

## Identification of Circulating Natural Antibodies against Endogenous Mediators in the Peripheral Blood Sera of Patients with Osteoarthritis of the Knee: A New Diagnostic Frontier

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### Abstract

**Introduction:** The presence of NA against EM regarding specificity and have gained increasing attention in proteome analysis for diagnosis, developing, monitoring and effective treatment of osteoarthritis of the knee (kOA). An understanding of the various regulatory systems controlling blood vessel growth, inflammation and pain in the joint should lead to help explain kOA disease progression. To investigate the specific presence of NA (IgM, IgG, IgA) against EM (BK, AII, VEGF, bFGF) in the sera of kOA patients and control and to correlate this with process of joint destruction.

**Methods:** In this study novel immunoconjugates were designed, synthesized and then used to develop a rapid, specific and sensitive ELISA method to directly detect immune complexes (NA-EM) in humans. Following this procedure, we examined variations in the levels of natural antibodies recognized a panel of self-antigens in the sera from healthy individuals and kOA patients. Blood samples were obtained from 250 patients with symptomatic kOA and 250 ages, sex-matched healthy individuals.

**Results:** NA against EM was detected with novel ELISA assay in the sera of kOA patients as well as in the sera of control. At time of inclusion kOA patients (100%) had significantly higher BK-IgG levels relative to normal sera. The over expression BK-IgG were positively associated with destructive changes (KL>4;  $r=0.75$ ;  $p<0.005$ ). kOA patients in whom KL scores progress rapidly tend to have higher BK-IgG levels at all time point. Serum BK-IgG over expression in kOA patients were positively associated with destructive changes (KL>4;  $r=0.75$ ;  $p<0.005$ ). Elevated BK-IgG was significantly correlated with VAS ( $r=0.85$ ;  $p<0.0001$ ) and loss of functions ( $r=0.69$ ;  $p<0.0003$ ) in kOA patients. Affinity chromatography yielded EM-specific NA from the sera of healthy individuals and kOA patients.

**Conclusions:** We showed that EM represents a group of novel self-antigens which are targeted by NA from kOA patients. Circulating BK-IgG in the sera has been proposed as a sensitive and specific marker of diagnosing kOA at early stages of the disease. Our results have potential applications for controlling unwanted angiogenesis, inflammation, pain and future response to therapy in kOA patients.

**Keywords:** Osteoarthritis of the knee; Angiogenesis; Vasoregulatory systems; Inflammation; Pain; Radiological damage; immune regulation; Natural antibodies; Endogenous mediators; Serodiagnosis; Biomarkers

**Abbreviations:** ABTS: 2,20-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid); AII: Angiotensin II; bFGF: Basic Fibroblast Growth Factor; BK: Bradykinin; BK-IgG: Natural IgG Antibodies against Bradykinin; BSA: Bovine Serum Albumin; CT: Computed Tomography; ELISA: Enzyme Linked Immunosorbent assay; EM: Endogenous Mediators; EM-IgG: Natural IgG Antibodies against Endogenous Mediators; HMW: High Molecular Weight; HAS: Human Serum Albumin; Ig: Immunoglobulin; IgA: Immunoglobulin A; IgA-HRP: Immunoglobulin A-Horseradish peroxidase Conjugate; IgG: Immunoglobulin G; IgG-HRP: Immunoglobulin G-Horseradish Peroxidase Conjugate; IgM: Immunoglobulin M; IgM-HRP: Immunoglobulin M-horseradish Peroxidase Conjugate; KL Grade: Kellgren-Lawrence grade; KKS: Kallikrein-kinin System; kOA: Osteo Arthritis of the knee; LMW: Low Molecular Weight; NA: Natural Antibodies; NHS: Normal Human Sera; OA: Osteoarthritis; PBS: Phosphate-Buffered Saline; PBST: phosphate-buffered saline with Tween20; RA: Rheumatoid Arthritis; RAS: Rennin-Angiotensin System; TMB: 3,3',5,5'-TetraMethylBenzidine; VAS: visual analogue scale; VEGF: Vascular Endothelial Growth Factor

### Introduction

Osteoarthritis (OA) is the most common disorder affecting synovial joints, with structural changes of osteoarthritis present in approximately half of the adult population in world [1]. The knee is the most commonly affected weight-bearing joint and various deformity is the most common misalignment of the knee associated with osteoarthritis.

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Osteoarthritis of the knee (kOA) can be a progressive disabling disease, which results from the pathological imbalance of degradative and reparative processes, with concomitant inflammatory changes [2]. The clinical features of kOA include pain, stiffness, reduced motion, swelling, crepitus, and deformity [3-5].

Several factors are considered for the pathogenesis of kOA [6-14]. Complicated path biological interactions between the Kallikrein-Kinin System (KKS), the Rennin-Angiotensin (RAS), angiogenesis and natural immunity could contribute to joint destruction in the disease process of kOA [15-19]. It is also likely that biomarkers will be used in conjunction with imaging in order to establish stage of disease, predict progression, and assess improvement in the setting of clinical trials [20-22].

Vasoregulatory systems are developed and a fully functional microvasculature is formed in kOA [6,19,23,24]. Vasoregulatory systems, as the KKS and the RAS, play essential roles in the maintenance of vascular homeostasis [25,26]. An understanding of the various regulatory systems controlling blood vessel growth, inflammation and pain in the joint should lead to help explain kOA progression.

The KKS, most well-known as mediator of inflammation, also has role in the control of vessel diameter and growth [27,28]. Factors of KKS are thought to be key mediators in inflammatory joint disease [3]. Kinins released into synovial fluid by the proteolytic action of kallikreins on kininogens on the surface of neutrophils are likely to cause vasodilatation and pain, increase vascular permeability, promote leukocyte margination and stimulate cytokine release from monocytes [17,29]. Pain, the predominant symptom in kOA, is multidimensional in its nature and mediated through a variety of factors as bradykinin [30-33].

The RAS is best known as a major regulator of blood pressure, but it also is important at the micro vascular level in the regulation of neovascularization [34,35]. In addition, the RAS has important modulatory activities in the process of kOA [24]. Vascular inflammation is an independent risk factor for the development of kOA [19]. Angiotensin II (AII) augments vascular inflammation, induces endothelial dysfunction, and, in so doing, enhances the process of kOA [25]. AII the classical signs of inflammation-pain, redness, erythema, edema, and hyperthermia.

Angiogenesis, defined as the development of new capillaries from preexisting blood vessels, is an important the pathogenesis of kOA since it in the initiation and perpetuation of the disease [2,18]. Inflammation and angiogenesis are closely associated in kOA, modulating functions of chondrocytes, contributing towards abnormal tissue growth and perfusion, ossification and endochondral bone towards abnormal tissue growth and perfusion, ossification and endochondral bone development, leading to radiographic changes observed in the joint [36-39].

Almost all of the human autoimmune diseases are characterized by the generation of Natural Antibodies (NA) [40-44]. Identifying those antibodies is the cornerstone for the diagnosis of autoimmunity in humans [45-50]. In autoimmune rheumatic diseases, pathogenic auto antibodies are used for classification, development of diagnostic criteria, monitoring of disease activity and prediction of prognosis [51-53]. However, autoimmunity defined by the detection of auto antibodies does not necessarily imply the presence of an autoimmune disease. Furthermore, the normal immune system is able to produce, in relatively high amounts, antibodies that bind various self-antigens, i.e.

endogenous mediators. Those auto antibodies, defined as NA, have an important physiological regulatory role [54-57].

NA refers to antibodies that are present in the serum of healthy individuals in the absence of deliberate immunization with the target antigen [58,59]. A vast majority of NA react with one or more self antigens and are termed as natural auto antibodies [60,61]. The importance of NA in immune regulation has long been neglected, since tolerance to self was thought to be primarily dependent on the deletion of auto reactive clones, rather than on peripheral suppressive mechanisms [62,63]. Clonal deletion and energy cannot account, however, for the prevalence of natural auto reactivity among healthy individuals [64]. It is now well established that auto reactive antibodies and B cells, and auto reactive T cells, are present in healthy individuals, and in virtually all vertebrate species [65]. Auto reactive repertoires are predominantly selected early in ontogeny [66,67]. Questions pertaining to the role of NA in the regulation of the immune response and maintenance of immune homeostasis and to the distinction between natural auto reactivity and pathological autoimmunity have not been adequately addressed [68,69].

KOA is a chronic, destructive autoimmune disease of the joints [9,70,71]. It is characterized by the presence of NA that are reactive to various target molecules [72-76]. kOA is an autoimmune disease characterized by chronic synovitis, which manifests as joint pain and often progresses to bone and joint destruction [77-81]. Inflammation in kOA may result from a number of different mechanisms, including antibody-mediated complement activation and cellular injury, T-cell-mediated mechanisms and generation of pro-inflammatory mediators [82,83].

Differentiating between pathogenic, natural and other nonpathogenic auto antibodies is crucial for the definition, diagnosis and identification of a reliable biomarker of osteoarthritis [84-87]. The identification of NA that highly predicts the development of kOA is of great interest. This study focuses on human NA against EM discovered in our Unit [88,89]. Development technologies that permit assessment of potentially disease-modifying agents of vascularization and inflammation are the current approach to the management of kOA. It is an established fact that any physiological stress can interact with the immune system.

The analysis of the osteoarthritis-associated antigen-antibody systems in the normal peripheral blood has new approach to the patient at risk for or with newly diagnosed kOA. The presence of NA against EM regarding specificity and have gained increasing attention in proteome analysis for diagnosis, developing, monitoring and effective treatment of kOA. Previous retrospective studies in different countries have shown that NA can be detected in patients with kOA several years before clinical symptoms occur [57,90-93]. Given the low prevalence of kOA, NA testing in the general population is of no clinical benefit. NA in kOA has been found to be quite useful in clinical practice for diagnosis and assessing prognosis. NA against EM has recently been shown to predict development of kOA as well as poor outcome in early kOA.

The aim of this study was to investigate the specific presence of NA (IgM, IgG, and IgA) against EM (bradykinin, angiotensin II, vascular endothelial growth factor, basic fibroblast growth factor) in the peripheral blood sera of patients with o kOA and healthy individuals. In addition, we are interested in investigating the functional properties of affinity-isolated NA against EM from the peripheral blood of healthy individuals and patients with kOA (focusing on isotype, affinity,

specificity). This study also further characterizes the markers of clinical relevance in patients with KOA.

## Methods

### Patients

The study included 250 patients with symptomatic KOA (age range 45-79 years) fulfilling the American College of Rheumatology criteria for KOA. All patients with KOA had involvement of the knee joint with typical radiographic changes graded Kellgren Lawrence classification. Pain was scored on a Visual Analogue Scale (VAS) immediately after walking 50 m. All patients with KOA are with persistent pain longer than 6 months. Parameters for function were performed by Lequesne's functional indexes. 250 age and sex-matched healthy Individuals. Characteristics of patients are listed in (Table 1). The patients and control had no associated organic disease and exhibited no evidence of autoimmune disease. They did not have immunological or other arthritic disease or any physical illness known to affect their immunological status.

### Control subjects

The general reference (normal) control samples consisted in age- and sex- matched 250 healthy individuals in Blood Transfusion Service of the National Institute of Rehabilitation (Mexico City, Mexico). The absence of disease was confirmed by physical examination, clinical history and routine laboratory tests.

### Blood sampling

Seven milliliters of the peripheral blood was drawn into a serum separator tube (Vacutainer Systems, code 607213 Becton-Dickinson, USA). Blood was allowed to clot for 1 h at Room Temperature (RT). Sera were obtained after centrifugation at 3000 rpm for 10 min at 4°C. All serum samples were stored in 300 µl aliquots at -80°C until analysis.

### Reagents

All reagents were of analytical grade and were obtained from Sigma-Aldrich Ltd, Poole, UK, unless otherwise indicated.

### Design and synthesis of immunoconjugates for ELISA

In this study novel immunoconjugates were designed, synthesized and then used to develop a rapid, specific and sensitive ELISA method to detect NA against EM directly in the peripheral venous blood sera of humans. Human low molecular weight EM was coupled with High Molecular Weight Matrix (HMWM: polyphenylacrilate) according to in-hous protocols provided by Tissue Engineering, Cell Therapy and Regenerative Medicine Unit (National Institute of Rehabilitation, Mexico City, Mexico). Briefly, 1 mg of HMWM was coupled to 1 mg of EM in 0.1 M buffer, pH 4.5, containing 2 mg of EDC in a final volume of 1.6 ml. The mixture was incubated for 2 h at RT. The conjugated EM-

VARIABLES	OA	CONTROL
Age (years)	54 ± 8.8	51 ± 11.1
Body mass index BMI (kg/m <sup>2</sup> )	26 ± 2.5	26 ± 3.3
Duration of OA (years)	13.0 ± 9.8	
Pain (visual analog) scores	5.46 ± 2.15	0.00 ± 0.00
Beck Depression Index	5.56 ± 5.69	1.00 ± 1.86
Mean interval in years between baseline and follow-up scan	2.67	

Table 1: Baseline characteristics of study population.

%	SELF ANTIGEN			
	BK	All	VEGF	bFGF
Reproducibility	96	97	90	90
Sensitivity				
Primary KOA	26	13	23	8
Secondary KOA	17	8	5	3
Specificity	96	95	93	91

Table 2: Reproducibility, sensitivity and specificity of detection of natural IgG antibodies in osteoarthritis of the knee.

HMWM was then dialyzed against Phosphate Buffered Saline (PBS), pH 7.4 at 4°C.

### Development of ELISA for rapid detection natural antibodies against endogenous mediators

Polystyrene micro titer ELISA plates with 96-wells (Maxi-sorb, NUNC, Rochester, NY, USA) were incubated overnight at 4°C with EM-HMWM (1 µg/ml) in 0.1 M carbonate/bicarbonate buffer, pH 9.6. The final volume of this as well as of all other steps was 100 µl per well, unless stated otherwise. After washing the plates twice with PBS, residual binding sites were blocked (1 h at RT) with 200 µl per well of PBS containing 2%, w/v, Human Serum Albumin (HAS). Human sera were appropriately diluted in assay buffer (veronal buffer containing 0.1%, w/v, HSA, 2 mM CaCl<sub>2</sub>, 0.1%, w/v, Tween-20, pH 7.4), and incubated for 1 h at RT. After this and the subsequent incubation steps, the plates were washed with PBS containing 0.1%, w/v, Tween-20 (PBST). IgM, IgG, IgA bound to EM-HMWM was quantified with horseradish peroxidase labeled anti-human IgM, IgG, IgA diluted in assay buffer. Finally, horseradish peroxidase activity was visualized by incubation with 100 µg/ml 3,3',5,5'-Tetra-Methylbenzidine (TMB), in 0.11 M sodium acetate, pH 5.5, containing 0.003%, v/v, H<sub>2</sub>O<sub>2</sub>. The reaction was stopped after 10 min by addition of 2 M H<sub>2</sub>SO<sub>4</sub>, and the absorbance at 450 nm was measured in a micro titer plate reader (Bio-Kinetics Reader; Bio-Tek Instruments, Winooski, VT, USA). Tests were performed in duplicate. All measurement (patients and control subjects) were made on the same day and under the same experimental conditions.

Dilutions of a pool of normal sera, obtained from 250 healthy volunteers, were used to generate a standard curve in each micro titer plate. This standard was arbitrarily proposed to contain EM-Ig. Results with serum samples were related to this standard and expressed as EM-Ig. The specificity of the binding of EM-Ig to EM-HMWM was determined by competition immunoassay (Table 2). The standard curve was pre-incubated with increasing amounts of the competitors. After 1 h incubation, the standard with or without competitors was added to the EM-HMWM-coated plates and tested as described above.

### Purification of human natural antibodies against endogenous mediators

Immunoabsorbent columns were prepared with antigen of interest coupled to cyanogens bromide-activated Sepharose (Pharmacia Biotech). Two milligrams of protein were used for coupling to 1.5 ml of bed volume of CNBr-activated Sepharose.

One gram of Ig in 100 ml of PBS was loaded on the immunoabsorbent column and run twice on the column at a speed of 1 ml/min at RT, followed by washing with PBS until the absorbance of the flow-through at 280 nm reached baseline values. Bound antibodies were eluted using glycine-HCl (0.1 M) buffer of pH 2.8, 2 M NaCl followed by PBS and then diethanolamine (0.1 M) buffer, pH 11; 2 M NaCl. The eluates obtained at different pH were brought to pH 7.0 and pooled. Two

milliliters of the flow-through fractions were allowed to run through the sorbents for two more cycles and further used as effluent fractions. Eluates and effluents were dialyzed against PBS.

### Affinity of purification of natural antibodies against endogenous mediators

Immunoabsorbent columns were prepared with the antigens of interest coupled with cyanogens bromide-activated Sepharose (Pharmacia Biotech) [94,95]. One milligram of protein was used for coupling with 1.5 ml of bed volume of CNBr-activated Sepharose. One gram of IVIG in 100 ml of PBS was loaded on the immunoabsorbent column and run twice on the column at a speed of 1 ml/min at RT, followed by washing with PBS until the absorbance of the flow-through at 280 nm reached baseline values. Bound antibodies were eluted using a glycine-HCl (0.1 M) buffer, pH 2.8 and 2 M NaCl followed by PBS and then diethanolamine (0.1 M) buffer, pH 11; 2 M NaCl. The eluates obtained at different pH levels were brought to pH 7.0 and pooled. Two milliliters of the flow-through fractions were allowed to run through the sorbents for two more cycles and then used as effluent fractions. Eluates and effluents were dialyzed against PBS.

### Determination of total IgM and IgG levels

We used the CovaLink ELISA system to determine the total IgM and IgG levels of the patients and controls. For each plate standard curves were drawn using known amounts of no conjugated human IgG and IgM.

### Radiological method

Plain X-ray films were performed on the small joints of the knee in all osteoarthritis patients at baseline (n=250) and after two years (n=250). These films were examined by expert radiologists. Radiological progression was defined as an increase in the LS score from the baseline to endpoint that was greater than the median value for each patient. The Kellgren-Lawrence (KL) grade, an integer index ranging from 0 to 4, is a standard radiographic measurement of joint degradation used in diagnosing osteoarthritis of the knee [96]. Radiographic osteoarthritis can be defined simply as a KL grade of 2 or higher.

### Statistical analysis

The data was analyzed on an IBM computer using SPSS. Quantitative variables were described as mean, standard deviation (SD) and range. Qualitative variables were described as number and percentage. The Chi-square test was used to compare qualitative variables between groups. The Kruskal-Wallis test was used instead of ANOVA in non-parametric data (SD>50% mean). Spearman's correlation test was used to rank different variables against each other. Receiver Operator Characteristic Curve (ROC) was drawn to find out the best cut-off value of natural antibodies against endogenous

mediators in diagnosing osteoarthritis of the knee and to test for its statistical efficacy. P-value >0.05 was considered insignificant, p<0.05 was significant and p<0.01 was highly significant.

### Ethical approval

All patients and healthy controls provided informed, written consent and the study was approved by the Ethics Committee of National Institute of Rehabilitation, Mexico City, Mexico.

### Results

Serological identification of natural antibodies against endogenous mediators in healthy Individuals and patients with osteoarthritis of the knee by the novel ELISA Natural self-reactive antibodies of the IgM, IgG and IgA isotype are present in the serum of healthy individuals and kOA patients. Different classes of NA (IgM, IgG, IgA) against EM (BK, ANII, VEGF, bFGF) were detected with novel ELISA protocol in the sera of kOA patients as well as in the sera of healthy individuals (Table 3). These EM represent a group of novel self antigens which are targeted by NA from kOA patients.

In this study, we found BK-IgG expression most abundantly in testis among the kOA sera tested. No significant difference in binding of serum EM-IgA levels in kOA patients in comparison with that in control. Characterization of the functional properties of natural antibodies that recognize human endogenous mediators.

### Isotypes of natural antibodies against endogenous mediators

NA belong mainly to the immunoglobulin M class and are characterized by several features, including the ability to bind self and non-self antigens, low affinity (monovalent antigenic binding to a small single epitope), high avidity (overall force that binds multivalent antibody to a macromolecule carrying multivalent epitopes), and polyreactivity (binding different epitopes). Pathogenic auto antibodies are antigen driven and belong mainly to the IgG isotype. Affinity chromatography yielded two isotypes (IgM, IgG) of EM-specific NA from the sera of healthy individuals and kOA patients. EM-IgA was not useful for the presence kOA in humans. Affinity-purified NA against EM displayed the expected characteristics and was functionally fully active.

### Affinity of natural antibodies against endogenous mediators

The affinity constant of NA against self-antigens from both kOA patients and normal sera were determined in table 4. Low affinity EM-IgM was predominantly isotype of Ig which present in healthy individuals. Deficiency in the sera EM-IgM predisposes to development expression of high affinity EM-IgG in kOA patients. The secondary immune response is characterized by the rapid production of high affinity EM-IgG in kOA patients.

The Study Group	Self Antigens							
	BK		All		VEGF		bFGF	
	ISOTOPE of NATURAL ANTIBODIES							
	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
Patients with osteoarthritis of the knee	689 ± 161	287 ± 84	409 ± 111	289 ± 99	669 ± 241	559 ± 198	689 ± 245	521 ± 222
	100%	77%	71%	66%	59%	79%	35%	61%
Healthy Individuals	501 ± 98	407 ± 115	274 ± 84	455 ± 101	471 ± 189	366 ± 181	455 ± 115	705 ± 239
Significance P value	0.0001	0.0004	0.0005	0.0001	0.0003	0.005	0.009	0.007

**Table 3:** Serum expression of natural antibodies against endogenous mediators.



The Study Group	VEGF		BK		All	
	AFFINITY CONSTANTS RANGING BETWEEN, M					
	IgM	IgG	IgM	IgG	IgM	IgG
Healthy Individuals	10 <sup>-6</sup> -10 <sup>-7</sup>	10 <sup>-5</sup> -10 <sup>-8</sup>	10 <sup>-6</sup> -10 <sup>-7</sup>	10 <sup>-5</sup> -10 <sup>-8</sup>	10 <sup>-6</sup> -10 <sup>-8</sup>	10 <sup>-5</sup> -10 <sup>-7</sup>
Patients with osteoarthritis of the knee	10 <sup>-10</sup> -10 <sup>-11</sup>	10 <sup>-4</sup> -10 <sup>-6</sup>	10 <sup>-9</sup> -10 <sup>-11</sup>	10 <sup>-3</sup> -10 <sup>-5</sup>	10 <sup>-9</sup> -10 <sup>-10</sup>	10 <sup>-4</sup> -10 <sup>-5</sup>



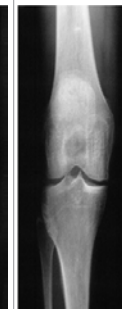
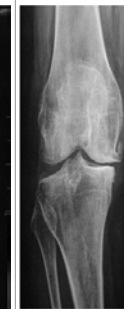
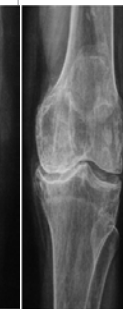
**Table 4:** The affinity of natural antibodies against endogenous mediators from both osteoarthritis of the knee and normal sera.

Structurally % Significant Inhibition Similar Inhibitors	% Significant Inhibition			
	BK-IgG	BK-IgM	All-IgG	All-IgM
Angiotensin II	0.12	0.01	100	100
Bradykinin	100	100	0.02	0.01
Vasopressin	0.003	0.01	0.07	0.001
Dermorphin	0.001	0.001	0.003	0.001
Beta-Endorphin	0.007	0.001	0.001	0.02

**Table 5:** The cross-reactivity of affinity-purified natural antibodies against endogenous mediators from osteoarthritis of the knee and normal sera.

Variables	Spearman's r	P
BK-IgG	0.897	0.0005
All-IgG	0.489	0.0027
VEGF-IgG	0.381	0.0045
bFGF-IgG	0.291	0.0001

**Table 6:** Correlations between Kellgren Lawrence grading and serum markers.

KELLGREN-LAWRENCE STAGE				
CONTROL	I	II	III	IV
				
SERUM BK-IgG LEVEL, ODx1000				
501 ± 98	555 ± 131	635 ± 141	685 ± 181	781 ± 211

**Table 7:** Correlations of bk-IgG titer with kellgren-lawrence grading.

### Specificity of natural antibodies against endogenous mediators

The cross-reactivity of affinity-purified natural antibodies against self-antigens from both kOA and normal sera were determined (Table 5). Serum BK-IgG was detectable in 85% of kOA patients, with 100% specificity for kOA. NA against BK and AII showed no cross reactivity to other structurally similar EM.

Natural antibodies against endogenous mediators in relation to radiographic progression assessed by Kellgren-Lawrence scoring the many measurable outcomes in OA include pain, function, synovitis, and serum and urine biomarkers or imaging biomarkers. Currently, radiographic outcomes are used to establish diagnosis and follow structural progression of the disease. While current radiographic

techniques (X-rays) are useful for the diagnosis of established disease, they have shortcoming with respect to the assessment of progressive disease.

Radiographic impairment was assessed by Kellgren-Lawrence score at baseline and again after 2 years. The mean Kellgren-Lawrence score in the whole group at presentation was 2.5 and the mean progression was 4. The relationship between the Kellgren-Lawrence grading and serum markers is also shown in table 6.

A significant relationship was noted for BK-IgG (Table 7). We examined associations between serum BK-IgG and radiographic KOA status. Levels of serum BK-IgG were positively associated with all definitions of radiographic kOA ( $p < 0.0001$ ). KOA Patients in whom KL scores progress rapidly tend to have higher serum BK-IgG levels at all time point. Serum BK-IgG Over expression in kOA patients were positively associated with destructive changes ( $KL > 4$ ;  $r = 0.75$ ;  $p < 0.005$ ). No correlations were found to conventional parameters for pain and function with expression of NA (isotypes IgA, IgM) against EM.

Table 8 shows the correlations between the serum markers in all of the subjects as determined by the Spearman rank test. These were significant correlations between BK-IgG and VEGF-IgG, between bFGF-IgG and VEGF-IgG, and between BK-IgG and AII-IgG. Table 9 shows the correlations between the joint space width and endogenous markers. All markers had negative correlations with the joint space width, but only serum BK-IgG had a significant correlation ( $p < 0.0005$ ).

### Discussion

Diagnostic immunology is a collective term for a variety of diagnostic techniques that rely on the specificity of the bond between antibodies and antigens [97-100]. The pathologic role of natural antibodies in many autoimmune diseases is widely accepted [101-103]. Clinically detectable joint inflammation may predict a worse radiological outcome in osteoarthritis of the knee [104-106].

Immunoassays are of general interest for all proteomic and diagnostic approaches in which several parameters have to be determined simultaneously from a limited amount of sample material. Improved analytical methods are required to accommodate the analysis of large numbers of samples for biological and epidemiological monitoring [44,107-112]. A sensitive assay to identify markers that can accurately determine the onset of osteoarthritis-especially if the technique is of low risk to the patient, such as blood drawing -is ideal for early detection of osteoarthritis of the knee. An enzyme immunoassay was used for measurement of antibodies against disease-specific antigens and etiologic agents for cross-reactive antigens associated with them. This natural antibodies assay was applied to a panel of antigens for the detection of osteoarthritis of the knee. Novel

Marker	BK-IgG	All-IgG	VEGF-IgG	bFGF-IgG
BK-IgG	-		0.855	
All-IgG	0.881	-		0.293
VEGF-IgG		0.213	-	
bFGF-IgG			0.791	-

**Table 8:** Correlations between natural antibodies.

Variables	Spearman's r	P
BK-IgG	0.897	0.0005
All-IgG	0.489	0.0027
VEGF-IgG	0.381	0.0045
bFGF-IgG	0.211	0.0001

**Table 9:** Correlations between joint space width and markers.

antigen-specific ELISA will be used in additional studies that will prove its clinical efficacy, not only for the early diagnosis osteoarthritis of the knee, but also for prognosis and the implementation of preventive steps for osteoarthritis of the knee.

Natural antibodies, which bind self-protein as endogenous mediators, are keys to the homeostasis of the immune system, particularly relating to B-lymphocytes and autoimmunity [113-116]. Serological evidence of the presence natural antibodies against endogenous mediators is an early future in osteoarthritis of the knee and not restricted to patients with end-stage disease undergoing joint replacement surgery. An understanding of the properties of natural antibodies, which characterizes their activity in relation to self-antigens, is important for binding capacity, functional activities, and immune recognition.

In this first study, we demonstrate the presence of natural polyreactive antibodies in normal human IgG that recognize bradykinin. Natural self-reactive antibodies belong to IgM, IgG and IgA isotypes. Here we found that natural IgG antibodies to bradykinin are predominantly of the IgG isotype. This may be of interest in view of previous findings indicating that natural antibodies specific for bradykinin in healthy individuals are of IgM class, while in patients osteoarthritis of the knee they are mostly IgG.

Kinins are thought to be key mediators in inflammatory joint disease. Bradykinin may exert influence on multiple players of the immune system. Bradykinin modulates the activation, proliferation, migration and effectors functions of these cells. The possible impact of bradykinin in human immune-mediated diseases could be emphasis on autoimmune neuroinflammation, osteoarthritis and infection. However, recent studies suggest a specific role of the bradykinin system in adaptive, i.e., antigen-specific immune reactions. Expression patterns of the bradykinin receptors on key cellular players within the adaptive immune system, and provide an overview of evidence so far indicating the possible involvement of the Kallikrein-kinin system in antigen-specific immune responses, including osteoarthritis conditions.

In healthy patients, chronic elevations of circulating BK-IgG or its biomarkers are predictors for increased risk in the development and progression of osteoarthritis of the knee disease. Predicting disease requires specific tests as well as a population in which a reasonable proportion of patients will develop disease [117]. The presence of BK-IgG was an important predictor for osteoarthritis of the knee.

In the present work we have shown that all IgM anti-EM antibodies and certain IgG-type anti-EM antibodies were significantly elevated in sera of patients with osteoarthritis of the knee compared with adult controls. Serum anti-EM antibody ratios were consistently low for IgM antibodies and were relatively high in the case of IgGs. In contrast to IgM, IgG gets into the inflamed synovial fluid readily, and thus, our data argue against local production of IgM type anti-EM antibodies.

The next important step was to test if the levels of BK-IgG antibodies showed any correlation with the disease activity in osteoarthritis of the knee. Intriguingly, using a multistep approach, our work has demonstrated that BK-specific IgG antibody levels show a clear inverse correlation with the activity of osteoarthritis of the knee. Thus, we suggest that BK-specific IgG is a disease-state biomarker in osteoarthritis of the knee. We found a similar relation when we analyzed the connection between disease activity and BK-IgG concentrations.

## Conclusions

We showed that novel ELISA assay was able to demonstrate

immune responses to each of the 4 type specific self-antigens in kOA patients. EM represents a group of novel self-antigens which are targeted by NA from kOA patients. Self-reactive NA of IgM, IgG, IgA classes are present in the sera of healthy individuals and kOA patients. This results show a specific imbalance of immunoglobulin's content in kOA patients. Serum NA profiling is a promising approach for early detection and diagnosis of kOA. Additionally, a serum expression profiling study identified 4 self-antigens specifically expressed in kOA patients, which were identified by affinity chromatography. High-affinity, BK-IgG has demonstrated a direct role for BK in kOA development. The isolated IgG fractions of patients suffering from kOA had higher anti-BK reactivity than those detected in normal individuals. Serum BK-IgG is a promising candidate as kOA-specific disease antibodies. Identification of novel broadly cross-reactive kOA-neutralizing NA against EM in the sera has major implications for the development of treatment, angiogenesis inhibitors, and tools to study mechanisms.

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