G-CSF	Control plasma	50.4	20.5	48.4	32.3–62.4	26.5–82.6
	OA plasma	95.7	69.4	86.8	42.7–136.3	0.4–328.1
	OA SF	3.8	4.5	2.9	1.0–4.1	0.4–15.4
PECAM	Control plasma	160.5	87.9	159.7	116.0–168.8	23.6–323.1
	OA plasma	644.0	520.4	501.6	319.6–714.7	4.3–2427.4
	OA SF	1256.1	1998.8	436.1	132.5–1090.7	4.3–6261.1
HGF	Control plasma	74.2	37.9	69.0	46.8–100.4	25.1–146.3
	OA plasma	251.6	176.6	262.5	124.2–307.8	10.4–800.8
	OA SF	338.2	966.9	57.7	25. 9–153.9	5.5–4055.0
VEGF	Control plasma	7.8	3.2	7.6	5.8–9.6	4.3–13.9
	OA plasma	18.5	9.8	18.4	13.3–23.6	1.4–47.2
	OA SF	95.2	174.8	53.6	26.1–90. 6	7.9–999.6
Leptin	Control plasma	800.0	666.6	1185.1	88.8–1265.1	60.7–1400.4
	OA plasma	2509.3	2576.7	1610.4	915.2–3010.4	345.5–10572.8
	OA SF	2959.2	3796.2	1647.4	997.3–4435.7	372.7–15930.9
PDGF-BB	Control plasma	19.9	–	19.9	19.9-19.9	19.9-19.9
	OA plasma	34.3	20.6	32.0	19.6–48.6	2.3–66.3
	OA SF	–	–	–	–	–
Angiopoietin-2	Control plasma	167.9	49.9	173.7	132.4–198.3	73.6–261.1
	OA plasma	403.7	195.5	367.6	267.7–470.9	190.8–1072.0
	OA SF	539.7	1412.1	87.4	31.6–130.3	12.4–5355.9
Follistatin	Control plasma	87.4	29.7	80.5	67.4–94.8	50.1–171.5
	OA plasma	153.6	73.5	134.3	106.4–166.2	76.6–369.7
	OA SF	13.6	16.6	2.0	2.0–20.9	1.4–8.6
IL-8	Control plasma	3.6	1.7	3.8	2.1–4.8	1.2–7.2
	OA plasma	36.7	138.7	11.0	8.0–13.3	1.7–757.3
	OA SF	75.8	242.5	10.7	4.5–29.5	0.6–1292.7

Values (pg/ml) where appropriate. *IQR* interquartile range

significantly higher percentage of VEGF-positive chondrocytes in cartilage of OA than that of controls [2]. The previous study used a larger sample size which may have influenced the statistical significance threshold of the results. In recent years, angiopoietin-2 and VEGF-D were found to be significantly higher in rheumatoid arthritis (RA) patients with synovitis compared to those without synovitis [16]. Both VEGF and angiopoietin-2 are known to upregulate Notch signalling, which is critical to vascularisation. Additionally, notch signalling mediates VEGF and angiopoietin-2-induced IL-8 and IL-6 production in synovial explants from RA patients [17]. In this study, both plasma and synovial fluid levels of IL-8 were shown to be associated with corresponding VEGF levels and angiopoietin-2 in synovial fluid (data not shown). Furthermore, VEGF and angiopoietin-2 are also known to act on the tyrosine kinase with immunoglobulin-like and endothelial growth factor-like domains (TIE)-2 pathway. Whilst VEGF activates the TIE-2 receptors, angiopoietin-2 is known to regulate angiogenesis by inhibition of the pathway, but in certain conditions can act as an agonist to the same TIE-2 receptors [18, 19]. TIE-2 receptors have been shown to be expressed in proliferating capillaries and lining of RA synovial membranes [20]. Moreover, VEGF regulates angiopoietin-2 expression at a transcriptional level in endothelial cells [21, 22].

Follistatin

Plasma follistatin levels were significantly higher in knee OA patients than controls. Moreover, plasma follistatin levels were correlated with the disease severity of knee OA.

Follistatin, encoded by the *FST* gene, is a bone morphogenetic protein (BMP) antagonist, notably BMP-7, BMP-2 and BMP-4 [23]. BMPs are highly involved not only in embryonic development but also in adult tissue homeostasis. Through binding to BMP type-1 receptors which activate Smad signalling pathways, BMPs induce bone and cartilage formation and repair [24, 25]. Our results show increased plasma levels of follistatin in early stage OA. By inhibiting BMP signalling,

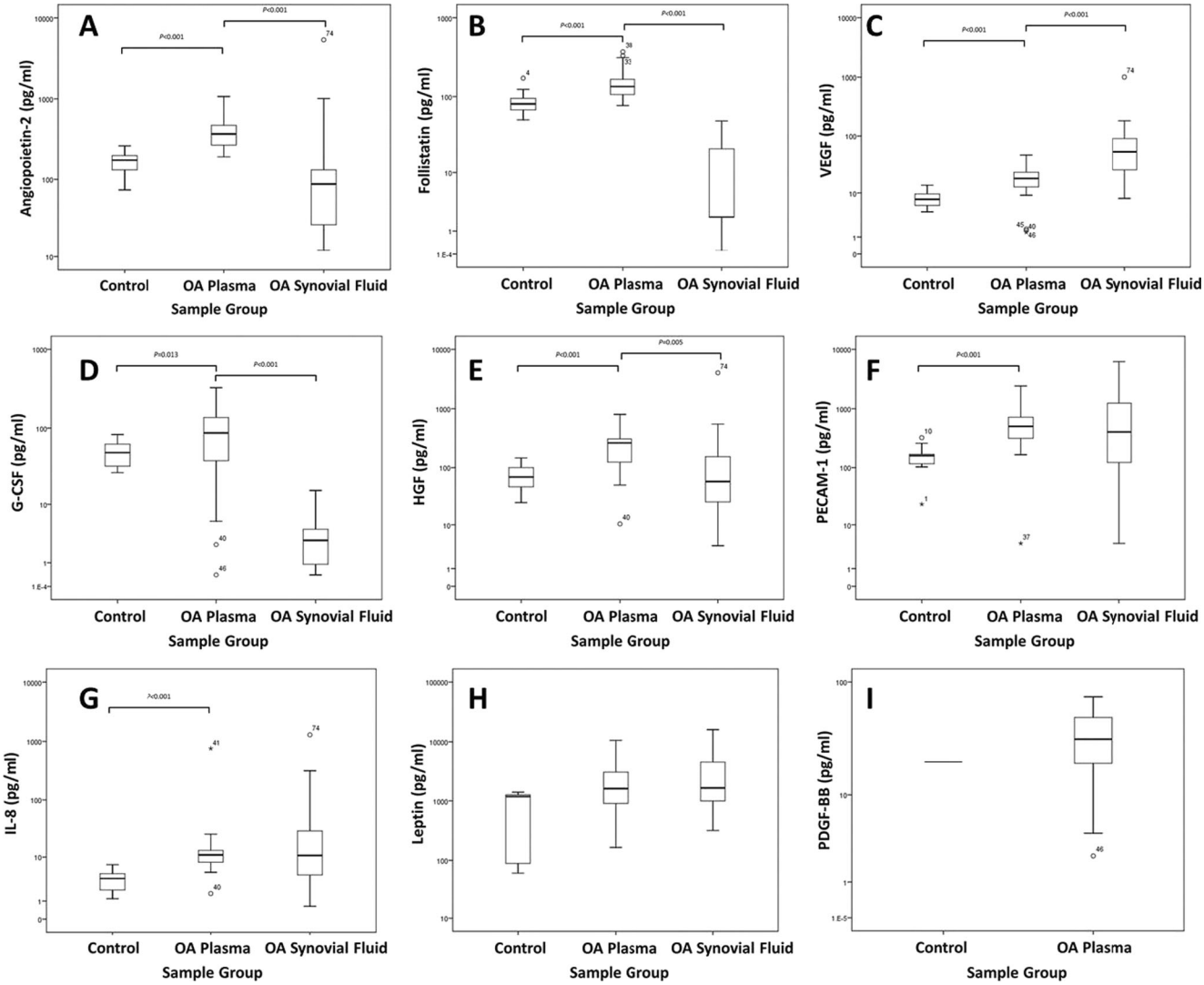

Fig. 1 Box plots of angiogenic cytokines in healthy controls and OA patients. **a** Angiopoietin-2. **b** Follistatin. **c** VEGF. **d** G-CSF. **e** HGF. **f** PECAM-1. **g** IL-8. **h** Leptin. **i** PDGF-BB

Table 3 Median cytokine values and interquartile range (IQR) of plasma controls and OA patients

Cytokines	Controls		Early OA		Advanced OA	
	Median	IQR (Q1-Q3)	Median	IQR (Q1-Q3)	Median	IQR (Q1-Q3)
G-CSF	48.4	32.3–62.4	90.2	83.5–129.8	78.3	32.3–148.9
PECAM-1	159.7	116.0–168.8	488.7	387.5–540.5	549.8	315.5–752.0
HGF	69.0	46.8–100.4	251.0	137.3–295.1	262.5	119.7–351.1
VEGF	7.6	5.8–9.6	15.6	10.9–18.9	19.0	14.8–26.4
Leptin	1185.1	88.8–1265.1	1316.2	778.4–2068.4	1695.8	967.0–3299.7
PDGF-BB	19.9	19.9–19.9	30.2	5.7–31.3	39.0	20.7–54.7
Angiopoietin-2	173.7	132.4–198.3	294.1	237.0–307.4	414.9	298.1–528.2
Follistatin	80.5	67.4–94.8	173.9	131.8–204.2	129.5	99.9–150.2
IL-8	3.8	2.1–4.8	9.8	7.7–11.4	11.6	8.0–14.6

IQR interquartile range, *OA* osteoarthritis

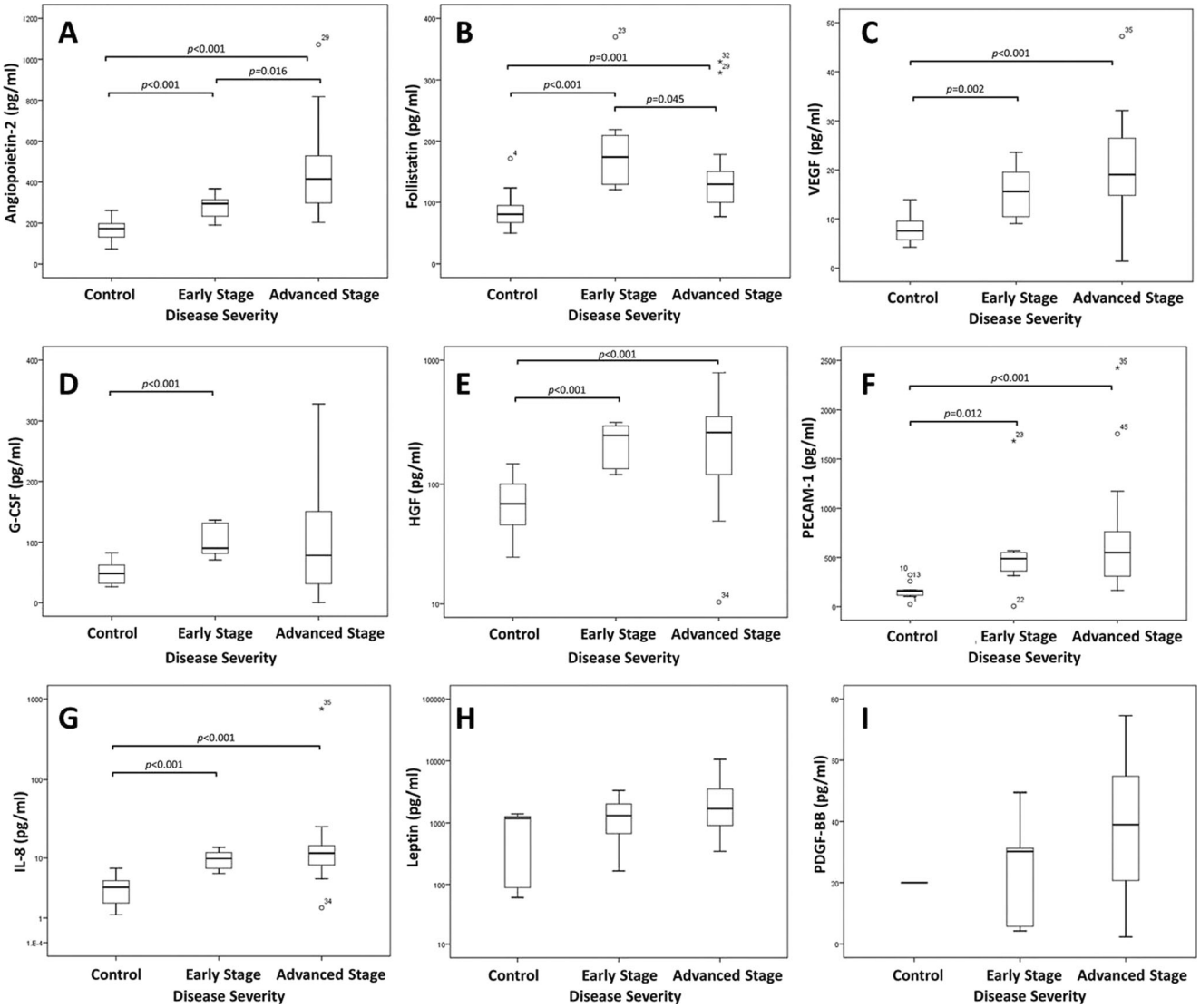

Fig. 2 Comparison of plasma angiogenic cytokines in controls and OA patients (control, early stage OA, and advanced stage OA). **a** Angiopoietin-2. **b** Follistatin. **c** VEGF. **d** G-CSF. **e** HGF. **f** PECAM-1. **g** IL-8. **h** Leptin. **i** PDGF-BB

follistatin may be hindering the repair of skeletal tissue damage within affected joints, and thus contribute to the pathogenesis of OA. However, synovial fluid follistatin levels were significantly lower than paired circulating levels. A possible explanation for this might be the result of clearance kinetics or membrane permeability with osteoarthritic knee joints.

Follistatin is also known to bind to activin. Activin is, like BMPs, a member of the transforming growth factor (TGF)-β superfamily and up-regulates follistatin expression [26]. Activin is involved with a range of physiological processes including tissue repair, anti-inflammation and cell proliferation. Circulating levels of activin were higher in OA patients than healthy controls and lower in synovial fluid compared with RA patients [27]. Activin has been shown to exhibit possible anti-catabolic effects in cartilage during inflammation [28]. As a result, the inhibitory binding of follistatin and activin could disrupt the tissue repair in articular cartilage. The equilibrium of anabolic and catabolic processes in an affected joint may be shifted, disrupting the limited repair properties of cartilage leading to accelerated cartilage loss and disease progression. Furthermore, follistatin expression was also found to be upregulated by a number of pro-inflammatory cytokines, namely, tumour necrosis factor-α and interferon-γ, and down-regulated by a range of growth and development factors and cytokines including BMP-3, BMP-4, IL-13, and activin A [26]. With inflammation an important part of early OA, such cytokine levels are often higher in early stages but decrease with disease progression. These changes in follistatin-expression mediators may help to explain the results found in this study in which levels were highest in the early stages of OA but were reduced significantly in the latter stages.

Leptin

Leptin was the only cytokine tested in this study to show a significant correlation between plasma and corresponding synovial fluid samples in OA patients. This is in accordance with Lübbeke et al. [29]. They also showed increased pain associated with higher synovial fluid leptin levels in OA patients but, similar to our study, no significant association with KL grading of OA severity. Conversely, Staikos et al. [30] found that synovial fluid leptin levels were correlated with Ahlback scores. It was also associated with an increased prevalence and incidence of knee OA [31]. Through the induction of IL-6 and IL-8, leptin is thought to play an inflammatory role in OA cartilage [32]. This supports our findings which found a strong positive correlation between circulating leptin and IL-8 levels.

IL-8

A recent study has shown that multiple cytokines including IL-8 can be highly detected in drainage fluid and are involved in the inflammatory response after total hip arthroplasty [33]. Synovial fluid IL-8 levels in hip OA have been shown to be higher in advanced hip OA patients than early OA patients [34]; however, our data did not show any relationships in either early or advanced knee OA. In concordance with our finding, Kaneko et al. showed that circulating IL-8 levels were not found to be associated with Japanese Orthopaedic Association scores in OA patients [35]. Bon et al. have investigated inflammatory mediators associated with bone metabolism in plasma from chronic obstructive pulmonary disease (COPD) patients [36]. They did not find an association with bone metabolism and IL-8, G-CSF, or PDGF levels. Our results support those of Huebner et al. who found that G-CSF was increased in OA-prone guinea pig models compared with an OA-resistant strain [37, 38].

PECAM-1

PECAM-1 (also known as CD31) has previously been found to be expressed in endothelial cells and synovial lining cells of OA [11, 39]. This could be an explanation as to why PECAM-1 levels were higher in OA plasma than controls. However, our study did not show an increase in synovial fluid PECAM-1 levels compared with OA plasma. Neovascularisation in rheumatoid arthritis was observed as showing a more intense PECAM-1 expression when compared with OA [11].

Limitations and considerations

This study had limitations which should be considered when evaluating the results and conclusions presented herein. Firstly, the study was cross-sectional in design with a relatively small sample size. Accordingly, cause-and-effect relationships cannot be determined and require additional longitudinal studies to elucidate any relationships. Secondly, angiogenic cytokines in some samples were undetectable, particularly for synovial fluid samples. A possible explanation for this finding is likely attributed to limited assay sensitivity of the kit used. Reduced data limit the statistical power of these results. Furthermore, due to ethical considerations, synovial fluid samples were not collected from healthy controls resulting in no direct comparison between the local environments of joints being made between controls and OA patients. Instead circulating cytokine levels reflect the systemic nature of the condition. In future studies, this may be overcome by collecting synovial fluid samples from traumatic knee patients. Finally, tissue expression of angiognic cytokines has not been investigated. Further immunohistochemical studies of osteoarthritic cartilage and synovial tissues may provide useful information on the pathogenic role of these cytokines in OA.

In conclusion, angiogenic cytokines in plasma can differentiate knee OA patients from controls. Follistatin, VEGF, and angiopoietin-2 may have potential as biochemical markers for the assessment of severity in knee OA patients. However, more research on this topic needs to be undertaken before the association between angiogenic cytokines and the severity of OA is more clearly understood.

Acknowledgments This investigation has been supported by the Ratchadapiseksompotch Fund (RA55/22), Faculty of Medicine, Chulalongkorn University, and the 90th anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund). The authors commemorate the 100th Anniversary of the King Chulalongkorn Memorial Hospital, Thai Red Cross Society and gratefully acknowledge Dr. Chintana Chirathaworn for kindly providing the facilities.

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