Inflammatory and oxidative stress biomarkers in alkaptonuria: data from the DevelopAKUre project

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Abstract

Objective: The aim of this work was to assess baseline serum levels of established biomarkers related to inflammation and oxidative stress in nearly 200 serum samples from AKU subjects enrolled in SONIA1 and SONIA2 clinical trials (DevelopAKUre project).

Methods: Levels of Serum Amyloid A (SAA), IL-6, IL-1β, TNFα, CRP, cathepsin D, IL-1ra, and MMP-3 were determined through commercial ELISA assays. Chitotriosidase activity was assessed through a fluorimetric method. Adavnced Oxidation Protein Products (AOPP) were determined by spectrophotometry. Thiols, *S*-thiolated proteins and Protein Thiolation Index (PTI) were determined by spectrophotometry and HPLC. Patients' quality of life was assessed through validated questionnaires.

Results: We found that SAA serum levels were significantly increased compared to reference threshold in 57.5% and 86% of the analysed samples in SONIA1 and SONIA2, respectively. Similarly, chitotriosidase activity was above the reference range in half of the tested SONIA2 samples, whereas CRP levels were increased only in a minority of the tested AKU subjects. AOPP, thiols, *S*-thiolated protein and PTI showed no differences from control population. We provided evidence that AKU patients presenting with significantly higher SAA, chitotriosidase activity and PTI reported more often a decreased quality of life. This suggests that worsening of symptoms in AKU is paralleled by increased inflammation and oxidative stress, which might play a role in disease progression.

Conclusions: Monitoring of SAA may be suggested in AKU to evaluate inflammation. Though further evidence is needed, SAA, chitotriosidase activity and PTI might be proposed as disease activity markers in AKU.

Keywords: Amyloidosis; Biomarker; Chitotriosidase; Protein thiols; Serum; Serum amyloid A

1 Introduction

Alkaptonuria (AKU) is a rare autosomal recessive metabolic disorder (MIM 203500) causing an early onset, chronically debilitating spondylo-arthropathy due to high circulating homogentisic acid (HGA, 2,5-dihydroxyphenylacetic acid) [1]. Accumulation of HGA is due to mutations of the *HGD* gene causing the production of a defective HGD enzyme in tyrosine and phenilalanine catabolic pathways [2]. Excess HGA is partly eliminated in the urine, partly contributes to the production of an ochronotic pigment deposited in cartilagineous tissues, which leads to a range of clinical manifestations. AKU causes considerable morbidity in adulthood, and cases of acute fatal metabolic complications (oxidative haemolysis and/or methaemoglobinaemia) were reported [3]. So far, no correlation between genotype and HGA circulating levels has been found.

AKU still lacks appropriate biomarkers to monitor progression excepting for an AKU Severity Score Index (AKUSSI) [4]. The use of nitisinone (NTBC) was suggested in AKU to lower circulating HGA levels, and clinical trials were undertaken in Europe (DevelopAKUre - Clinical Development of Nitisinone for Alkaptonuria) [5]. Recent evidence pointed out also that AKU is a multisystem disease involving secondary (AA) amyloidosis due to high circulating Serum Amyloid A (SAA) promoting inflammation, oxidative stress and amyloidosis [6, 7]. The presence of SAA and Serum Amyloid P (SAP) in *in vitro* and *ex vivo* AKU models highlighted the amyloid nature of ochronotic pigment [6, 8, 9]. So far, AA amyloid has been reported in AKU in several tissues:

a. cartilage [9-11]
b. synovia [9, 11]
c. cardiac valve [8, 12]
d. salivary gland [11]

and high circulating levels of SAA have been found in a small cohort of Italian AKU patients [6, 8, 9, 13]. Furthermore, HGA-induced oxidative stress was highlighted in AKU [6-8, 13-19].

In this framework, we undertook this work to monitor the presence of established biomarkers related to inflammation and oxidative stress in serum of a high number of AKU subjects who were/are enrolled in DevelopAKUre clinical trials.

2 Material and Methods

2.1 Samples

This study was carried out as a part of the inflammatory and oxidative marker analysis of DevelopAKUre project [5] for SONIA1 (Suitability of Nitisinone in Alkaptonuria 1), and SONIA2 (Suitability of Nitisinone in Alkaptonuria 2) clinical studies. In SONIA1, serum samples were collected from 40 AKU subjects under fasting conditions at baseline (i.e., when they first entered the study) at the investigative sites of Liverpool (UK) and Piešťany (SK). Details on inclusion/exclusion criteria can be found in [5]. Serum samples from healthy volunteers were collected at Siena University Hospital and used as controls. Demographics of SONIA1 AKU and control cohorts are reported in **Table 1S**.

In SONIA2, serum samples were collected from 138 AKU subjects under fasting conditions at baseline at the investigative sites of Liverpool (UK), Piešťany (SK) and Paris (F). Demographics of SONIA2 patients are reported in **Table 2S**.

2.3 ELISA

Assays for pro-inflammatory markers were carried out by means of commercial ELISA kits according to manufacturer's instruction, as follows: SAA (KHA0012), IL-1β (KHC0011), IL-6 (KHC0062); TNFα (KHC3013), CRP (KHA0031), MMP-3 (KAC1541) (all from Invitrogen-Life Technologies), CATD (ab119586, abcam), IL-1ra (KAC1181, BioSource Europe). Plates were read on a VersaMax microplate reader (Molecular Devices) using Ascent software (Thermo Scientific). Quantification of analytes was obtained against polynomial standard curves generated with appropriate standards.

2.4 Laboratory tests

Cholesterol and triglycerides were determined through an enzymatic colorimetric method, and HDL-cholesterol and LDL-cholesterol were determined through a homogeneous enzymatic colorimetric method on a Cobas® 6000, Roche/Hitachi cobas c system. Serum HGA levels were previously determined in [5].

2.5 Serum chitotriosidase activity assay

Chitotriosidase activity was determined according to [20]. Briefly, 2.5 µL of serum were incubated with 50 µL of 22 µM 4-methylumbelliferyl-l-β-D-N,N',N''-triacetylchitotriose (Sigma) in McIlvain's phosphate-citrate buffer (pH 5.2) for 1 hour at 37°C. Reactions were terminated by adding 1.4 mL of 0.2 M glycine buffer (pH 10.8); fluorescence of 4-methylumbelliferone was read in a fluorimeter (Perkin Elemer; excitation 365 nm, emission 435 nm).

2.6 Advanced Oxidation Protein Products

AOPP were measured according to [21] by spectrophotometry on a microplate reader (VersaMax, Molecular Devices) using Softmax Pro software (Molecular Devices). Calibration was performed with chloramine-T (Sigma) solutions that in the presence of potassium iodide absorb at 340 nm. Blank wells were prepared with 200 µL of PBS; standard wells were prepared with 200 µL of chloramine-T solution (range 5–100 µmol/L); test wells were prepared with 200 µL of serum samples diluted 1:10 or 1:20 in PBS. Then, 10 µL of 1.16 M potassium iodide (Sigma) was added

to each well followed 2 minutes later by bolus addition of acetic acid (20 µL). The chloramine-T absorbance was immediately read at 340 nm; being linear within the range of 0 to 100 µmol/L, AOPP concentrations were expressed as micromoles per litre of chloramine-T equivalents.

2.7 Thiols, S-thiolated proteins and Protein Thiolation Index (PTI)

Quantitative determination of free thiols and *S*-thiolated proteins (used to calculate PTI) in serum samples was carried out according to [22]. Briefly, one aliquot of serum (0.03 mL) was used to measure thiol levels by colorimetric reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [23]. One additional aliquot of serum (0.1 mL) was treated with 0.1 mL of 2 mM N-ethylmaleimide (NEM, dissolved in 0.2 M phosphate buffer pH 7.4) for 2 min and then deproteinized by addition of 18 µl of 60% (w/v) trichloroacetic acid (TCA). This second aliquot of serum was used to measure the level of *S*-thiolated proteins.

The content of mixed disulfides between low molecular mass thiols and protein thiols (*S*-thiolated proteins) was determined by HPLC after release of the protein-bound thiols with dithiotreitol (DTT) and their labeling with monobromobimane (mBrB) [24]. The protein pellet obtained by centrifugation at 10,000g for 2 min was washed three times with 1.5% (w/v) TCA, in order to remove excess NEM and free low molecular mass thiols. Then, it was resuspended by gyratory shaking with 400 µl of 1 mM K_3EDTA containing 16 µl of 50 mM DTT and 15 µl of 2 M Tris [15]. Supernatants (0.1 mL) were then spiked with 15 µl of 40 mM mBrB and brought to a pH of 8.0 using 20 µL of 2 M Tris. After a 10-min incubation in the dark, samples were acidified with 1% (v/v, final concentration) HCl and loaded onto HPLC. HPLC separation was performed on a C18 column (Zorbax Eclipse XDB-C18, 4.6 mm 150 mm, 5 mm, Agilent Technologies). Elution conditions were as follows: solvent A, sodium acetate 0.25% (v/v), pH 3.09; solvent B, acetonitrile; 0–5 min, 94% solvent A/6% solvent B; 5–10 min linear gradient from 6% to 100% solvent B. A constant flow rate of 1.2 mL/min was applied. Detection was performed at 390 nm excitation and at 480 nm emission wavelengths [15]. All measurements were carried out with an Agilent series 1100 HPLC.

PTI was calculated as the molar ratio between total *S*-thiolated proteins (RSSP, where RS is usually cysteine, cysteinylglycine, homocysteine, γ-glutamylcysteine and glutathione) and the concentration of free, DTNB-titrable protein thiol groups [15].

2.8 Patients' health questionnaires

In SONIA2, quality of life of AKU patients was assesses through the following validated questionnaires:

- Knee injury and Osteoarthritis Outcome Score (KOOS), evaluating both short- and long-term consequences of knee injury. It holds 42 items in five separately scored subscales [pain, other symptoms, function in daily living, function in sport and recreation, and knee-related quality of life (QoL)]. Scores are normalized to a "0–100" scale, with "0" representing extreme knee problems and "100" representing no knee problems.
- Health Assessment Questionnaire (HAQ), including a disability index (haqDI) and a global pain visual analog scale (hapVAS). Eight categories are assessed: dressing and grooming, arising, eating, walking, hygiene, reach, grip, common daily activities. Results are scored from 0 (no difficulties) to 3 (unable to do).
- Short Form-36 (SF-36), a multi-purpose short-form with 36 questions addressing both physical and mental status that measures patients' QoL across eight domains: vitality, physical functioning, bodily pain, general health perception, physical role functioning, social functioning, emotional role functioning, mental health. A score of "0" indicates maximum disability, while a score of "100" indicates no disability.
- AKUSSI, which incorporates multiple, clinically meaningful AKU outcomes combined with medical photography imaging investigations, and detailed questionnaires into a single score [4]. In this work, we limited to non-spine rheumatology (pain in 14 joints) and spine rheumatology (pain in four clinical spine regions) scores, expressed as percentages.

These scores were used to undertake correlation analyses with the measured markers, as detailed below.

2.9 Statistical analysis

Results were processed through Excel and GraphPad 6.0. Normal distribution was analysed with D'Agostino-Pearson or Shapiro Wilk test depending on sample size, and summary statistics was

obtained for each analysed dataset. Mann-Whitney, Kruskal-Wallis followed by Dunn's multiple comparisons, and Spearman's rank correlation analysis were used as appropriate.

3 Results

The overall aim of this work was to assess baseline levels of established biomarkers related to inflammation and oxidative stress in serum from alkaptonuric patients who were/are enrolled in DevelopAKUre clinical trials. The tested biomarkers included well-known mediators of inflammatory responses (IL-6, IL-1β, TNFα and CRP) and SAA, which play also a role in inflammation, oxidative stress, and secondary (AA) amyloidosis. Serum levels of the following biomarkers were also tested: cathepsin D (CATD), a lysosomal aspartic protease taking part in intracellular digestion of proteoglycan in the initial stages of osteoarticular inflammation [25] and involved in degradation of SAA, preventing amyloid deposition [26]; IL-1 receptor antagonist (IL-1ra), which is specific for preventing the activity of IL-1α and IL-1β by competing with IL-1α and IL-1β for binding to the ligand-binding chain, termed type I (IL-1RI); metalloproteinase 3 (MMP-3), which is involved in extracellular matrix remodelling and whose serum levels are increased in inflammatory rheumatic diseases [27].

AOPP were tested as oxidative stress and potential inflammatory mediators, as they are found in several human diseases where these events are involved, such as chronic renal failure and [21, 28], diabetes mellitus [29], obesity and insulin resistance [30] and their pro-inflammatory activity was demonstrated [31]. Free serum protein thiols (PSH), *S*-thiolated proteins, and PTI were measured to assess oxidative stress.

3.1 SONIA1

The majority of AKU patients (23/40; 57.5%) enrolled in SONIA1 presented with SAA levels above the reference threshold of 10 mg/L [32]; conversely, only a minority (7/40; 17.5%) had CRP levels above the reference limit (**Table 1**). All the other tested inflammatory markers were not statistically different (CATD, IL-1ra, TNFα, and MMP-3) or were slightly lower in AKU (IL-1β, P=0.011 and IL-6, P=0.046) compared to a control age-matched healthy population (**Table 1**). Routinely assessed

hematological parameters such as: glucose, cystatin C, alkaline phosphatase (data not shown), cholesterol, tryglicerides and LDL-cholesterol (**Table 3S**) were generally in range, whereas HDL-cholesterol scored below the reference range in 90% of the tested AKU subjects (**Table 3S**).

[suggested position for Table 1]

The possible dependence of the tested inflammatory biomarkers from age, BMI, smoking and drinking habits, gender and site of sample collection was evaluated. No differences according to gender or cigarette smoking habits could be highlighted (**Table 2**). As for the other confounding factors, we found that CATD serum levels were significantly increased in subjects drinking alcohol ($P<0.0001$) and that IL-1ra, TNFα and CRP serum levels were higher in overweight/obese subjects compared to those with a normal BMI (**Table 2**). Interestingly, there were also some biomarkers that showed a different distribution according to the clinical site: CATD ($P<0.0001$), IL-1β ($P=0.005$) and MMP-3, ($P<0.0001$). Since AKU patients enrolled in Liverpool were from different European nations, a common trait in different lifestyle or eating habits explaining such a difference could not be identified yet [33]. Due to the chronic and progressive nature of AKU, several AKU patients enrolled in SONIA1 presented with concomitant pathologies and/or reported the use of concomitant medications. The possible effect of such concomitant medications on the levels of the tested biomarkers was ruled out (**Figure 1S**). A positive and significant correlation was found for SAA and CRP (**Table 3**), and several inflammatory biomarkers were positively correlated to BMI (SAA, IL-6, IL-1ra, TNFα and CRP). Conversely, none of the tested biomarkers was correlated to serum HGA levels (**Table 3**).

[suggested position for Table 2 and Table 3]

3.2 SONIA2

SAA was the only marker, among those tested in SONIA1, that was measured also in SONIA2. Chitotriosidase activity was included as an additional marker of non-infectious inflammation [34]. Since increased AOPP [13] and PTI [15] were reported previoulsy in smaller cohorts of AKU patients, AOPP, thiols, *S*-thiolated proteins and PTI were investigated in SONIA2.

AOPP ranged between 1.60-60.12 μmol/dL chloramine T equivalents (mean 12.45 ± 8.57 μmol/dL) and were above the reference value (set at 30 μmol/dL) in six out of the 138 analysed samples

(4%) (**Figure 1A**). No differences were found once AOPP were stratified according to subjects' age (**Figure 1B**), sex (**Figure 1C**) or BMI (**Figure 1D**). No significant correlation was found with age (r=0.08913, P=0.2985) (**Figure 1E**) or BMI (r=0.1349, P=0.1146) (**Figure 1F**).

[suggested position for Figure 1]

SAA ranged between 1.5-311.9 mg/L (mean value 57.01 ± 64.80 mg/L). Interestingly, SAA serum levels ranged between 3 and 10 mg/L in 18 subjects (13%) and were above the threshold of 10 mg/L [32] in 119 out of the 138 analysed samples (86%) (**Figure 2A**). SAA levels showed no differences once stratified according to subjects' age (**Figure 2B**), or sex (**Figure 2D**), whereas a small but significant difference between underweight and obese AKU subjects was found. Nevertheless, similar ranges were observed for SAA in normal (3.8-311.9 mg/L), overweight (1.5-305.7 mg/L) and obese (9.0-298.6 mg/L) AKU sujects (**Figure 2E**). SAA serum levels were also positively and significantly correlated to subjects' BMI (r=0.3556, P<0.0001) (**Figure 2F**) but not age (r=0.1268, P=0.1382) (**Figure 2C**).

[suggested position for Figure 2]

Chitotriosidase activity ranged between 8.2-187 nmoL/mL/h (mean value 60.32 ± 33.87 nmoL/mL/h) and was above the reference value (set at 51 nmoL/mL/h) in 72 out of the 138 tested samples (52%) (**Figure 3A**). Increasing chitotriosidase activity was observed stratifying patients according to their age (**Figure 3B**) and a positive correlation was found with age (**Figure 3C**). Conversely, no differences were observed according to sex (**Figure 3D**) or BMI classification (**Figure 3E**), and no correlation was found with BMI (**Figure 3F**).

[suggested position for Figure 3]

Levels of free thiols and *S*-thiolated proteins, ultimately combined into PTI, did not differ significantly beween control and AKU subjects (**Figure 2S**). However, a positive and significant correlation was found bewteen PTI and AKU subjects' age [**Figure 4(B)**], and PTI values were statistically different when stratified according to age [**Figure 4(A)**]. Conversely, no differences were observed according to sex [**Figure 4(E)**] or BMI classification [**Figure 4(C)**], and no correlation was found between PTI and BMI [**Figure 4(D)**].

[suggested position for Figure 4]

Concomitant medications were not found to alter significantly the levels of the tested markers (Figure 3S).

When inflammatory and oxidative marker levels were correlated to the outcomes of health questionnaires, we found weak but statistically significant correlations indicating that high levels of SAA were more frequently associated both to a higher degree of difficulties in sport activities as well as to a reduced perceived knee-related quality of life (KOOS questionnaire). Similarly, patients with high PTI and chitotriosidase activity reported more frequently an increased severity of pain and symptoms, difficulties in daily activities and sport, and a reduced perceived knee-related quality of life (KOOS questionnaire) (**Table 4**). We also found that high serum levels of SAA, PTI and chitotriosidase activity were more frequently associated to an increased perception of disability (haqDI, HAQ questionnaire) and to a reduced perceived physical health (i.e., lower levels of functioning according to SF-36) (**Table 4**). Higher PTI values were positively associated to pain in multiple spine regions, and higher chitotriosidase activity was positively associated to joint and spinal pain (AKUSSI questionnaire) (**Table 4**). Positive correlations were also found between PTI-SAA ($r=0.187$, $P=0.032$) and PTI-chitotriosidase ($r=0.392$, $P<0.0001$).

[suggested position for Table 4]

4 Discussion

Serum represents an excellent and easily accessible source of protein biomarkers that can reflect physiological/pathological conditions [35, 36]. Though AKU represents the iconic prototype “inborn error of metabolism” and shares features with other more common rheumatic diseases, it still lacks appropriate biomarkers to monitor severity and progression. Hence, this work was undertaken with the main aim of analysing levels of established biomarkers related to oxidative stress and inflammation in a large cohort of alkaptonuric patients. Due to the ultra-rarity of the disease (affecting 1:250,000-1,000,000 [1]), we were given an invaluable opportunity, as we were able to test for the very first time a high number of alkaptonuric serum specimens that were collected and stored under standardised procedures (agreed among the involved clinical centres). Our analyses were carried out at baseline, i.e. before randomisation into untreated (control) or treated-arm.

Confirming previous evidence from ours [6-9, 37], the major finding of this study was that SAA seemed the most promising biomarker to be assessed in AKU to monitor inflammation. SAA serum levels were significantly increased compared to reference threshold in the vast majority of samples. A similar trend was observed for another inflammatory biomarker, namely chitotriosidase, whose activity was above the reference range in half of the tested samples. These findings suggest that sub-clinical inflammation may be relevant in AKU and connected with the development of disease-related complications, similarly to other rheumatic conditions where increased SAA levels can be found, such as: osteoarthritis (OA) [38], rheumatoid arthritis [39-43], Familial Mediterranean Fever (FMF) [44, 45], Juvenile Idiopathic Arthritis (JIA) [46], systemic lupus erythematosus (SLE) [43]. Conversely, serum AKU-related oxidative stress markers that were shown to be increased in smaller cohorts of AKU subjects such as AOPP [13] and PTI [15], in this work were not significantly different from a control population.

Since CRP levels were increased only in a minority of the tested AKU subjects, superiority of SAA and chitotriosidase compared to CRP to monitor subclinical inflammation might be suggested in AKU. This is similar to what observed for SAA in patients suffering from FMF [44, 47, 48] and is further supported by recent works where SAA was proposed as a better biomarker than CRP to monitor rheumatic disease activity [41, 46, 49, 50] or response to pharmacological treatment [43, 44, 51].

Additionally, since plasma SAA levels correlate with SAA levels in synovial fluid, passive diffusion of SAA from systemic circulation to synovial joint may be speculated [38]. This is particularly relevant due to the role that SAA might play in joint destruction through induction of metalloproteinases and collagen [41] although different functions have been suggested for systemic and locally-produced SAA isoforms, as well as for acute and constitutive SAA [41]. SAA may thus be considered a mediator of “danger signal” driving inflammatory processes in AKU.

The serum concentration of SAA closely reflects the activity and severity of OA [38], FMF [44, 52], ankylosing spondylitis [50], JIA [46], polymyalgia rheumatica [53] and early RA [54, 55]. We provided evidence that AKU patients presenting with significantly higher SAA and chitotriosidase activity (enhanced inflammation) and higher PTI (enhanced oxidative stress) reported more often a

decreased quality of life (as assessed through patients' health questionnaires) and scored higher in the AKUSSI scale for joint and spinal pain. This suggests that worsening of symptoms in AKU is paralleled by increased inflammation and oxidative stress, which might play a role in AKU progression. Consequently, SAA, chitotriosidase activity and PTI might be proposed as disease activity markers in AKU, although further evidence is needed.

The positive association between SAA and BMI that we found in the tested AKU subjects is not new [41, 56-58] and might be justified by the fact that SAA is expressed both in liver and adipose tissue [56]. In particular, in obesity (where low-grade inflammation is found), adipose tissue is the major source of SAA, which can be considered an obesity-related inflammatory protein [57, 59]. It is known that HDL counter-regulates SAA and other pro-inflammatory mediators [60]. Interestingly, we found that 90% of the tested AKU subjects enrolled in SONIA1 had lower levels of HDL than what established by reference guidelines. Chronic inflammation, as outlined in FMF, RA and SLE subjects [48, 61-63] might alter the structure and functions of HDL, overall impairing HDL properties. In particular, a decreased antioxidant activity of HDL might follow displacement of ApoA-I from HDL due to high SAA. Since altered profiles in apolipoproteins were documented by comparative proteomics of AKU serum [13], this topic deserves further investigations in AKU.

Reactive systemic AA amyloidosis can complicate chronic inflammatory disorders that are associated with a sustained acute phase response. AA amyloid fibrils are derived from the acute-phase reactant SAA through a process of cleavage, misfolding, and aggregation into a highly ordered abnormal β-sheet conformation (amyloid) [32]. Sustained overproduction of SAA is a prerequisite for the development of AA amyloidosis [32].

Persistently elevated SAA levels represent a risk factor for the development of amyloidosis due to deposition of amyloid aggregates in several organs and tissues. However, physiological and pathological functions of SAA are still partly unclear and differences between recombinant and endogenous SAA have been highlighted in *in vitro* assays, probably due to a difference in association to lipids [41, 59]. Pathological SAA serum levels were found to fall within a wide range in the tested AKU subjects. This finding becomes particularly relevant in the light of a very recent

work [64] where HGA was found to act as an amyloid aggregation enhancer *in vitro* (in a time- and dose- dependent fashion) for amyloidogenic proteins and peptides, such as: Aβ(1-42), transthyretin, atrial natriuretic peptide, α-synuclein and SAA. In particular, the pro-aggregating effect of HGA towards SAA was found even at nearly physiological HGA concentrations [64]. Thus, based on the results presented in this work, pharmacological control of SAA circulating levels in AKU seems appropriate to be suggested.

We believe our study presents a number of strengths. Considering the rarity of the disease, the number of tested samples (nearly 200) and biomarkers is noteworthy. Furthermore, homogenous study samples were collected thanks to tight coordination between the involved clinical sites. Lastly, this was the first time that inflammatory and oxidative stress biomarkers could be investigated *in vivo* in AKU. Conversely, since AKU is not life-threating, the presence of concomitant pathologies or medications has to be taken into account due to the chronic/progressive nature of the disease. In this respect, it should be underlined that all the possible confounding factors collected during the studies (smoking, drinking, concomitant use of drugs) were considered in our analysis. All the data obtained within this work could hence be used to populate a dedicated database integrating biomarker levels, demographics, patient's quality of life, environmental and life-style data, and clinical outcomes. Such a database could represent an optimal tool with potential relapses for the study of AKU and the development of a precision medicine approach for AKU and other more common rheumatic disorders [65].

In the light of data presented here showing increased serum SAA in AKU, an appropriate pharmacological treatment able to address this feature of the disease could be suggested as well. Low dose methotrexate (MTX) can down-regulate inflammation acting on several steps triggering and perpetuating inflammation [66]. In particulat, thanks to its ability to lower SAA production, MTX at low dosages is the anchor drug to treat rheumatic diseases and the associated AA amyloidosis [32, 67, 68]. Control of the acute phase response is currently the standard of care in amyloidosis and rheumatic disorders [32, 69]. Efficacy of low dose MTX in lowering several inflammatory

mediators in serum or synovial fluid of RA patients can be observed, associated with prolonged survival, reversal of amyloid deposition and recovery of organ function when SAA concentration are kept below 10 mg/L [55, 70].

5 Conclusions

Increased SAA and chitotriosidase activity were detected in the vast majority of AKU samples, indicating increased systemic inflammation. Conversely, oxidative stress biomarkers were not significantly different when compared to a normal population. SAA, but especially PTI and chitotriosidase activity were correlated to AKU severity, as assessed through validated health questionnaires and AKUSSI, indicating a role for both oxidative stress and inflammation in AKU progression and severity. Prospectively, routine assessment of SAA should be recommended in AKU so that proper interventions could be put in place to address the inflammatory-pro-amyloidogenic component of the disease. This is particularly relevant in view of the recent *in vitro* reports indicating that even nearly physiological HGA concentrations might enhance SAA aggregation [64].

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Authors' contribution

All authors contributed to the conception and design of the study, acquisition, analysis or interpretation of the data. All authors were also involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version. Annalisa Santucci (annalisa.santucci@unisi.it) as the corresponding author, takes responsibility of the integrity of the work as a whole, from inception to finished article.

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7 Competing interest statement

The authors have no conflicts of interest to declare.

Figure legends

Figure 1: AOPP serum levels (expressed as µmoL/dL chloramine T equivalents) at baseline in SONIA2 subjects. M: males; F: females; U: underweight; N: normal; OW: overweight; O: obese.

Figure 2: SAA serum levels (mg/L) at baseline in SONIA2 subjects. M: males; F: females; U: underweight; N: normal; OW: overweight; O: obese.

Figure 3: Chitotriosidase activity (nomL/mL/h) at baseline in SONIA2 subjects. M: males; F: females; U: underweight; N: normal; OW: overweight; O: obese.

Figure 4: PTI values at baseline in SONIA2 subjects. M: males; F: females; U: underweight; N: normal; OW: overweight; O: obese.

Figure 1

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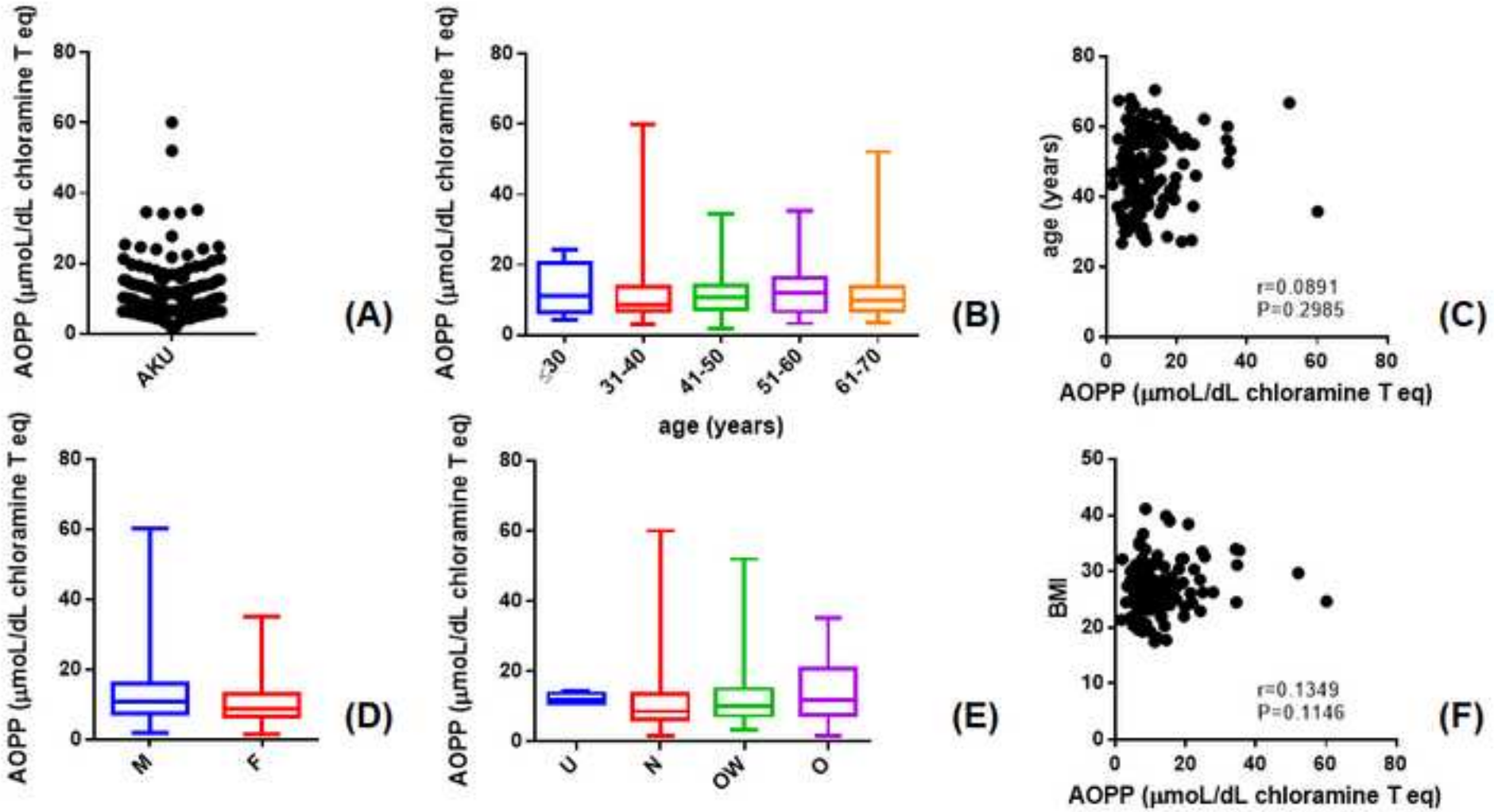

Figure 2

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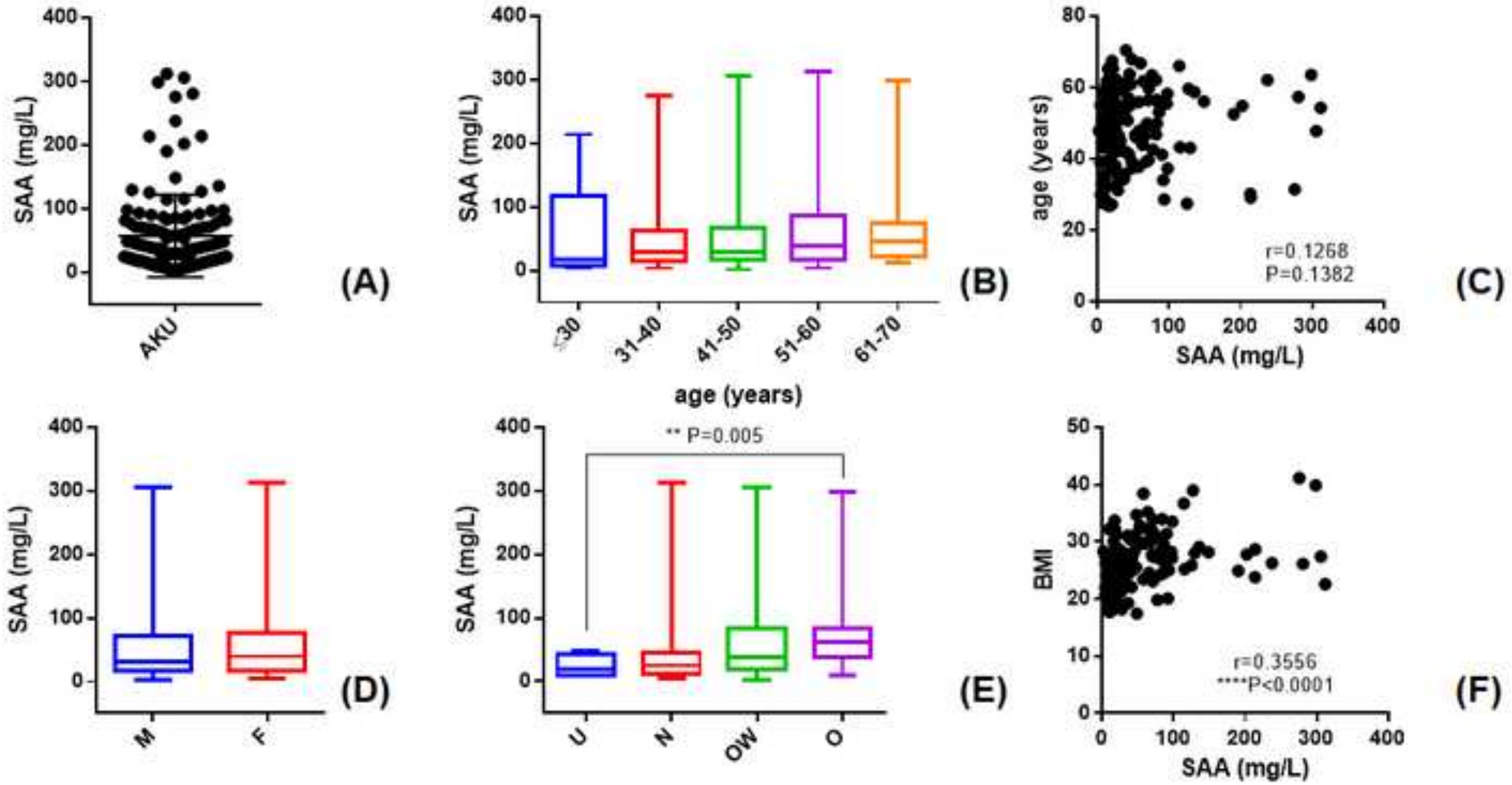

Figure 3

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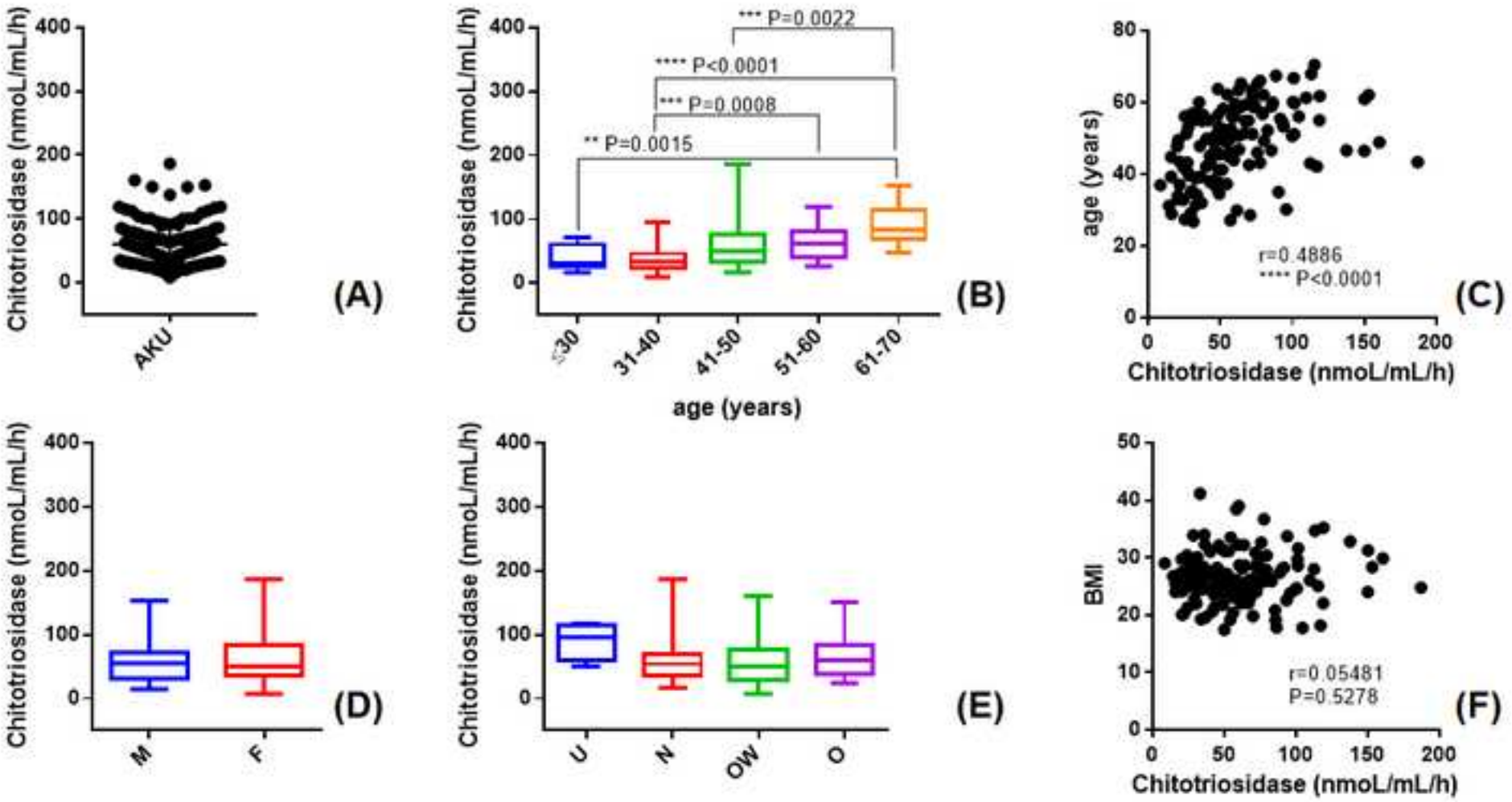

Figure 4

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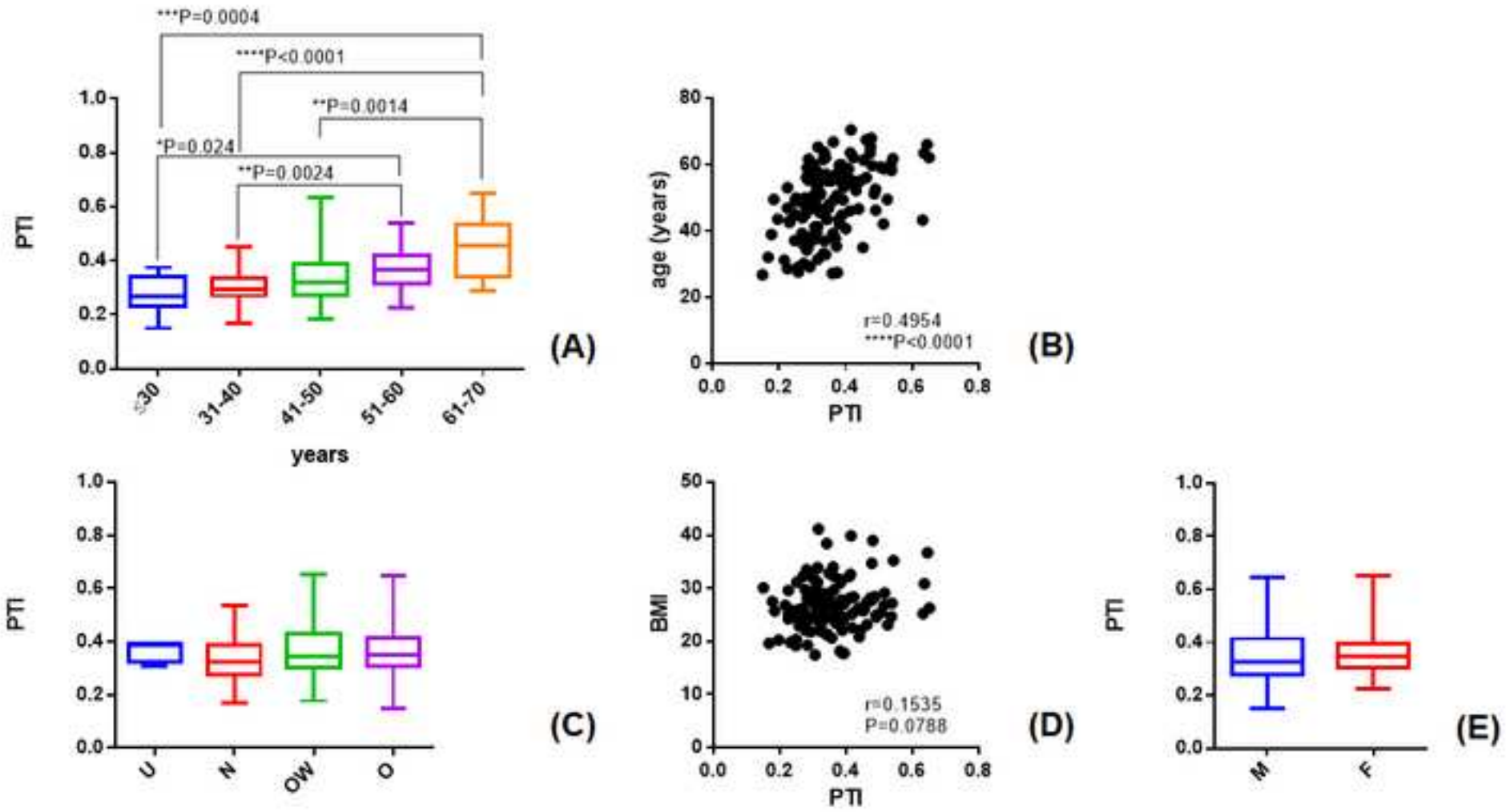

Table 1: SONIA1 inflammatory markers. Data are expressed as mean±stdev

	SAA (mg/L)	CATD (ng/mL)	IL-1ra (ng/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)	TNFα (pg/mL)	CRP (mg/L)	MMP-3 (ng/mL)
AKU	3≥SAA>10 n=13 (32.5%) SAA≥10 n=23 (57.5%)	59.01±35.95	146.4±115.2	1.674±0.510	4.604±0.908	4.874±0.964	CRP≥5 n=7 (17.5%)	11.29±5.654
CTR	nd	46.68±5.881	81.29±19.25	1.774±0.101	4.927±0.133	5.109±2.653	nd	13.15±4.823
P value	na	0.551	0.093	**** 0.011**	*** 0.046**	0.546	na	0.642

na: not applicable; nd: not determined

Table 2

Table 2: SONIA1 inflammatory markers according to gender, BMI classification, patients' smoking and drinking habits, and clinical site. Data are expressed as mean±stdev.

Variable		SAA (mg/L)	CATD (ng/mL)	IL-1ra (ng/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)	TNFα (pg/mL)	CRP (mg/L)	MMP-3 (ng/mL)
gender	male	25.71±40.33	64.99±36.95	146.7±108.5	1.686±0.555	4.874±0.964	4.721±0.882	1.830±2.023	12.41±5.85
	female	53.25±56.81	47.06±31.88	145.9±132.6	1.648±0.418	5.109±2.653	5.191±1.083	3.988±5.621	8.98±4.60
	P value	0.082	0.075	0.829	0.881	0.286	0.226	0.316	0.059
BMI	normal	26.08±34.73	55.37±29.89	95.89±32.27	1.692±0.533	4.695±1.287	4.491±0.892	1.295±1.652	11.32±5.99
	overweight	42.30±65.20	55.76±32.87	114.7±46.59	1.542±0.266	4.513±0.603	4.713±0.841	3.143±5.207	10.60±4.84
	obese	34.46±26.40	68.95±48.13	267.9±173.7	1.859±0.726	4.622±0.738	5.667±	3.284±2.383	12.35±6.76
	P value (N *vs.* OW) (N *vs.* O) (OW *vs.* O)	 > 0.999 > 0.999 > 0.999	 > 0.999 > 0.999 > 0.999	 > 0.999 **** 0.004** *** 0.031**	 > 0.999 > 0.999 > 0.999	 0.972 0.589 > 0.999	 > 0.999 **** 0.0099** 0.0759	 0.616 *** 0.025** 0.359	 > 0.999 > 0.999 > 0.999
drinking alcohol	yes	26.68±31.15	80.00±37.38	177.1±141.5	1.692±0.536	4.550±0.969	4.777±0.905	1.949±1.898	12.01±6.21
	no	41.89±58.27	41.02±23.06	118.6±78.39	1.657±0.497	4.653±0.870	4.961±0.991	3.058±4.739	8.29±5.17
	P value	0.507	****** <0.0001**	0.078	0.170	0.673	0.507	0.802	0.533
smoker	yes	37.68±62.41	66.26±30.99	155.1±101.3	1.775±0.691	4.413±0.318	5.004±0.999	2.600±2.731	13.18±6.69
	no	33.78±43.37	56.84±37.52	143.9±120.3	1.644±0.454	4.659±1.015	4.836±0.967	2.511±3.944	10.74±5.32
	P value	0.903	0.255	0.424	0.615	0.508	0.479	0.678	0.371
clinical site	UK	30.56±27.74	92.22±35.21	167.3±119.4	1.888±0.672	4.634±1.058	4.767±0.980	1.927±1.874	16.09±5.86
	SK	37.12±56.46	40.42±19.27	133.9±113.1	1.545±3.336	4.586±0.828	4.938±0.969	2.894±4.418	8.41±2.98
	P value	0.379	******<0.0001**	0.132	**** 0.005**	0.940	0.539	0.814	******<0.0001**

ns: not significant

Table 3

Table 3: Correlation matrix for inflammatory markers measured in SONIA1 study. Spearman's rank correlation analysis was carried out; r and P values are reported.

		CATD	IL-6	IL-1β	IL-1ra	TNFα	CRP	MMP-3	age	BMI	HGA	cholesterol	triglycerides	HDL	LDL
SAA	**r**	0.148	0.0450	0.135	0.257	0.066	0.604	0.108	0.351	0.344	0.030	0.362	0.073	0.209	0.336
	P	0.369	0.783	0.407	0.109	0.684	****** <0.0001**	0.508	*** 0.026**	*** 0.030**	0.854	*** 0.022**	0.653	0.195	*** 0.034**
CATD	**r**		-0.163	0.191	0.226	-0.101	-0.003	0.392	-0.0213	-0.006	0.270	0.181	-0.317	0.173	0.188
	P		0.320	0.243	0.166	0.541	0.987	*** 0.014**	0.193	0.970	0.097	0.269	*** 0.049**	0.293	0.252
IL-6	**r**			0.187	0.466	0.203	0.166	-0.005	0.287	0.350	0.256	-.0164	-0.064	-0.079	-0.073
	P			0.249	***** 0.002**	0.208	0.305	0.975	0.073	*** 0.027**	0.111	0.312	0.696	0.629	0.657
IL-1β	**r**				0.217	0.232	-0.047	0.197	-0.133	0.136	0.213	-0.020	-0.054	0.056	-0.030
	P				0.178	0.150	0.775	0.224	0.413	0.402	0.187	0.904	0.743	0.732	0.855
IL-1ra	**r**					0.121	0.439	0.226	0.059	0.467	0.121	-0.044	0.008	-0.221	-0.002
	P					0.457	**** 0.005**	0.160	0.716	***** 0.002**	0.456	0.788	0.962	0.171	0.989
TNFα	**r**						0.028	-0.025	0.056	0.429	0.258	-0.257	0.099	-0.290	-0.219
	P						0.863	0.876	0.734	**** 0.006**	0.108	0.110	0.544	0.069	0.174
CRP	**r**							-0.070	0.358	0.485	-0.030	0.098	0.269	-0.147	0.117
	P							0.668	*** 0.023**	***** 0.001**	0.856	0.548	0.093	0.366	0.471
MMP-3	**r**								0.144	0.012	0.225	0.172	-0.052	-0.027	0.188
	P								0.374	0.941	0.163	0.289	0.750	0.867	0.244
age	**r**									0.301	0.032	0.330	0.184	0.138	0.282
	P									0.059	0.844	*** 0.037**	0.256	0.396	0.078
BMI	**r**										0.394	-0.267	0.270	-0.503	-0.205
	P										*** 0.012**	0.096	0.092	*****0.001**	0.204
HGA	**r**											-0.143	-0.099	-0.249	-0.008
	P											0.379	0.542	0.122	0.959
cholesterol	**r**												0.144	0.400	0.918
	P												0.375	**** 0.01**	****** <0.0001**
triglycerides	**r**													-0.509	0.004
	P													*****0.001**	0.979
HDL	**r**														0.284
	P														0.076

Table 4

Table 4: Correlation matrix between markers measured in SONIA2 study and output of patients' questionnaires

		KOOS					HAQ		SF-36		AKUSSI	
		pain	symptoms	activity of daily living	sport	QoL	hapVAS	haqDI	physical	mental	joint pain	spinal pain
AOPP	**r**	0.043	0.054	0.048	-0.022	-0.040	-0.082	0.077	-0.042	-0.039	0.056	-0.024
	P	0.624	0.537	0.584	0.809	0.650	0.340	0.367	0.627	0.655	0.512	0.784
SAA	**r**	-0.132	-0.134	-0.169	-0.177	-0.226	0.084	0.209	-0.137	-0.137	0.091	0.057
	P	0.129	0.123	0.051	*** 0.044**	**** 0.009**	0.329	*** 0.015**	**** 0.006**	0.111	0.288	0.510
CHITOTRIOSIDASE	**r**	-0.314	-0.273	-0.303	-0.367	-0.330	0.055	0.336	-0.181	-0.076	0.244	0.228
	P	***** 0.0003**	**** 0.002**	***** 0.0004**	****** <0.0001**	***** 0.0001**	0.531	****** <0.0001**	*** 0.038**	0.387	**** 0.004**	**** 0.008**
PTI	**r**	-0.190	-0.265	-0.199	-0.312	-0.288	0.129	0.350	-0.228	-0.024	0.104	0.220
	P	*** 0.032**	**** 0.003**	*** 0.024**	***** 0.0004**	***** 0.001**	0.144	****** <0.0001**	**** 0.009**	0.783	0.237	*** 0.011**

Supplemental Material

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