Serum Amyloid A Truncations in Type 2 Diabetes Mellitus

Hussein N. Yassine[1]*, Olgica Trenchevska[2], Huijuan He[1], Chad R. Borges[2], Dobrin Nedelkov[2], Wendy Mack[1], Naoko Kono[1], Juraj Koska[3], Peter D. Reaven[3] Randall W. Nelson[2]

1 University of Southern California, Los Angeles, CA, United States of America, 2 Arizona State University, Tempe, AZ, United States of America, 3 Phoenix VA Health Care System, Phoenix, AZ, United States of America

* hyassine@usc.edu

Abstract

Serum Amyloid A (SAA) is an acute phase protein complex consisting of several abundant isoforms. The *N*- terminus of SAA is critical to its function in amyloid formation. SAA is frequently truncated, either missing an arginine or an arginine-serine dipeptide, resulting in isoforms that may influence the capacity to form amyloid. However, the relative abundance of truncated SAA in diabetes and chronic kidney disease is not known.

Methods

Using mass spectrometric immunoassay, the abundance of SAA truncations relative to the native variants was examined in plasma of 91 participants with type 2 diabetes and chronic kidney disease and 69 participants without diabetes.

Results

The ratio of SAA 1.1 (missing *N*-terminal arginine) to native SAA 1.1 was lower in diabetics compared to non-diabetics (p = 0.004), and in males compared to females (p<0.001). This ratio was negatively correlated with glycated hemoglobin (r = –0.32, p<0.001) and triglyceride concentrations (r = –0.37, p<0.001), and positively correlated with HDL cholesterol concentrations (r = 0.32, p<0.001).

Conclusion

The relative abundance of the *N*-terminal arginine truncation of SAA1.1 is significantly decreased in diabetes and negatively correlates with measures of glycemic and lipid control.

Introduction

Serum Amyloid A (SAA) proteins are members of the acute phase response protein group that play a significant role in inflammatory processes [1]. Structurally, proteins in the SAA complex

are encoded by four genes; SAA 1 and SAA 2 genes code acute phase SAA protein isoforms, SAA 3 is not expressed in humans, while SAA 4 is constitutively expressed under homeostatic conditions but does not vary significantly in the acute phase [2]. SAA 1 and SAA 2 genes give rise to five SAA 1 (SAA 1.1, SAA 1.2, SAA 1.3, SAA 1.4 and SAA 1.5) and two SAA 2 (SAA 2.1 and SAA 2.2) protein isoforms, respectively. All these protein products share a high degree of homology and differ only by a few amino acids in their sequences. Only SAA 1.1, SAA 1.2, SAA 1.3, SAA 2.1 and SAA 2.2 have been identified in humans [3] .

Following an inflammatory stimulus, SAA proteins act as apolipoproteins and become a major component of high density lipoproteins (HDL). This process removes cholesterol and extracellular lipid deposits at the inflammation site, but can reduce the ant-inflammatory capacity of HDL [4–6]. SAA is also chronically elevated in the setting of secondary amyloidosis leading systemic deposition of SAA-derived truncated fibrils (AA proteins) in tissues [7]. Secondary amyloidosis has been associated with chronic inflammatory conditions such as rheumatoid arthritis [8] or multiple sclerosis [9].In addition, SAA is recognized as a potential biomarker for a variety of chronic diseases including diabetes, cardiovascular disease (CVD), different types of cancers, sickle-cell disease, traumatic tissue damage and infections [10–13].

The role of SAA in each of these many conditions is generally believed to be a consequence of increases in total plasma concentration. However, not all studies find associations between disease and concentrations of total SAA. The discrepant results may be accounted for by the fact that most SAA studies do not discriminate between different molecular species of SAA, and do not consider the posttranslational modifications of the protein [14]. In fact, some studies demonstrate variations in the relative abundance of SAA truncated at the *N*-terminus [3,15,16]. This may have important clinical implications as the *N*-terminus of SAA is critical for amyloid fibrils formation [17,18]. The clearance of this truncated SAA variant is different compared to native SAA suggesting that these truncations may affect the capacity to form amyloid [16]. Therefore, it would be important to study if the relative abundances of the truncated forms differ in patients with chronic conditions such as diabetes or chronic kidney disease (CKD).

Current analytical techniques for SAA based on conventional immunoassay approaches provide total protein quantification. However, several mass-spectrometry based methods can distinguish between the different protein variants and posttranslational SAA protein modifications [12,19,20] . Mass spectrometric immunoassay (MSIA) is a high throughput methodology that is well suited to identify and quantify molecular variants and posttranslational modifications of plasma proteins [21,22]. MSIA is based on the isolation of protein moieties from a biological milieu by immobilized antibodies, which is followed by mass spectrometry assay. In our previous work using MSIA, we identified several novel *N*-terminal truncations of SAA 1.1, SAA1.2 and SAA 2.1 in human plasma [3]. In the present study, we investigate the association of SAA truncations with type 2 diabetes and chronic kidney disease (CKD).

Materials and Methods

Clinical Samples

The study was approved by the University of Arizona Institutional Review Board, and all patients provided written informed consent prior to testing. Two groups of adult participants (>18 years of age) were recruited: participants with type 2 diabetes (n = 91; 47 males and 44 females) and non-diabetic controls (n = 69, 29 males and 40 females). Participants reported to the Center for Clinical and Translational Sciences after an overnight fast. Blood was collected for clinical laboratory measurements (lipid profile, HbA1c, CRP, fasting insulin). Additional samples were collected in EDTA tubes, and plasma from these samples was separated and

immediately frozen at −80°C for all other assays. Demographic information (age, sex, ethnicity), physical exam measurements (blood pressure, waist circumference, weight, height, body mass index), medication use, and medical history (hypertension, hyperlipidemia, smoking, type and duration of diabetes) were also recorded. The glomerular filtration rate (GFR) was estimated using the Modification of Diet in Renal Disease Study (MDRD) equation [23]. Assignment of CKD (stages 1–5) was based on GFR levels as described [24]. Exclusion criteria included the following: type 1 diabetes, participation in an active weight loss program, history of cancer, HIV, active infection, other ongoing serious illness or current steroid use. Diabetes classification was based on clinical and medication history, or glycated hemoglobin greater than 6%. For the SAA MSIA analysis, frozen plasma samples were briefly thawed on ice, centrifuged for 5 min at 3000 rpm, and 50 μL aliquoted in low profile 96-well trays. Prior to the analysis, samples were diluted with 100 μL PBS containing 0.1%Tween.

Reagents

Monoclonal mouse anti-human antibody to serum amyloid A (SAA) was obtained from ANOGEN (Mississauga, ON, Canada, Catalog No. MO-C40028A, 500 mg). Protein calibration standard I (Cat. No. 206355) was purchased from Bruker (Billerica, MA). Phosphate buffered saline (PBS) buffer (Cat. No. 28372), MES buffered saline (28390), 1, 1'carbonyldiimidazole (97%) (CDI, 530-62-1), acetonitrile (ACN, A955-4), hydrochloric acid (HCl, A144-212), N-Methylpyrrolidinone (NMP, BP1172-4) and affinity pipettes fitted with porous microcolumns (991CUS01) were obtained from Thermo Scientific (Waltham, MA, USA). Tween 20 (Cat. No. P7949), trifluoroacetic acid (TFA, 299537-100G), sinapic acid (85429-5G), sodium chloride (S7653), HEPES (H3375), ethanolamine (ETA, 398136), albumin from bovine serum (BSA, A7906), ammonium acetate (A7330), octyl β-D glucopyranoside (NOG, 08001), polyethylene glycol (P3015-250G), calcium chloride (C1016-100G), and glycine buffer (G8898-500G) were obtained from Sigma Aldrich (St. Louis, MO, USA). Acetone (Cat. No. 0000017150) was obtained from Avantor Performance Materials (Center Valley, PA, USA). Plasma SAA was measured by an ELISA Kit (Novex, Cat. No. KHA0012).

Mass spectrometric immunoassay affinity pipette derivatization

The first step in MSIA method development is derivatization of the affinity pipettes with corresponding antibody toward the targeted protein. Using a Multimek 96 pipettor (Beckman Coulter, Brea, CA) affinity pipettes were derivatized with antibody toward SAA by initial rinse with 200 mM HCl (2 × 20 cycles, each cycle consisting of an aspiration and dispense of a 120 μL volume), followed by an acetone rinse (1×20 cycles). The surface of the microcolumns was activated by immersing the pipettes into a tray containing 100 mg/mL 1, 1'carbonyldiimidazole (in N-Methylpyrrolidinone), and 450 cycles of 100 μL aspirations and dispenses through each of the affinity pipettes were performed. Two rinses with NMP (10 cycles each, 150 μL volume) followed. The affinity pipettes were then immersed into a microwell plate containing the antibody solution (5 μg Ab per tip) and 750 cycles of aspirations and dispenses of 50 μL volume were performed to bind the antibody to the activated surfaces. One rinse with 60 mM HCl followed (50 cycles each, 100 μL), ending with two final rinses with assay buffer (PBS w/0.1% Tween, 50 cycles each, 100 μL). Antibody-derivatized pipettes were stored at +4°C until used.

Mass spectrometric immunoassay

Prior to sample protein extraction, the derivatized affinity pipettes were pre-rinsed with assay buffer (PBS, 0.1%Tween, 50 aspiration/dispense cycles, 100 μL each), followed by two 60 mM HCL rinses (50 aspiration/dispense cycles, 100 μL each) and another assay buffer rinse. Based

on prior experiments, pre-rinsing reduces non-specific binding to the tips during protein extraction. Pipettes were then immersed into a microplate containing the samples and 250 aspirations and dispense cycles were performed (100 µL each) allowing for affinity capture of SAA from the samples. The pipettes were then rinsed with mixture of 1 M ammonium acetate and acetonitrile (AA: ACN = 3:1 (v/v)) (100 cycles), and twice with water (10 cycles and 20 cycles respectively, 100 µL aspiration/dispense each). SAA-loaded tips were then exposed to six-microliter aliquots of MALDI matrix solution (25 g/L sinapic acid in aqueous solution containing 33% (v/v) acetonitrile, and 0.4% (v/v) trifluoroacetic acid). After a 10 second delay (to allow for the dissociation of the protein from the capturing antibody), the eluates were dispensed directly onto a 96-well formatted MALDI target. Following drying, linear-mode mass spectra were acquired from each sample spot, each consisting of ten thousand laser shots using an *Autoflex* III MALDI-TOF mass spectrometer (Bruker, Billerica, MA). The mass spectra were internally calibrated using low molecular mass protein standards, and further processed (baseline subtracted and smoothed) with the Flex Analysis software (Bruker Daltonics). Antibody specificity was verified by confirming the absence of SAA-related mass spectral peaks from negative control tips on which 1) no antibody was present, and 2) on which monoclonal antibodies specific to other proteins were immobilized. The peak areas for all SAA signals were measured in Zebra software (Beavis Informatics, Ltd.). Relative percent abundance of every SAA isoform was calculated in correspondence to the total SAA in the corresponding mass spectra.

SAA quantification

Total SAA concentrations were measured using a commercial ELISA from Invitrogen Life technologies (Novex, Cat. KHA0012) that has been extensively used [25–29] and tested on serum, plasma and tissue culture samples. The kit is based on antibody affinity to SAA 1.1, and thus does not capture the other forms of SAA.

Statistical Analysis

Mean (SD) or median (25th and 75th percentiles) were calculated for continuous variables. The diabetes and non-diabetes groups were compared by independent t-tests (normally distributed variables) or Wilcoxon rank sum tests (non-normally distributed variables). Categorical variables were compared using the chi-square test. The relationship between SAA variants and diabetes status was measured by logistic regression, with diabetes status as the binary dependent variable. The relation between SAA truncations and clinical variables was modeled using linear regression; all regression models used log-transformed SAA 1.1R as the dependent variable, and included age, BMI, and GFR as adjusting covariates. Regression models were performed in the total sample and by gender; a product interaction term tested whether the associations of clinical variables with SAA variants differed in males vs. females. Statistical analyses used SAS version 9.3 software package; a p-value of < 0.007 (0.05 divided by 7) was used to define significance levels in the SAA variants between the diabetes and non-diabetes groups accounting for the seven SAA variants (SAA 1.1R, SAA 1.1RS, SAA 2.1R, SAA 2.1 RS, SAA 2.2R, SAA 1.3R and SAA 1.3 RS truncations) of interest in this study. All other statistical tests used a p-value of 0.05.

Results

Participants' demographic and biochemical characteristics are summarized in Table 1. Participants with diabetes were on average older, had greater BMI, and demonstrated several metabolic characteristics of type 2 diabetes, including low HDL cholesterol, increased fasting insulin and elevated CRP levels when compared to participants without diabetes. In addition, participants with diabetes had a lower GFR and a greater urine albumin excretion ($p < 0.01$ for both)

Table 1. Demographic and clinical characteristics.

Characteristic	N_1/ N_2	No diabetes	Diabetes	p-value
Age, years	**69 / 91**	**49.4 (15.1)**	**56.5 (12.8)**	**0.002**
Sex	69 / 91			
Male		29 (42.0%)	47 (51.6%)	0.23
Female		40 (58.0%)	44 (48.4%)	
Race	69 / 91			
Caucasians		45 (65.2%)	50 (54.9%)	0.41
Hispanics		21 (30.4%)	35 (38.5%)	
Others		3 (4.4%)	6 (6.6%)	
Body mass index, kg/m^2	69 / 86	**27.8 (24.4, 32.8)**	**33.9 (28.2, 39.6)**	**<0.001**
Waist circumference, cm	68 / 80	**98.2 (17.2)**	**115.0 (19.8)**	**<0.001**
Glucose, mg/dL	69 / 85	**101.0 (93.0, 110.0)**	**144.0 (118.0, 202.0)**	**<0.001**
Hemoglobin A1C, (%)	65 / 77	**5.3 (5.1, 5.8)**	**7.8 (6.5, 9.8)**	**<0.001**
LDL cholesterol, mg/dL	68 / 84	117.3 (33.1)	107.7 (38.4)	0.11
HDL cholesterol, mg/dL	68 / 84	**52.6 (14.2)**	**45.2 (12.3)**	**<0.001**
Total cholesterol, mg/dL	68 / 84	196.6 (38.7)	186.1 (45.7)	0.14
Triglycerides, mg/dL	68 / 84	**131.5 (97.5, 182.5)**	**163.5 (134.0, 267.0)**	**0.001**
Glomerular FiltrationRate (GFR), L/min/1.73 m^2	65 / 76	**104.9 (25.1)**	**81.6 (38.1)**	**<0.001**
Fasting Insulin, IU		**9.0 (7.0, 14.0)**	**16.0 (8.0, 26.0)**	**0.004**
Urine Microalbumin,mg/mg creatinine	66 / 78	**5.0 (5.0, 9.0)**	**12.5 (5.0, 61.0)**	**<0.001**
Diabetes duration, years	0 / 85	-	9.0 (4.0, 15.0)	-
CRP, mg/dL	34 / 64	**2.8 (1.0, 6.1)**	**6.8 (3.0, 12.1)**	**0.006**
Total SAA, ng/mL*	21 / 21	28.7 (17.3, 44.5)	21.2 (9.9, 38.6)	0.21

Mean (SD) or median (25th percentile, 75th percentile) shown for continuous variables (normally distributed or non-normal, respectively).
*Only a subset from the whole sample was evaluated for total SAA concentration by ELISA.

doi:10.1371/journal.pone.0115320.t001

compared with the non-diabetic controls. Males (in the combined group) had significantly lower GFR than females (mean $\pm$ SD: male: 85.6 $\pm$ 32.5 L/min/1.73m^2; female: 98.4 $\pm$ 35.6 L/min/1.73m^2, p = 0.03).

Total SAA concentration was evaluated using a commercial ELISA in an unselected subset of samples (non-diabetes n = 21 vs. diabetes n = 21). There was no significant difference in total SAA concentration between the non-diabetes and diabetes groups: (median (25th percentile, 75th percentile), non-diabetes: 28.7 (17.3, 44.5), diabetes: 21.2 (9.9, 38.6), p = 0.21).

Characteristics of known SAA variants are summarized in Table 2. The SAA variants that can be resolved by MSIA are summarized in Table 3. A total of four protein isoforms, SAA 1.1, SAA 1.3, SAA 2.1 and SAA 2.2 (reflecting protein products from SAA1 and SAA2 genes) were detected among the samples, expressed either as single or as multiple variants. MALDI-MSIA-mass spectra of SAA from five different plasma samples showing all the variant forms and truncations (missing terminal –R and/or terminal –RS) are shown in Fig. 1. All samples that expressed SAA 1.1, presented with R and RS truncations. The MSIA analysis confirmed that the SAA variant 1.1 (MW = 11682.68) was the most abundant and it was detected in all but one (159/160) individual. The distribution of the other identified SAA protein entities within the cohort was as follows: 58.8% for SAA 2.1, 13.1% for SAA 2.2 and 11.9% for SAA 1.3. In addition to these native forms, two N-terminal truncation isoforms were noted for all the variants: those missing arginine (des-R; Δm = −156.19 Da) and/or arginine-serine dipeptide (des-RS; Δm = −243.27 Da).

The relative abundance of each of the truncated isoforms was calculated as the ratio of truncated SAA to native SAA and compared between subjects with and without diabetes (SAA

Table 2. SAA isoforms detected by MSIA.

SAA gene	SAA proteins	Amino acid sequence	native form	-R truncated form	-RS truncated form
Allelic genes			MW	MW	MW
SAA1	SAA 1.1	RS FFSFLGEAFD GARDMWRAYS DMREANYIGS DKYFHARGNY DAAKRGPGGV WAAEAISDAR ENIQRFFGHG AEDSLADQAA NEWGRSGKDP NHFRPAGLPE KY	11682.7	11526.5	11439.4
	SAA 1.2	RS FFSFLGEAFD GARDMWRAYS DMREANYIGS DKYFHARGNY DAAKRGPGG**A** WAAEVISDAR ENIQRFFGH**D** AEDSLADQAA NEWGRSGKDP NHFRPAGLPE KY	11740.7	11584.5	11497.5
	SAA 1.3	RS FFSFLGEAFD GARDMWRAYS DMREANYIGS DKYFHARGNY DAAKRGPGG**A** WAAEAISDAR ENIQRFFGHG AEDSLADQAA NEWGRSGKDP NHFRPAGLPE KY	11654.6	11498.4	11411.4
	SAA 1.4*	RS FFSFLGEAFD GARDMWRAYS DMREANYIGS DKYFHARGNY DAAKRGPGG**A** WAAEVISNAR ENIQRFFGHG AEDSLADQAA NEWGRSGKDP NHFRPAGLPE KY	11681.7	11525.5	11438.4
	SAA 1.5*	RS FFSFLGEAFD GARDMWRAYS DMREANYIGS DKYFHARGNY DAAKRGPGG**A** WAAEVISDAR ENIQRFFGHG AEDSLADQAA NEWGRSGKDP NHFRPAGLPE KY	11682.7	11526.5	11439.4
SAA2	SAA 2.1	RS FFSFLGEAFD GARDMWRAYS DMREANYIGS DKYFHARGNY DAAKRGPGGA WAAEVISNAR ENIQRLTG**H**G AEDSLADQAA NKWGRSGRDP NHFRPAGLPE KY	11628.7	11472.5	11385.4
	SAA 2.2	RS FFSFLGEAFD GARDMWRAYS DMREANYIGS DKYFHARGNY DAAKRGPGGA WAAEVISNAR ENIQRLTGRG AEDSLADQAA NKWGRSGRDP NHFRPAGLPE KY	11647.7	11491.5	11404.5
SAA4	SAA 4**	ES WRSFFKEALQ GVGDMGRAYW DIMISNHQNS NRYLYARGNY DAAQRGPGGV WAAKLISRSR VYLQGLIDCY LFGNSSTVLE DSKSNEKAEE WGRSGKDPDR FRPDGLPKKY	12802.2		

SAA exists in multiple forms. The main isoforms expressed are SAA 1.1 and 2.2. MSIA can detect the SAA 1.1, 1.2, 1.3, 2.1 and 2.2. SAA 1.4 and 1.5 cannot be resolved. SAA 3 is a pseudo gene (not expressed). SAA 4 is constitutively expressed. MSIA: Mass Spectrometric Immunoassay.

* These variants can't be distinguished in the mass spectra due to close proximity (overlapping) of the mass in spite of the changes in the amino acid sequence.

** SAA 4 is constitutively expressed.

doi:10.1371/journal.pone.0115320.t002

Table 3. Comparison of SAA variant ratios between the diabetes and non-diabetes groups.

SAA Variant Ratio	% variants detected	N_1 / N_2	Non-Diabetes	Diabetes	p-value
SAA 1.1R	99.4% (159/160)	69 / 90	0.93 (0.76, 1.17)	0.84 (0.65, 0.99)	0.004*
SAA 1.1RS	99.4% (159/160)	69 / 90	0.22 (0.18, 0.28)	0.21 (0.15, 0.29)	0.11
SAA 2.1R	57.5% (92/160)	34 / 58	0.88 (0.76, 1.10)	0.90 (0.78, 1.04)	0.73
SAA 2.1RS	29.4% (47/160)	16 / 31	0.25 (0.20, 0.30)	0.25 (0.20, 0.32)	0.96
SAA 2.2R	13.1% (21/160)	6 / 15	0.70 (0.53, 0.89)	0.81 (0.76, 0.93)	0.32
SAA 1.3R	11.9% (19/160)	6 / 13	0.79 (0.74, 1.24)	0.96 (0.80, 1.18)	0.94
SAA 1.3RS	10.6% (17/160)	5 / 12	0.23 (0.23, 0.27)	0.28 (0.22, 0.36)	0.42

SAA isoforms were heavily truncated at the N terminus missing either arginine (R) or arginine serine (RS). The truncation abundance and the differences in participants with diabetes and without diabetes are summarized. The data are presented as medians (25th percentile, 75th percentile) by diabetes status. The comparison between the two groups was performed with a logistic regression model after adjusting for age.

*Indicates statistically significant at the threshold of 0.007. After adjusting for age and BMI, the association of diabetes and SAA 1.1R was significant at the .05 alpha level: OR(95 CI) = 0.24 (0.07 to 0.78), p = 0.02.
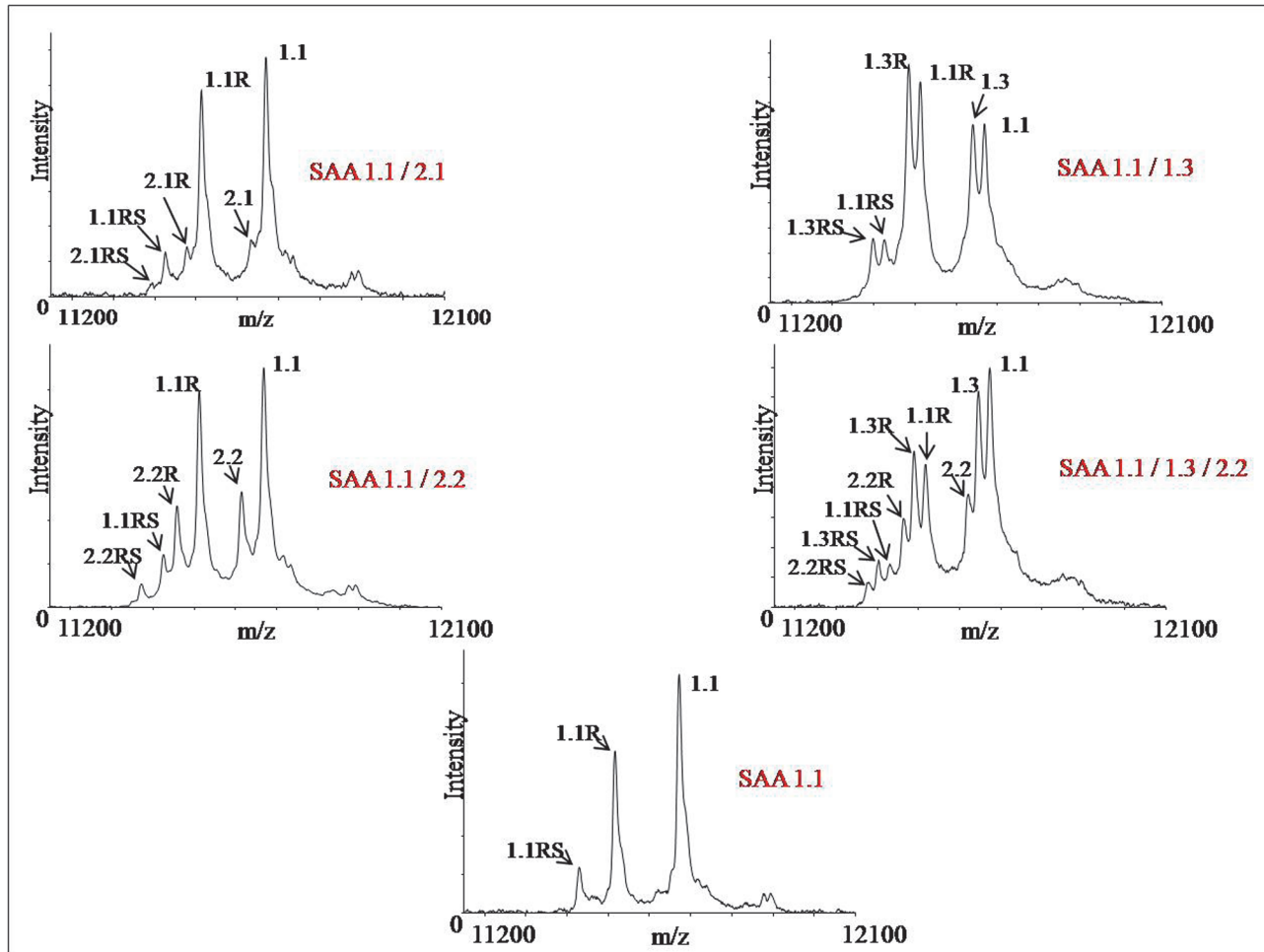
doi:10.1371/journal.pone.0115320.t003

Figure 1. MALDI-MSIA-mass spectra of SAA from five different plasma samples showing all the variant forms and truncations (missing terminal –R and/or terminal –RS). All samples express SAA 1.1 with R and RS truncations.

variants R and RS ratios). The log of SAA1.1R ratio was higher in the non-diabetic group compared to the diabetic group, (p = 0.02). The log of SAA1.1R was significantly associated with age (r = 0.16, p = 0.03), but not with BMI (r = −0.12, p = 0.11). As shown in Table 3, after adjustment for age, SAA 1.1R was lower in the diabetes group compared with the non-diabetes group (p = 0.004); the association remained significant with adjustment for both BMI and age (p = 0.02). In contrast, the ratio of other SAA truncation variants to native (SAA 1.1RS, 2.1R, 2.1RS, 2.2R, 1.3R and 1.3 RS truncations) did not differ between the diabetes and non-diabetes groups (all p>0.05, Table 3). In addition, in the whole group (diabetes and non-diabetes combined) the SAA 1.1R ratio to native was lower in males compared with females (median (IQR): male: n = 76, 0.78 (0.29); female: n = 84, 0.97 (0.43), p<0.001), Fig. 2).

An inverse correlation was observed between glucose levels and log-transformed SAA 1.1R (r = −0.3, p<0.001). The correlation between log-transformed SAA1.1R and glucose was significant in males (r = −0.44, p<0.001, Fig. 3B) but not in females (r = −0.15, p = 0.17, Fig. 3C). Table 4 summarizes the linear associations of log-transformed SAA 1.1R ratio and several metabolic measures in males and females, adjusting for age, BMI and GFR. Both fasting glucose and glycated hemoglobin were strongly inversely correlated with log-transformed SAA 1.1R ratio. Although none of these associations significantly differed in males and females (all
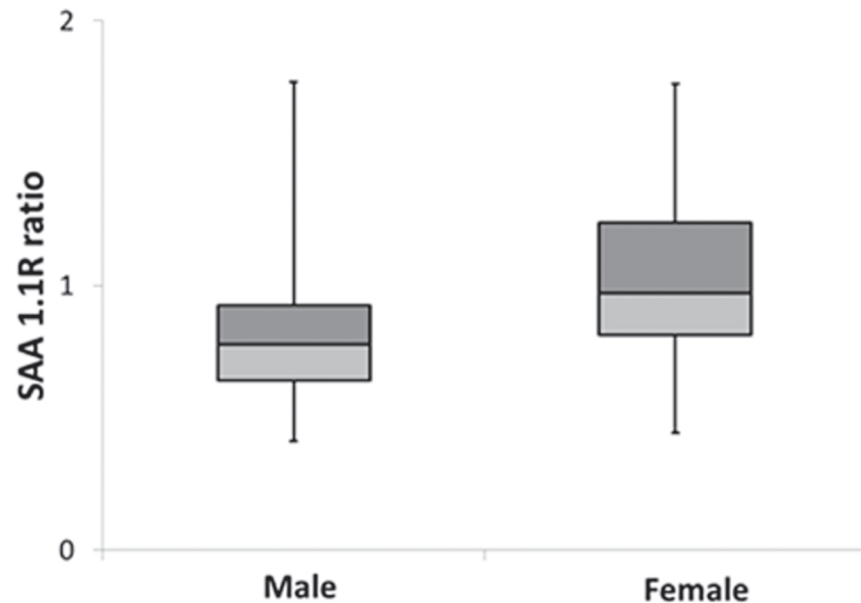
Figure 2. SAA variant ratios (median, IQR) among males (n = 76) and females (n = 84) group. SAA 1.1R (missing *N*-terminal R arginine) ratio to SAA 1.1 was significantly lower in males compared to females (p<0.001).

interaction p-values > 0.05), the SAA 1.1R ratio was most strongly and inversely correlated with glucose levels in males. There was no correlation between total SAA concentrations measured by ELISA and the SAA1.1R ratio determined by MSIA.

Since males had a lower GFR than females, the relative abundance of SAA truncations could be explained by differences in kidney clearance rate. To address this possibility, the relationship between log-transformed SAA 1.1R and GFR in both males and females was examined. In males, log-transformed SAA 1.1R was not significantly correlated with GFR (r = 0.21, p = 0.09); the association was reduced with adjustment for glucose (r = 0.12, p = 0.37). In females, log-transformed SAA1.1R and GFR were inversely but not significantly correlated (r = −0.221, p = 0.06); adjustment for glucose did not alter this association (r = −0.21, p = 0.08). These glucose-adjusted associations did not significantly differ among males and females (p-value for interaction = 0.09).

The study group was categorized by CKD stage with the non-CKD group defined based on a GFR > 90 L/min/1.73m^2. The distribution of CKD stages in the diabetes and non-diabetes groups is presented in Table 5. There were 61 patients in the CKD group and 80 patients in the non-CKD group. The majority of non-diabetic participants did not have evidence of CKD, whereas the diabetic participants recruited in this study had different stages of CKD with 9 participants in stages 4 and 5. The inverse association of log-transformed SAA1.1R with glucose did not differ among persons with CKD vs. non-CKD (interaction p-value = 0.12, adjusted for age and BMI). These findings confirm that glucose levels had an inverse correlation with SAA 1.1R, with the association apparent in persons with and without CKD.

The peak area of SAA 1.1 was highly correlated to the peak area of SAA 1.1R and SAA 1.1RS (Table 6). The correlation was strongest (r> 0.9, p <0.001, all subjects) among the SAA 1.1 R and RS variants suggesting that the same process favors the formation of both truncations.
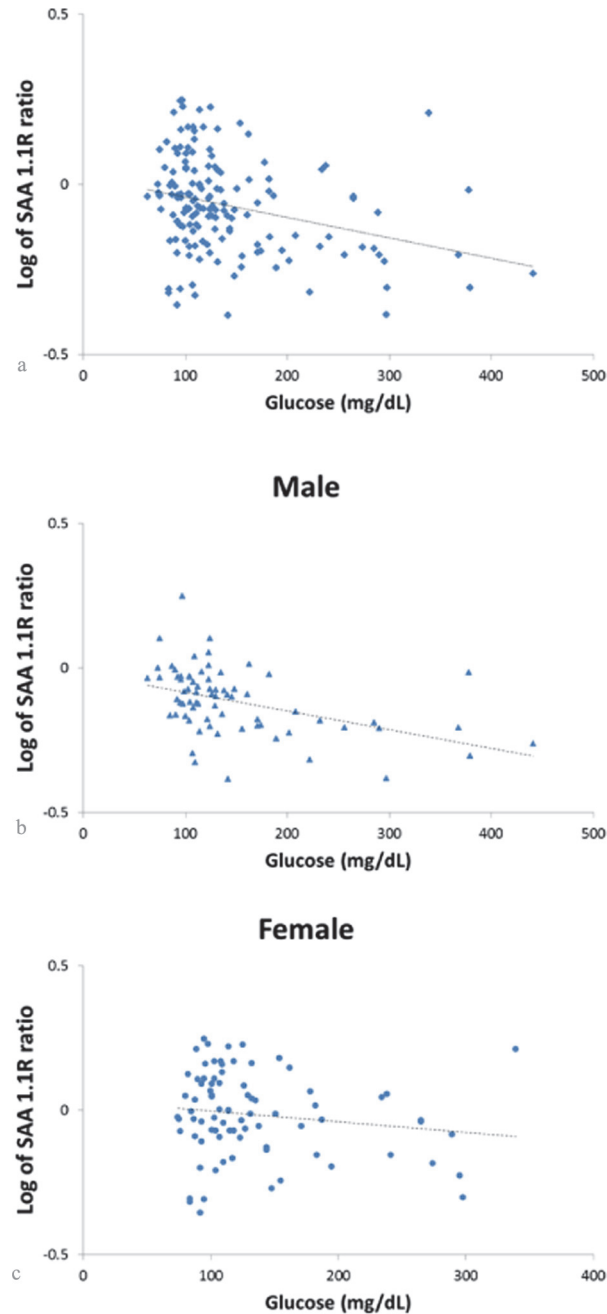
Figure 3. Correlation between glucose and the log of SAA 1.1R ratio in the entire group (panel a), in males (panel b), and in females (panel c). In the entire group, the log of SAA 1.1R was inversely related to glucose levels (panel a, r = −0.3, p<0.001). This correlation was driven by the males in the study. The log of SAA 1.1R was inversely correlated to glucose in males (panel b, r= −0.44, p<0.001), but not in females (panel c, r = −0.15, p = 0.17).

doi:10.1371/journal.pone.0115320.g003

Discussion

Our findings demonstrate that ratios of SAA 1.1R truncations to their native SAA variants are decreased in diabetes. The occurrence of *N*-terminally truncated SAA has been observed in several studies confirming that the relative abundance of this variant of SAA is quite high

Table 4. Relationship of SAA 1.1R variant ratios with clinical and laboratory characteristics.

	N/N$_1$/N$_2$	Combined sample		Male		Female	
		Regression Coefficient Beta (SE)	p-value	Regression Coefficient Beta (SE)	p-value	Regression Coefficient Beta (SE)	p-value
Waist circumference, cm	129/59/70	**−0.008(0.003)**	**0.02**	−0.004(0.006)	0.47	−0.002(0.004)	0.59
Glucose,mg/dL	134/62/72	**−0.001(0.0004)**	**0.004**	**−0.002(0.0004)**	**<0.001**	−0.0006(0.0007)	0.41
Glycated Hemoglobin %	128/61/67	**−0.040(0.011)**	**<0.001**	**−0.035(0.010)**	**0.001**	−0.028(0.019)	0.15
Triglycerides,mg/dL	135/62/73	**−0.0006(0.0002)**	**0.01**	−0.0003(0.0002)	0.23	−0.0004(0.0004)	0.33
HDL,cholesterolmg/dL	135/62/73	**0.007(0.002)**	**0.003**	0.003(0.004)	0.43	0.002(0.003)	0.59
CRP,mg/dL	82/42/40	−0.001(0.006)	0.85	0.0005(0.006)	0.94	−0.005(0.008)	0.52

Linear regression with log-transformed SAA 1.1R as the dependent variable with clinical and laboratory characteristics as the independent variables adjusted for age, BMI and glomerular filtration rate (GFR).

All samples in table include both diabetics and non-diabetics.

N = number of subjects in combined sample (male and female subjects)

N$_1$ = number of male subjects

N$_2$ = number of female subjects

doi:10.1371/journal.pone.0115320.t004

[3,12,15,16,30,31]. The population frequency of these truncations and their physiologic relevance are not known. Since the *N* terminus of SAA determines its amyloid fibril formation activity [17,18], assessing changes in SAA truncations might provide insight into diseases such as reactive amyloidosis where susceptibility to amyloid formation is dependent on the SAA functionality and clearance. We previously reported a lower relative abundance of SAA truncations in plasma of one subject with acute inflammation (rheumatoid arthritis) compared with subjects without evidence of inflammation [3]. Given that the *N*-terminal arginine and serine of SAA are highly conserved, these truncations are likely the result of posttranslational modifications [12,32]. Dipeptidyl aminopeptidase cleavage has been reported for several lipoproteins [33], and SAA is an apolipoprotein that forms HDL. In our study, glucose levels had the strongest correlation (inverse relationship) with the ratio of truncations in males, and this persisted after adjusting for changes in kidney function, age or BMI. This inverse relationship with hyperglycemia suggests that glucose might impair the peptidase activity. In one previous study, the clearance of truncated SAA at the *N*-terminus differed from native SAA suggesting that these truncations may impact SAA turnover [16].

Our findings also suggest that sex may independently modify the activity of this peptidase, leading to a lower truncated SAA to native SAA ratio. Of note, male sex was previously

Table 5. Numbers of participants in non-diabetes and diabetes groups based on their CKD stages.

CKD stage	Non-Diabetes N = 65	Diabetes N = 76
Stage1 (GFR ≥90 mL/min/1.73m^2)	46	34
Stage2 (60≤GFR<90 mL/min/1.73m^2)	18	21
Stage3 (30≤GFR<60 mL/min/1.73m^2)	1	12
Stage4 (15≤GFR<30 mL/min/1.73m^2)	0	6
Stage5 (GFR<15 mL/min/1.73m^2)	0	3

GFR values for 19 patients were unavailable.

Fisher's exact test was used to test for differences in CKD stages between diabetics and non-diabetics, p < 0.001.

doi:10.1371/journal.pone.0115320.t005

Table 6. Correlations between SAA 1.1 variants in diabetes group.

Variants	All subjects N = 159	No Diabetes N_1 = 69	Diabetes N_2 = 90
SAA 1.1 with SAA 1.1R	0.87	0.84	0.90
SAA 1.1 with SAA 1.1RS	0.81	0.78	0.83
SAA 1.1R with SAA 1.1RS	0.91	0.89	0.93

The peak area of log-transformed SAA 1.1 (unmodified) was highly correlated to the peak area of log-transformed SAA 1.1R and SAA 1.1RS (all p-values <0.001).

doi:10.1371/journal.pone.0115320.t006

identified as a risk factor for both amyloidosis and diabetes complications. In a study of Familial Mediterranean Fever, the male to female ratio was significantly higher (2:1) in patients with reactive amyloidosis, [34]. In the Pittsburgh Epidemiology of Diabetes Complications Study, males had worse diabetic complications after long term follow up [35]. The role of these truncations in chronic diabetic complications merits further study.

We measured total SAA concentrations by a validated commercial ELISA [25–29]. Total SAA concentrations did not differ between the diabetic and non-diabetic groups and SAA concentrations did not correlate with the ratio of SAA1.1R ratio. Although a previous study [36] demonstrated that SAA concentrations were increased in type 2 diabetes with chronic kidney disease, we did not find increased total SAA concentrations in persons with diabetes.

The relative abundance of the SAA RS truncation is lower than that of SAA R, and there has been speculation that it might be a biomarker of certain diseases, such as renal cancer [12]. However, the strong correlation of R and RS truncations with the unmodified SAA 1.1 variant suggest that the same process affects the formation of both of these truncations in patients with and without diabetes (Table 6). This may argue against RS truncation as a selective marker of diabetes complications.

It is worth noting that MSIA cannot distinguish SAA form 1.5 from 1.1 because their molecular weight differs by only 1 Da, which makes these forms difficult to differentiate from each other on most mass spectrometers. The expression frequencies of SAA 1.4 and 1.5 are not known, but based on previous genetic studies, they are likely uncommon variants [37]. Given the lower relative abundance of SAA 2.1, 2.2 and 1.3 truncations, this study could be underpowered to detect differences in these variants between the diabetes and non-diabetes groups.

In conclusion, our findings indicate that truncated SAA is less common in diabetes. SAA truncations may modify SAA function and clearance. Measuring SAA truncations may help us better understand the susceptibility to reactive amyloidosis in chronic conditions.

Author Contributions

Conceived and designed the experiments: HY CB PR RN. Performed the experiments: HY OT DN CB. Analyzed the data: HH WM NK. Contributed reagents/materials/analysis tools: RN CB OT DN. Wrote the paper: HY PR JK CB WM OT.

References

1. Malle E, De Beer FC (1996) Human serum amyloid A (SAA) protein: a prominent acute-phase reactant for clinical practice. European Journal of Clinical Investigation 26: 427–435. doi: 10.1046/j.1365-2362.1996.159291.x PMID: 8817153

2. Malle E, Steinmetz A, Raynes JG (1993) Serum amyloid A (SAA): an acute phase protein and apolipoprotein. Atherosclerosis 102: 131–146. doi: 10.1016/0021-9150(93)90155-N PMID: 7504491

3. Kiernan UA, Tubbs KA, Nedelkov D, Niederkofler EE, Nelson RW (2003) Detection of novel truncated forms of human serum amyloid A protein in human plasma. FEBS Lett 537: 166–170. doi: 10.1016/S0014-5793(03)00097-8 PMID: 12606051

4. Cabana V, Siegel J, Sabesin S (1989) Effects of the acute phase response on the concentration and density distribution of plasma lipids and apolipoproteins. Journal of lipid research 30: 39–49. PMID: 2493057

5. Liang J, Sipe J (1995) Recombinant human serum amyloid A (apoSAAp) binds cholesterol and modulates cholesterol flux. Journal of lipid research 36: 37–46. PMID: 7706946

6. Tölle M, Huang T, Schuchardt M, Jankowski V, Prüfer N, et al. (2012) High-density lipoprotein loses its anti-inflammatory capacity by accumulation of pro-inflammatory-serum amyloid A. Cardiovascular research 94: 154–162. doi: 10.1093/cvr/cvs089 PMID: 22328092

7. Elliott B, Liang, Sipe, Cathcart (1998) Catabolism of Lipid-Free Recombinant Apolipoprotein Serum Amyloid A by Mouse Macrophages In Vitro Results in Removal of the Amyloid Fibril-Forming Amino Terminus. Scandinavian Journal of Immunology 48: 241–247. doi: 10.1046/j.1365-3083.1998.00384.x PMID: 9743207

8. Cunnane G (2001) Amyloid precursors and amyloidosis in inflammatory arthritis. Current opinion in rheumatology 13: 67–73. doi: 10.1097/00002281-200101000-00011 PMID: 11148718

9. Ristori G, Laurenti F, Stacchini P, Gasperini C, Buttinelli C, et al. (1998) Serum amyloid A protein is elevated in relapsing-remitting multiple sclerosis. Journal of neuroimmunology 88: 9–12. doi: 10.1016/S0165-5728(98)00037-X PMID: 9688318

10. Malle E, Sodin-Semrl S, Kovacevic A (2009) Serum amyloid A: an acute-phase protein involved in tumour pathogenesis. Cell Mol Life Sci 66: 9–26. doi: 10.1007/s00018-008-8321-x PMID: 18726069

11. Zhao Y, He X, Shi X, Huang C, Liu J, et al. (2010) Association between serum amyloid A and obesity: a meta-analysis and systematic review. Inflamm Res 59: 323–334. doi: 10.1007/s00011-010-0163-y PMID: 20140694

12. Tolson J, Bogumil R, Brunst E, Beck H, Elsner R, et al. (2004) Serum protein profiling by SELDI mass spectrometry: detection of multiple variants of serum amyloid alpha in renal cancer patients. Laboratory Investigation 84: 845–856. doi: 10.1038/labinvest.3700097 PMID: 15107802

13. Tumblin A, Tailor A, Hoehn GT, Mack AK, Mendelsohn L, et al. (2010) Apolipoprotein AI and serum amyloid A plasma levels are biomarkers of acute painful episodes in patients with sickle cell disease. haematologica 95: 1467–1472. doi: 10.3324/haematol.2009.018044 PMID: 20378559

14. Avgerinos ED, Kadoglou NP, Moulakakis KG, Giannakopoulos TG, Liapis CD (2011) Current role of biomarkers in carotid disease: a systematic review. Int J Stroke 6: 337–345. doi: 10.1111/j.1747-4949.2011.00623.x PMID: 21745345

15. Beach CM, De Beer MC, Sipe JD, Loose LD, De Beer FC (1992) Human serum amyloid A protein. Complete amino acid sequence of a new variant. Biochem J 282 (Pt 2): 615–620. PMID: 1546977

16. Raynes JG, McAdam KP (1991) Serum amyloid A isoforms in inflammation. Scand J Immunol 33: 657–666.

17. Westermark GT, Engström U, Westermark P (1992) The N-terminal segment of protein AA determines its fibrillogenic property. Biochemical and biophysical research communications 182: 27–33. doi: 10.1016/S0006-291X(05)80107-X PMID: 1731787

18. Egashira M, Takase H, Yamamoto I, Tanaka M, Saito H (2011) Identification of regions responsible for heparin-induced amyloidogenesis of human serum amyloid A using its fragment peptides. Archives of biochemistry and biophysics 511: 101–106. doi: 10.1016/j.abb.2011.04.019 PMID: 21569756

19. Farwig ZN, McNeal CJ, Little D, Baisden CE, Macfarlane RD (2005) Novel truncated isoforms of constitutive serum amyloid A detected by MALDI mass spectrometry. Biochemical and biophysical research communications 332: 352–356. doi: 10.1016/j.bbrc.2005.04.129 PMID: 15910745

20. Ducret A, Bruun CF, Bures EJ, Marhaug G, Husby G, et al. (1996) Characterization of human serum amyloid A protein isoforms separated by two-dimensional electrophoresis by liquid chromatography/electrospray ionization tandem mass spectrometry. ELECTROPHORESIS 17: 866–876. doi: 10.1002/elps.1150170508 PMID: 8783012

21. Nelson RW, Borges CR (2011) Mass spectrometric immunoassay revisited. J Am Soc Mass Spectrom 22: 960–968. doi: 10.1007/s13361-011-0094-z PMID: 21953037

22. Nelson RW, Krone JR, Bieber AL, Williams P (1995) Mass spectrometric immunoassay. Anal Chem 67: 1153–1158. doi: 10.1021/ac00103a003 PMID: 15134097

23. Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, et al. (1999) A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Annals of internal medicine 130: 461–470. doi: 10.7326/0003-4819-130-6-199903160-00002 PMID: 10075613

24. Levey AS, de Jong PE, Coresh J, El Nahas M, Astor BC, et al. (2010) The definition, classification, and prognosis of chronic kidney disease: a KDIGO Controversies Conference report. Kidney international 80: 17–28.

25. Tajima M, Wakita D, Noguchi D, Chamoto K, Yue Z, et al. (2008) IL-6-dependent spontaneous proliferation is required for the induction of colitogenic IL-17-producing CD8+ T cells. The Journal of experimental medicine 205: 1019–1027. doi: 10.1084/jem.20071133 PMID: 18426983

26. Sung H-J, Jeon S, Ahn J-M, Seul K-J, Kim JY, et al. (2012) Large-scale isotype-specific quantification of Serum amyloid A 1/2 by multiple reaction monitoring in crude sera. Journal of proteomics 75: 2170–2180. doi: 10.1016/j.jprot.2012.01.018 PMID: 22300576

27. Igawa T, Ishii S, Tachibana T, Maeda A, Higuchi Y, et al. (2010) Antibody recycling by engineered pH-dependent antigen binding improves the duration of antigen neutralization. Nature biotechnology 28: 1203–1207. doi: 10.1038/nbt.1691 PMID: 20953198

28. Malik P, Berisha SZ, Santore J, Agatisa-Boyle C, Brubaker G, et al. (2011) Zymosan-mediated inflammation impairs in vivo reverse cholesterol transport. Journal of lipid research 52: 951–957. doi: 10.1194/jlr.M011122 PMID: 21335620

29. Vijay-Kumar M, Sanders CJ, Taylor RT, Kumar A, Aitken JD, et al. (2007) Deletion of TLR5 results in spontaneous colitis in mice. The Journal of clinical investigation 117: 3909–3921. doi: 10.1172/JCI33084 PMID: 18008007

30. Strachan AF, Brandt WF, Woo P, van der Westhuyzen DR, Coetzee GA, et al. (1989) Human serum amyloid A protein. The assignment of the six major isoforms to three published gene sequences and evidence for two genetic loci. Journal of Biological Chemistry 264: 18368–18373. PMID: 2808379

31. Dwulet FE, Wallace DK, Benson MD (1988) Amino acid structures of multiple forms of amyloid-related serum protein SAA from a single individual. Biochemistry 27: 1677–1682. doi: 10.1021/bi00405a044 PMID: 3130100

32. Skogen B, Natvig JB (1981) Degradation of amyloid proteins by different serine proteases. Scand J Immunol 14: 389–396. doi: 10.1111/j.1365-3083.1981.tb00579.x PMID: 6461061

33. Gordon JI, Sims HF, Strauss AW, Scanu AM, Edelstein C, et al. (1986) Proteolytic Processing and Compartmentalization of the Primary Translation Products of Mammalian Apolipoprotein Mrna. Critical Reviews in Biochemistry and Molecular Biology 20: 37–71. doi: 10.3109/10409238609115900 PMID: 3514123

34. Gershoni-Baruch R, Brik R, Lidar M, Shinawi M, Livneh A (2003) Male sex coupled with articular manifestations cause a 4-fold increase in susceptibility to amyloidosis in patients with familial Mediterranean fever homozygous for the M694V-MEFV mutation. The Journal of rheumatology 30: 308–312. PMID: 12563686

35. Orchard TJ, Dorman JS, Maser RE, Becker DJ, Drash AL, et al. (1990) Prevalence of complications in IDDM by sex and duration: Pittsburgh Epidemiology of Diabetes Complications Study II. Diabetes 39: 1116–1124. doi: 10.2337/diabetes.39.9.1116 PMID: 2384191

36. Tsun J, Shiu S, Wong Y, Yung S, Chan T, et al. (2013) Impact of serum amyloid A on cellular cholesterol efflux to serum in type 2 diabetes mellitus. Atherosclerosis 231: 405–410. doi: 10.1016/j.atherosclerosis.2013.10.008 PMID: 24267259

37. Steinkasserer A, Weiss EH, Schwaeble W, Linke RP (1990) Heterogeneity of human serum amyloid A protein. Five different variants from one individual demonstrated by cDNA sequence analysis. Biochem J 268: 187–193. PMID: 1971508