Development of a highly sensitive chemiluminescence immunoassay for quantification of aggrecanase-generated ARG5 aggrecan fragments in serum

Yi He a,*, Kamilla E. Jensen b, Anne Sofie Siebuhr a, Morten A. Karsdal a, Jonathan Larkin c, Anne C. Bay-Jensen a

a ImmunoScience, Biomarker and Research, Nordic Bioscience, Herlev, Denmark
b Assay Qualification and Validation, Biomarker and Research, Nordic Bioscience, Herlev, Denmark
c Novel Human Genetics Research Unit, GlaxoSmithKline, Collegeville, PA, USA

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ABSTRACT

Objective: Cartilage degradation is a hallmark of osteoarthritis (OA). Aggrecan, a major proteoglycan of articular cartilage extracellular matrix (ECM), is degraded by ADAMTS-5 resulting in the release of ARG5-G2 fragments to synovial fluid and circulation. The aim was to quantify ARG5-G2 in the serum of OA patients using the huARGS immunoassay.

Methods: The immunoassay was produced under GMP conditions and the technical performance was assessed. The biological relevance of the immunoassay was assessed in the conditioned media from a bovine full-depth cartilage explant (BEX) model. The diurnal and inter-day variations of ARG5 levels were evaluated in OA patients’ serum.

Post-hoc analysis of huARGS was conducted in a sub-cohort of a phase III OA trial testing the safety and efficacy of oral salmon calcitonin.

Results: Technical performance: huARGS demonstrated good technical performance. Biological relevance: ARG5 release was induced by inflammatory factors stimulation compared to the vehicle group, reaching a peak at day 3 and gradually decreasing to base level at day 12. The ARG5 release was suppressed by the addition of the ADAMTS-4/-5 activation inhibitor. Biological variation: No significant diurnal or inter-day effect was found. Phase III clinical trial: The participants in the lowest group (Q1) of baseline huARGS levels were more likely to progress radiographically than the highest group (Q4): OR 3.38[0.81-14.02].

Conclusions: The huARGS shows good technical performance and low biological variation. It has the potential to aid drug development in various stages, both as a PD biomarker and identifying progressors who might be likely to respond to an OA drug.

1. Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by cartilage degradation, osteophyte formation, subchondral sclerosis, and synovial hyperplasia [1]. It is one of the leading causes of chronic pain and old age disability and also affects the quality of life [2]. As an age-associated disease, OA poses a huge economic burden to societies with an aging population. Currently, there are no disease-modifying OA drugs (DMOADs) approved to slow down, stop, or reverse the course of the disease.

The cellular and molecular basis of OA initiation and rate of progression remain poorly understood. Proteolysis of aggrecan, a major component in the extracellular matrix of cartilage, is an early and critical feature of cartilage degradation [3]. Aggrecan is present as aggregates and is critical for the structure and proper functioning of cartilage. Its core protein is composed of three globular domains (G1, G2, and G3) and a small interglobular domain (IGD) between G1 and G2, and a large interglobular domain between G2 and G3, where glycosaminoglycan (GAG) chains attach [4]. Aggrecan in cartilage is degraded amongst others by A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), in particular, ADAMTS-4 and ADAMTS-5 that are upregulated and activated in OA [5-7]. One of the well-described ADAMTS-4/5 cleavage sites is at 373E–374A in the IGD domain resulting in the release of the N-terminal neoepitope ARG5. This cleavage is viewed as being extremely detrimental to cartilage functions as GAG chains are liberated from the core protein, therefore, leading to a reduced...
size of aggrecan [8]. The presence of increased ARGS fragments in synovial fluid or circulation can therefore serve as a biomarker of cartilage destruction in OA [9].

The quantification of aggrecan fragments carrying ARGS neopeptide has been of great interest. A sandwich ELISA assay was developed by Struglics et al. to detect and quantify ARGS fragments in the conditioned medium and human synovial fluid [10]. This assay was also applied to quantify ARGS fragments in synovial fluid and they found that its levels were increased in arthritis, OA and after knee injury compared to the reference group [11]. Later the same group found an association between synovial fluid levels of ARGS fragments and radiographic progression in knee OA by using an improved sandwich assay on a Meso Scale Discovery (MSD) platform [12]. Furthermore, this MSD ARGS assay was validated and measured in more complex biological matrices such as blood and urine from OA patients [13,14]. Another group has developed a similar sandwich ARGS assay using a different anti-ARGS neopeptide antibody BC3-C2 and also quantified ARGS fragments in human serum and urine [15].

We report here that a highly sensitive and robust immunoassay, huARGS was re-established. The immunoassay was manufactured under Good Manufacturing Practices (GMP) and fully validated as per the Food and Drug Administration guidelines [16]. The ARGS-G2 fragments were released in response to catabolic factors’ stimulation and when the activation of ADAMTS-4/5 was blocked by adding the anti-ADAMTS-5 nanobody M6495 [17,18]. In support, data presented at the American College of Rheumatology annual meeting in Atlanta (US) in 2019, has shown that huARGS was time- and dose-dependently reduced in response to M6495, when measured in serum samples of the single ascending dose study testing the safety and tolerability of the nanobody (manuscript submitted and in review) [19].

2. Materials and methods

All chemicals were obtained from either Sigma-Aldrich or Merck Millipore unless other stated. The streptavidin pre-coated ELISA plates were purchased from Roche (Mannheim, Germany). All ELISA plates were analyzed with the reader from Molecular Devices, SpectraMax M (CA, USA).

2.1. huARGS immunoassay

We developed a sandwich ELISA, huARGS, using a chemiluminescence substrate for measuring ARGS-G2 fragments. F78 against the conformational G2 domain was employed as a capture antibody and OA-1 (licensed for use by GlaxoSmithKline) targeting ARGS neopeptide was used as a detection antibody (see Fig. 1 for the schematic diagram of huARGS assay). Aggrecan from bovine cartilage (A1960-1 MG, Sigma-Aldrich) digested by human recombinant ADAMTS-5 (2198-AD, R&D system) was used as a calibrator.

The assay was manufactured according to GMP and technically validated as follows: Inter- and intra-assay variations were determined by using five samples in six runs spanning three days. Accuracy was defined as the agreement between the measured value and the true value and was performed in six runs using a pre-defined quality control panel (QC1-QC4). The lower limit of blank (LLOB) was the concentration expected to be found when 60 replicates of a blank sample (buffer only) were tested. The lower limit of quantitation (LLOQ) was defined as the lowest concentration at which the analyte was reliably quantified with acceptable precision. LLOQ was determined by the use of the low-level human serum samples. The upper limit of quantitation (ULOQ) was the highest standard concentration of the assay within acceptable precision of back-calculated concentrations. Parallelism aimed to demonstrate that the sample dilution response curve was parallel to the standard concentration curve. Parallelism was tested using three serum samples from healthy donors and seven serum samples from subjects with OA in two-fold serial dilutions. Selectivity was defined as the ability to assess the analyze of interest in the presence of interference substances (bikunin, hemoglobin, and intralipid). Selectivity was performed on 10 serum samples measured in duplicates.

The measurement range was between LLOQ and ULOQ. The kit stability was tested by storing the complete assay at 4 °C, 20 °C, and 37 °C for 4 h. Recovery (Re%) of the highest standard signal, kit controls, and QCs in percentage against the reference was calculated. The freeze/thaw stability of the kit was investigated after 3 freeze/thaw cycles. The effect of temperature for the short term on the stability of analyte was tested by incubating serum samples for 2hr, 4hr, 24hr, and 48hr at 4 °C and 20 °C. The recovery percentage (Re%) for each sample was determined against the non-stressed samples.

2.2. Assay setup

A 96-well streptavidin pre-coated microplate (cat#655995, White plate, Greiner Bio-one) was coated with 100 µL of biotinylated F78 diluted to 2 µg/mL in PBS-BTB buffer (10 mM phosphate-buffered saline with 1% bovine serum albumin (BSA) and 0.1% tween-20) for 30 min at 20 °C on a shaker (300 rpm). After five washes with wash buffer (20 mM Tris, 50 mM NaCl, pH 7.2), plates were incubated with 20µL/well of duplicates of standards (made by complete ADAMTS-5 digestion of bovine aggrecan), kit controls, or serum samples, followed by 100µL/well of HRP-OA-1 diluted to 35 ng/mL in assay buffer (10 mM PBS-BTB, 0.1% tween-20, 5% Liquid II) (Roche Diagnostics, Germany). Thereafter the plate was incubated overnight (20 ± 1 hr) at 4 °C. After five washes, 100µL/well of pre-mixed Lumi-phos HRP solution A + B (cat#PSA-1000, LUMIGEN) was added to the plate. The light emission was then read at 450 nm with 650 nm as reference. A 5-Parameter logistic (5-PL) model was used for curve fitting.

2.3. Bovine full-depth cartilage explants (BEX)

To assess the biological relevance of huARGS, bovine full-depth cartilage explants were set up. The ex vivo model is well-characterized and has been described in detail previously [20,21]. To mimic the breakdown of cartilage in OA disease, the explants were stimulated with catabolic factors: TNF-α (cat. 210-RA, R&D system) plus Oncostatin M

![Fig. 1. The schematic diagram for aggrecan and the antibody binding regions in huARGS immunoassay. ARGS neopeptide is generated by ADAMTS-4/5. Biotinylated F78 was used as a capture antibody and HRP conjugated OA-1 was used as a detection antibody.](image-url)

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2.4. Study participants

Human biological samples were obtained with written informed consent from all participants.

2.4.1. The GSK cohort

Intra- and inter-day variation was evaluated in a mild/moderate OA group (n = 20) recruited from the GSK clinical unit (Cambridge, UK) (Registry No. 10/H0301/61, GSK ADM114261) [14]. Confirmation of knee OA was confirmed by X-ray radiography. Serum samples were collected on 3 visits in this cohort. On visit 1st, subjects provided serum samples at 5-time points throughout the day between 08:00 to 18:00. Single blood was donated by subjects at 09:00 on visits 2nd and 3rd. The time interval between visits was approximate 1-2 weeks.

2.4.2. The SMC sub-cohort

The sub-cohort recruited from one study center (Aalborg, Denmark) was retrieved from a randomized, double-blind, multi-center, placebo-controlled phase III clinical trial SMC021-C2301 (clinicaltrial.gov: NCT00486434) evaluating the safety and efficacy of oral salmon calcitonin (sCT) in the treatment of subjects with knee OA [22]. Both knees were examined during the study, but a signal knee was chosen before randomization to fulfill the criteria of the American College of Rheumatology (ACR): Kellgren and Lawrence Grades 2-3 at the medial tibiofemoral joint; classification by American Rheumatism (ARA) criteria as Functional class I, II or III; A JSW ≥2.0 mm at medial tibiofemoral joint measured on the X-ray; a WOMAC pain subscale (5 questions) ≥150 mm; and/or a WOMAC function subscale (17 questions) ≥510 mm. Serum samples and radiographs were taken at the time of entry and 2-year follow-up. One-thousand-one-hundred-and-seventy-six subjects in total participated in the phase III study, while the sub-cohort comprised 200 participants. Due to fifty-one subjects missing X-ray radiography at a 2-year follow-up and four subjects missing sample serum at baseline, huARGS was measured in serum samples of 145 subjects from the sub-cohort (Fig. 2). The demographic characteristics of the sub-SMC cohort participants are presented in Table 2.

2.5. Statistical analysis

The non-parametric Mann-Whitney U test was used to compare the huARGS levels between T + O and WO groups on day3. Paired one-way ANOVA was used to analyze the difference in the mean of huARGS among different time groups or day groups. The difference was considered significant if p < 0.05, and displayed as *; if p < 0.01, and displayed as **; if p < 0.001, and displayed as ***; if p < 0.0001, and displayed as ****. Statistical analysis and graphical illustration were performed using GraphPad Prism 8.

3. Results

3.1. The technical performance of huARGS

A five-parameter logistic (5-PL) curve fitting was used for the quantitative analysis of samples. The full validation data are listed in Table 1. Precision: Intra-assay variation was 1.7-2.1%. Inter-assay variation was 4.8-9.6%. Accuracy: The mean recovery (%RE) for the four QC samples (QC1-4) was 8.025. Parallelism: CV% in healthy serum samples was 8.2-24.8%, and in serum samples from OA subjects were 4.6-64.6% Selectivity: No interference observed up to 90 ng/L of Biotin, 5 mg/mL.

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Fig. 2. The flow diagram for subjects included in the post-hoc analysis of huARGS: the SMC sub-cohort, 1176 subjects in total participated in the phase III SMC study at baseline, while the sub-cohort (Alborg site) comprised 200 participants. Due to fifty-one subjects missing X-ray radiography at 2-year follow-up and four subjects missing sample serum at baseline, huARGS was measured in serum samples of 145 subjects from the sub-cohort.
In the current study, we re-developed biomarker assay huARGS as a highly sensitive and robust chemiluminescence immunoassay. To our knowledge, it is the first version of the biomarker that was manufactured in compliance with GMP and technically validated for quantifying ARGS fragments in serum. The huARGS profiling in BEX treated by catabolic factors or a potential ADAMTS-4/5 activation inhibitor confirmed the link between ARGS neoepitope generation and ADAMTS-4/5 activity. Unlike type II collagen biomarkers, especially serum type II collagen carboxyterminal propeptide (sCPII), urinary type II collagen neoepitope (uCTX-II), and urinary c-terminal cross-linked telopeptide (uCTX-III), have been shown diurnal variation. No significant within-day and day-to-day variation in serum samples of OA patients were observed. Lack of diurnal and inter-day fluctuation also has been reported in another ARGS biomarker by other authors [24]. Therefore, the time of sample collection
during the day doesn’t have a significant impact on the huARGS levels, which is important information for clinical practice. In addition, low baseline levels of huARGS provide the potential to predict radiographic progression of OA over two years in a post hoc analysis in a subcohort from a phase III OA clinical trial.

There is a large unmet medical need for DMOADs for OA [25]. The currently available drugs for OA mainly focus on pain management. More treatment options that target the underlying disease pathogenesis and provide sustainable clinical benefits are needed for patients with OA. Potential therapeutics that target the catabolic pathways in OA are under development [26–28]. In our previous study, huARGS levels were dose-dependently reduced by M6495, a nanobody specifically inhibiting ADAMTS-5 in ex vivo cartilage models [18]. In line with this finding, huARGS levels were blocked by an ADAMTS-4/-5 activation inhibitor in a similar ex vivo cartilage model in the present study. In general, ADAMTS-5 is a key player in aggrecan catabolism during OA, and therefore a potential drug target for DMOADs [29]. Five cleavage sites on aggrecan by ADAMTS-5 have been found so far, one within the IGD domain and four in the C-terminal chondroitin sulfate-rich (CS) domain [30]. The cleavage site (373E-374A) in the IGD domain plays a fundamental role in cartilage degradation due to the loss of glycosaminoglycan (GAG) side chains. The other four cleavage sites toward the CS region leave aggrecan still functional within the tissue. A study from mice study has shown that knock-in mice bearing a mutated IGD domain are protected from aggrecan loss and cartilage erosion in the surgical model of OA [31], indicating blocking aggrecanase cleavage in the IGD domain has beneficial effects. HuARGS measuring the ARGS fragment might be utilized as a pharmacodynamic biomarker for evaluating the target engagement of anti-ADAMTS-5 inhibitors. Monitoring all these cleavage sites by using neo-epitope-specific antibodies may help to better understand aggrecanolysis in OA pathogenesis and cartilage homeostasis. However, developing immunoassays for the other four cleavage sites would be more difficult than huARGS, because measuring analytes with sugar chains will encounter new challenges.

As a product of ADAMTS-5 enzymatic activity, ARGS as a biomarker has been mainly studied in the synovial fluid where its levels have been observed to increase in acute inflammatory arthritis, acute knee injury, and OA compared to the reference group [9], and be associated with...
radiographic progression in knee OA\cite{12}. However, due to the difficulty of collecting synovial fluid, there has been growing interest in the quantitation of ARGS concentrations in other biological fluids, such as serum. According to a previous study on 36 subjects at different time points after a knee injury, the ARGS concentrations were highest in synovial fluid, 20 times lower in the matched blood samples, and 80 times lower in the matched urine samples \cite{13}. Thus, it has been extraordinarily challenging to determine the levels of ARGS in systemic circulation because of its low levels and therefore high sensitivity and accuracy for the assay are required. In the current study, the assay was validated taking into account the Food and Drug Administration (FDA) guidance for bioanalytical method validation \cite{16}.

Larsson et al. have reported that low levels of synovial fluid ARGS were associated with the progression of radiographic knee OA defined by JSN and or osteophyte grades \cite{12}. Similarly, low levels of blood ARGS provided an odds ratio (OR) of 3 for the identification of fast radiographic progressors (JSN$\geq$0.5 mm) over 2 years in the present study. A possible explanation for this could be that the lower serum levels reflect a lower aggreganasc activity, but also a lower tissue-formation/repair capacity as measured by a type II collagen formation biomarker, PRO-C2, in our previously published paper \cite{32}. We speculated that there is a subgroup of OA indicated by lower serum ARGS levels in combination with lower PRO-C2 levels that are likely to progress radiographically and deteriorate over time. OA is a relatively slowly progressive disease and above 50% of the participants in clinical trials don’t undergo structural progression with sufficient sensitivity to be detected \cite{33}. The huARGS biomarker combined with formation biomarkers has the potential to facilitate the stratification of patients at high risk of rapid progression of OA. Finding these patients will enable a more efficient DMOAD trial design \cite{34–36}.

There are some limitations to this study. First, it includes a relatively small sample size per cohort. The findings of baseline ARGS as a prognostic biomarker for the rapid structure progression of OA from the subcohort would need to be verified in the whole phase III SMC cohort. Second, additional clinical samples to determine circadian variation if any in the levels of serum ARGS are needed. Third, the investigation of matched synovial fluid and serum samples to determine the correlation between local and systemic levels of ARGS is needed.

To summarize, we have re-developed a highly sensitive and reliable huARGS immunoassay which was produced under GMP and fully validated according to the FDA guidance. The huARGS has shown its potential as a PD biomarker for drug efficacy and a biomarker for identifying patients at risk of rapid structure progression and most likely to respond to a DMOAD treatment.

Author contributions

MAK, ACBJ, JLA, and YHE were in charge of the conception and design of the study. YHE carried out the key experiments and took the leading role in writing the manuscript. KEJ was responsible for the validation of huARGS. ASS was involved in the early assay development of huARGS. Moreover, JLA provided a guide on the OA-1 cell line culture and contributed to the preparation of the GSK ADM114261 cohort. All authors have reviewed and approved the version to be submitted.
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Declaration of competing interest

MAK, ACBJ are full-time employees and shareholders of Nordic Bioscience A/S. YH, KEJ and ASS are full-time employees at Nordic Bioscience A/S. JLA is a full-time employee and shareholder of GlaxoSmithKline.

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