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Original Article

# Correlation between systemic S100A8 and S100A9 levels and severity of diabetic retinopathy in patients with type 2 diabetes mellitus

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ABSTRACT

*Aims:* S100A8 and S100A9 are myeloid-related damage-associated molecular patterns (DAMPs) primarily involved in the modulation of innate immune response to cellular injury. This study evaluated the correlation between circulating concentrations of S100A8 and S100A9 proteins with the severity of diabetic retinopathy (DR) in patients with type 2 diabetes (T2DM).

*Methods:* T2DM patients with HbA1c levels >7%, fasting blood glucose >126 mg/dl and history of diabetes were included in this study. DR severity was graded based on ETDRS and Gloucestershire classifications. Plasma samples were evaluated for S100A8 and S100A9 levels using ELISA.

*Results:* In this comparative study, DR patients (n = 89) had increased plasma S100A8 and S100A9 proteins compared to age-matched T2DM controls (n = 28), which was directly related to the severity of DR. Female DR subjects had increased S100A8 expression compared to their male counterparts. Substantial retention of S100A8 and S100A9 production was seen in DR patients above 50 years of age. Duration of T2DM was not found to affect protein levels, however T2DM onset at >50 years old significantly increased S100A8 and S100A9 concentrations.

*Conclusions:* Our findings suggest that systemic circulation levels of S100A8 and S100A9 are correlated with the progression of DR in T2DM patients, indicating their potential role in DR pathogenesis.



## 1. Introduction

Diabetic retinopathy (DR) is a consequence of long-term diabetes (DM) and a significant cause of blindness in the adult population worldwide [1,2]. Recent data suggest that the prevalence of DR, particularly in severe vision-threatening diseases such as proliferative DR (PDR) and diabetic macular edema (DME), has increased affliction up to ~7% of diabetes patients worldwide [3,4]. These advanced stages of disease exhibit neovascular and degenerative changes within the retina leading to vision loss and blindness [5–7]. Therapeutic strategies for DR are limited to varying degrees of effectiveness. Anti-VEGF therapy [8] and laser photocoagulation [9] are most commonly used to prevent neovascularization in the advanced-stages of DR. There are no treatments available for the early-stages of DR.

The variations in the onset and severity of retinopathy amongst diabetic patients are the most perplexing features of DR pathogenesis. Disease prediction tools rely on a myriad of traditional DM risk factors such as duration of diabetes [10], hyperglycemia [11], hypertension [12,13] and blood HbA1c levels [14] to estimate the

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probability of developing DR. However, these established factors remained insufficient. The ADVANCE trial reported that intensive glucose control to reduce glycosylated hemoglobin to 6.5% or lower did not affect the 5-year incidence of DR [15], Furthermore, lowering of blood pressure to near normal levels did not achieve a reduction in the progression of DR [11], reiterating the need for a deeper understanding of the disease in human subjects.

Chronic hyperglycemia can result in damage to the retinal microvasculature resulting in diabetic retinopathy (DR). Progressive retinal damage in DR is characterized by capillary occlusion, tissue ischemia, increased vascular permeability, neovascularization and the breakdown of the blood-retinal barrier [16–18]. Thus, sterile inflammation caused due to the long-term hyperglycemic insult is thought to play a central role in the early- and late-stages of DR pathogenesis [19–22]. Several inflammatory markers including advanced glycation end products (AGEs), matrix metalloproteinases (MMP)-9, interleukin 1 beta (IL-1β), interleukin 6 (IL-6), intercellular adhesion molecule 1 (ICAM-1) and tumor necrosis factor-α (TNF-α) have been reported in the serum, vitreous, and retina tissue of DR subjects [21,23–25]. These inflammatory mediators are also suggested as potential sources of insult to the retina during DR. Local cellular stress stimulates the release of endogenous danger signals called damage-associated molecular patterns (DAMPs). They contribute to the amplification of acute inflammatory response via interaction with pattern recognition receptors (PRRs) or specific receptors on resident immune cells, thereby modulating innate immune response locally [26,27]. The best-known DAMPs include the heat shock proteins (HSPs), high-mobility group box 1 protein (HMGB1) and S100 proteins [28,29].

S100 proteins are $Ca^{2+}$ binding proteins with a helix-loop-helix binding domain. In humans, the S100 family of 24 proteins are involved in a myriad of cellular functions including proliferation, motility, apoptosis and inflammatory responses [30,31]. Of all, myeloid-related proteins, S100A8 (also known as Myeloid-related protein 8) and S100A9 (Myeloid-related protein 14) are constitutively expressed in human neutrophils and macrophages [32]. High concentrations of circulating S100A8 and S100A9 proteins have been widely predicted as a marker for inflammatory diseases [33]. These include peritonsillar abscess [34], inflammatory bowel disease (IBD) [35], rheumatic diseases [36], cardiovascular diseases [37], lupus nephritis [38] and diabetes [39]. In the eye, we have described the role of S100A8 and S100A9 proteins in ocular surface diseases such as dry eye and pterygium [40]. Recently, S100A8 and S100A9 were found to be associated with arthritis-associated uveitis [41] and acute anterior uveitis [42] in humans, which was targeted for therapy in experimental autoimmune uveitis [43] in rats. In the posterior part of the eye, autoantibodies against S100A9 was observed in patients with age-related macular degeneration (AMD) [44], and S100A9 protein was reportedly upregulated in vitreous of patients with PDR [45]. This cohort study was designed to study the correlation, if any, between plasma concentrations of S100A8 and S100A9 proteins with the severity of DR with and without maculopathy in T2DM patients.

## 2. Materials and methods

### 2.1. Study population

This was an observational study of DR subjects with pre-existing T2DM (n = 117) where age-matched patients without retinopathy served as controls. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Narayana Nethralaya Multispecialty Eye Hospital, Bangalore, India (C/2014/

**Table 1**
Cohort characteristics of control and diabetic retinopathy subjects.

| | Ctrl | DR | ETDRS Grading | | Gloucestershire Diabetic Eye Screening Program Classification | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | NPDR | PDR | R1M0 | R1M1 | R2M0 | R2M1 | R3M0 | R3M1 |
| Number of Patients | 28 | 89 | 44 | 45 | 16 | 4 | 11 | 12 | 29 | 17 |
| Age | 62 | 60 | 61 | 59 | 61 | 49 | 61 | 60 | 59 | 60 |
| | (43–79) | (44–89) | (45–89) | (44–75) | (50–89) | (45–62) | (52–71) | (46–70) | (44–75) | (45–68) |
| Sex (M/F) | 21/7 | 62/27 | 28/16 | 34/11 | 11/5 | 3/1 | 8/3 | 5/7 | 24/5 | 11/6 |
| Body mass index (kg/m$^2$) | 26.5 | 26.5 | 26.7 | 26.3 | 26.4 | 27.2 | 27.3 | 26 | 25.9 | 26.9 |
| | (23.2–30.1) | (22.1–31.9) | (22.8–31.6) | (22.1–31.9) | (22.8–31.2) | (26.3–29.5) | (24.1–31.6) | (23.5–31.0) | (22.3–29.0) | (22.1–31.9) |
| Fasting blood sugar (mg/dl) | 132.5 | 152 | 157 | 152 | 176.5 | 162.5 | 145 | 159 | 158 | 148 |
| | (65–303) | (48–325) | (88–325) | (48–321) | (96–300) | (102–236) | (99–325) | (88–283) | (90–321) | (48–280) |
| Postprandial blood sugar (mg/dl) | 180 | 250* | 250 | 252 | 271 | 255 | 237 | 200 | 266 | 250 |
| | (102–379) | (100–400) | (116–400) | (100–390) | (149–400) | (190–320) | (116–362) | (145–320) | (107–390) | (100–340) |
| HBA1C (%) | 7 | 8.2* | 8.6† | 8 | 8.7 | 8.4 | 8.9 | 8.3 | 8 | 8.5 |
| | (5.0–14.0) | (3.1–14.5) | (6.7–14.5) | (3.1–12.2) | (6.7–14.5) | (7.4–10.5) | (7.0–11.2) | (6.8–11.1) | (3.1–11.4) | (6.5–12.2) |
| Haemoglobin (gm/dl) | 13.6 | 13.3 | 13.5 | 12.9 | 14 | 15.1 | 13.3 | 12.7 | 12.9 | 13 |
| | (10.5–16.7) | (7.8–35) | (7.8–35) | (8.4–16.8) | (12.7–35) | (7.8–15.5) | (11.4–18.1) | (10.0–14.9) | (8.4–16.8) | (10.7–15.6) |
| Age of onset of diabetes mellitus (yrs) | 52.5 | 45.0* | 47 | 43 | 44.5 | 44 | 49 | 49.5 | 40* | 48 |
| | (23–69) | (24–79) | (25–79) | (24–64) | (25–79) | (40–55) | (36–61) | (35–60) | (24–64) | (27–61) |
| Duration of diabetes mellitus (yrs) | 11 | 12 | 12 | 12 | 15 | 5.5 | 15 | 11 | 15 | 10 |
| | (1–32) | (0.25–40) | (0.25–40) | (2–30) | (0.25–40) | (1–10) | (3–25) | (3–25) | (4–30) | (2–30) |

Data represented as Median (Range); *P < 0.05, Mann-Whitney test/Unpaired *t*-test (compared to controls); †P < 0.05, Kruskal-Wallis test (compared to controls); DR - Diabetic retinopathy; NPDR - Non-proliferative diabetic retinopathy; PDR - Proliferative diabetic retinopathy; RM - Gloucestershire Diabetic Eye Screening Program based classification.

07/04) with the regulations of Indian Council of Medical Research (ICMR).

### 2.2. Clinical evaluation of study participants and grading of diabetic retinopathy

Patients attending the Vitreoretinal Clinic at the Narayana Nethralaya Hospital underwent the following procedures as a part of routine clinical care. Detailed medical history collected by trained medical professionals included: (a) demographic information: age, sex; (b) duration of diabetes in years and onset of diabetes by age. Inclusion for study participation required the patient to fulfill at least 1 of the following criteria: (i) clinically diagnosed type II diabetes with HbA1c levels above 7%; (ii) fasting blood sugar >120 mg/dl or random blood sugar >200 mg/dl; (iii) history of diabetes and on antidiabetic medication (oral hypoglycemic agents or insulin). Patients with co-existing glaucoma, history of any previous intraocular surgery other than cataract, and any other co-existing degenerative ocular disorders such as age-related macular degeneration (AMD) were excluded from the study.

### 2.3. Retinal screening and classification of diabetic retinopathy

For retinal screening, the patients were given mydriatic drops for 10 min to allow pupil dilation. Fundus examination was performed by fundus imaging (TRC 50DX, Topcon, Japan), and OCT image was taken on SpectralisTM (Heidelberg, Germany). DR grading was performed according to the Early Treatment Diabetic

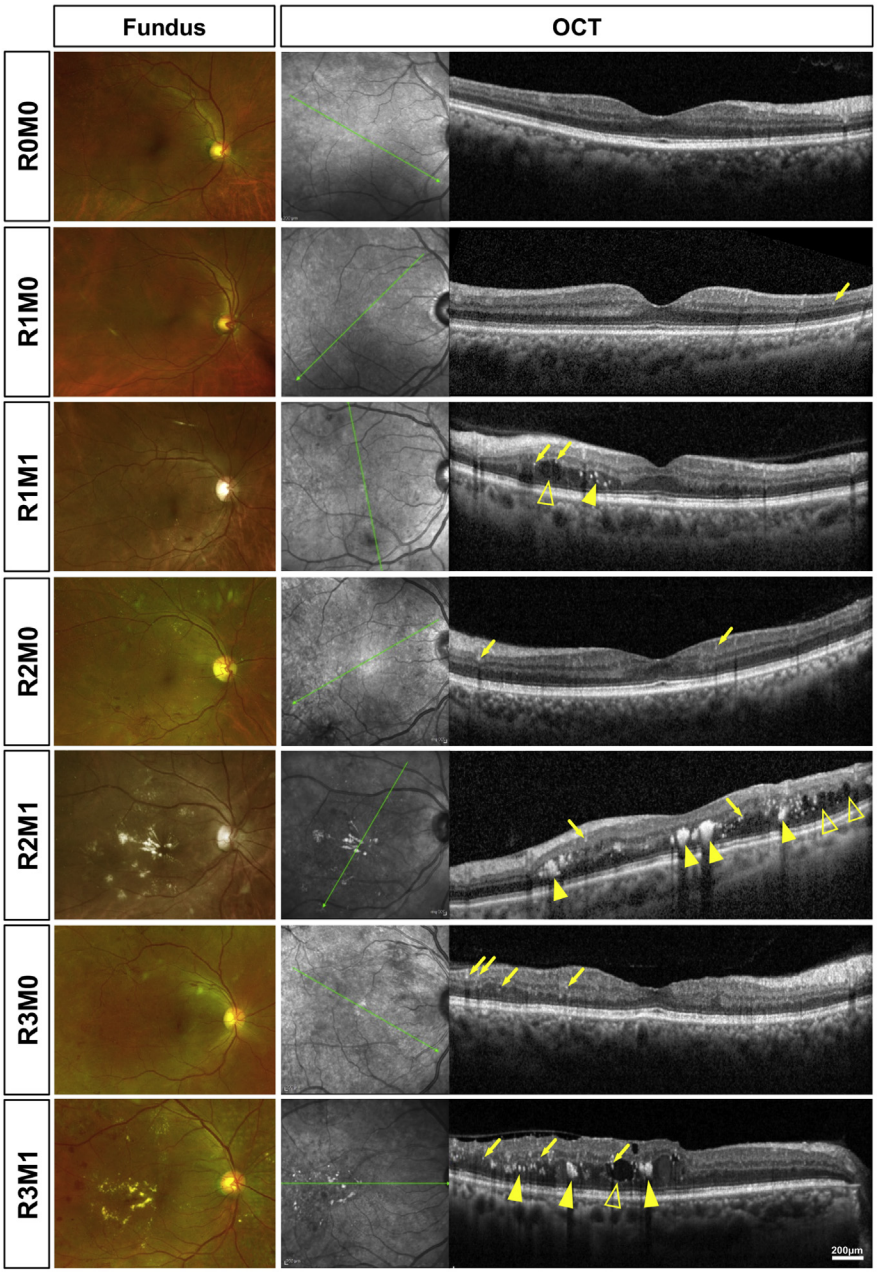


**Fig. 1.** Fundus photography and OCT images of retinopathy and maculopathy. Representative fundus photography and OCT images of diabetic patients retina. DR graded according to Gloucestershire classification. The degree of retinopathy as follows: R0 = no retinopathy, R1 = mild NPDR, R2 = moderate to severe NPDR, R3 = PDR. Maculopathy was denoted: M0: absent, M1: present. ▲, hard exudate; ↑, microaneurysm; Δ, intraretinal edema. Scale bar, 200 μm.

Retinopathy Study (ETDRS) classification system [46] and the Gloucestershire Diabetic Eye Screening Program [47]. According to ETDRS, the degree of retinopathy was classified into clinically defined non-proliferative DR (NPDR) and proliferative DR (PDR) sub-groups. In brief, NPDR was divided into mild, moderate and severe based on the degree of severity and location of the intra-retinal lesions. Mild NPDR had at least one microaneurysm and hemorrhage in all four fundus quadrants. Moderate NPDR had microaneurysms and hemorrhages of greater severity, as well as mild intra-retinal microvascular abnormalities (IRMA), venous bleeding and cotton wool spots. Severe NPDR had at least one severe microaneurysm and hemorrhage in all four fundus quadrants, or venous bleeding in two quadrants, or severe IRMA in one quadrant. PDR was defined as the stage when the proliferation of new vessels was seen in the retina. Further classification was made within both sub-groups depending on the presence of clinically significant macular edema (CSME), and PDR sub-group was distinguished by pan-retinal photocoagulation (PRP) and status post (s/p) PRP. The Gloucestershire clinical grades are as follows: R0-diabetes without DR; R1-mild NPDR; R2-moderate to severe NPDR; R3-PDR including vitreous hemorrhage and fibrous proliferation. Any associated maculopathy was classified: M0-no macular edema and M1-macular edema (hemorrhage or exudate within 1DD of fovea).

### 2.4. Plasma sample collection

Following medical history survey, 5 ml blood was collected from each subject into K2 EDTA lined vacutainers (BD, Franklin Lakes, NJ, USA). Blood tubes were transported to the research lab and processed within 2 h of collection. Plasma was separated from whole blood by centrifugation at 3000 rpm for 10 min. Samples were stored at −80 °C in aliquots until all patient samples were collected for ELISA.

### 2.5. S100A8 and S100A9 measurements

Human S100A8 and S100A9 DuoSet ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA) and used according to manufacturer's instructions. In brief, 96-well microplate was coated overnight with capture antibody, then blocked with reagent diluent for 1 h at room temperature. Samples and standards were incubated for 2 h at room temperature. Following the wash, the detection antibody was kept for 2 h, and streptavidin-HRP for additional 20 min at room temperature. The substrate solution was given for 20 min in the dark, and optical density was measured immediately after addition of stop solution, at 450 nm with wavelength correction at 540 nm. 100 μl of plasma samples diluted to 50-fold was used per sample for each ELISA assay.

### 2.6. Statistical analysis

Shapiro-Wilk normality test was used to determine the distribution of the data set. Unpaired *t*-test was used to analyze normally distributed data. Mann-Whitney test and Kruskal-Wallis test were used to analyze data sets with non-normal distribution. $p < 0.05$ was considered to be statistically significant. GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) and MedCalc® version 12.5 (MedCalc Software, Belgium) were used to perform the statistical analysis. All data presented as mean ± SEM.

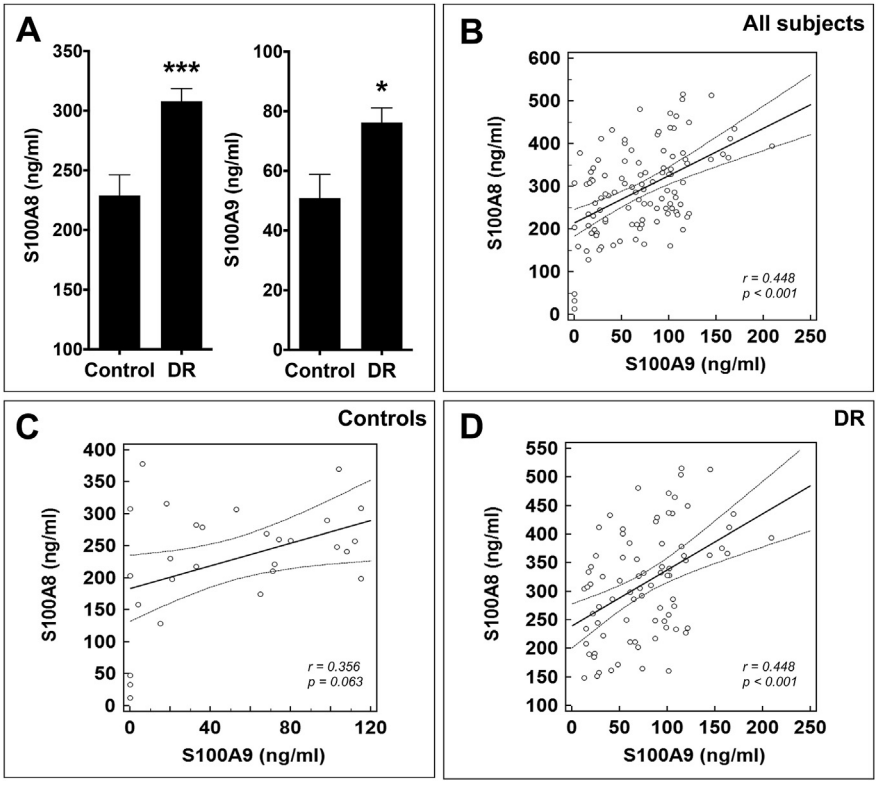


**Fig. 2.** Positive correlation between S100A8, S100A9 levels and DR. DR patients had significantly higher levels of plasma S100A8 (***, $p < 0.001$, unpaired two tailed *t*-test, $n = 89$) and S100A9 (*, $p < 0.05$, Mann Whitney test, $n = 80$) as compared to controls ($n = 28$) (**A**). Correlation analysis showed the positive relation between S100A8 and S100A9 levels in all subjects (**B,** $p < 0.001$, $r = 0.448$), which was contributed not by the controls (**C,** $p = 0.063$, $r = 0.353$) but by DR patients (**D,** $p < 0.001$, $r = 0.448$). 'r' is the Spearman's rank correlation coefficient. Bar graphs represent mean ± SEM.

## 3. Results

### 3.1. Study cohort characteristics

Clinical parameters of the study cohort, which fulfilled all inclusion and exclusion criteria are listed in Table 1. Of the enrolled subjects, 28 subjects were controls, and 89 subjects had retinopathy. The control T2DM subjects were aged 43–79 (median = 62) years old, and DR subjects aged 44–89 (median = 60) years old. The sex distribution in the cohort was 21/7 (M/F) in control and 62/27 (M/F) in DR subjects. Body mass index (BMI) showed no difference between the two groups. Of note, DR subjects had significantly higher postprandial blood sugar ($p = 0.012$) and HbA1c levels ($p = 0.003$) compared to T2DM controls. Fasting blood sugar levels were found to be higher in DR subjects but were not statistically significant. Interestingly, DR subjects had an earlier onset of diabetes ($p = 0.02$), although the duration of DM did not appear to affect retinopathy presentation. Representative fundus photography and OCT images of retinopathy and maculopathy according to Gloucestershire grading shown in Fig. 1.

### 3.2. Increased plasma S100A8 and S100A9 levels were correlated with DR severity

Significantly increased levels of S100A8 and S100A9 were observed in the plasma of subjects with DR (S100A8 = 308.1 ± 10.5 ng/ml; S100A9 = 76.2 ± 4.8 ng/ml) compared to that of T2DM controls (S100A8 = 229.1 ± 17.1 ng/ml; S100A9 = 50.8 ± 7.9 ng/ml) (Fig. 2A). An overall positive correlation between plasma S100A8 and S100A9 (Fig. 2B) was observed in the entire cohort. However, the significant correlation was primarily contributed by DR subjects (Fig. 2D) and not T2DM controls (Fig. 2C). Within the DR groups, an increase in S100A8 (Fig. 3A) and S100A9 (Fig. 4A) was found to be dependent on the severity of the disease. Based on Gloucestershire classification, subjects with R1M0, R2M1, R3M0 and R3M1 grading had significantly higher levels of plasma S100A8 compared to controls (Fig. 3B). The increase in R2M1, R3M0 and R3M1 was also significant to R2M0 group. Similarly, the elevated level of plasma S100A9 was seen in R3M0 and R3M1 grades, which was significant when compared to R0M0, R1M1 and R2M0 (Fig. 4B). When analyzed based on ETDRS classification, PDR subjects likewise showed significant levels of S100A8 (Fig. 3C) and S100A9 (Fig. 4C), whereas NPDR subjects only had elevated S100A8 in their circulation (Fig. 3C). Further analysis of S100A8 within subgroups revealed no difference between CSME in NPDR or PDR patients, however, the increase in S100A8 in the NPDR appeared to be contributed more by mild NPDR patients (Fig. 3D). The increase in S100A8 in PDR subjects was also significantly higher compared to NPDR subjects (Fig. 3D). In contrast, S100A9 in the PDR group was not significantly elevated in patients with CSME, but there was overall significance compared to the NPDR group (Fig. 4D).

### 3.3. DR patients showed gender-based differences in S100A8 and S100A9 levels

Gender-based difference between control (21 male, 7 female) and DR subjects (62 male, 27 female) is shown in Fig. 5. A significant difference in both plasma S100A8 (Fig. 5A) and S100A9 (Fig. 5B) levels was observed in male subjects with and without DR. However, in female subjects, a significant difference was only seen in

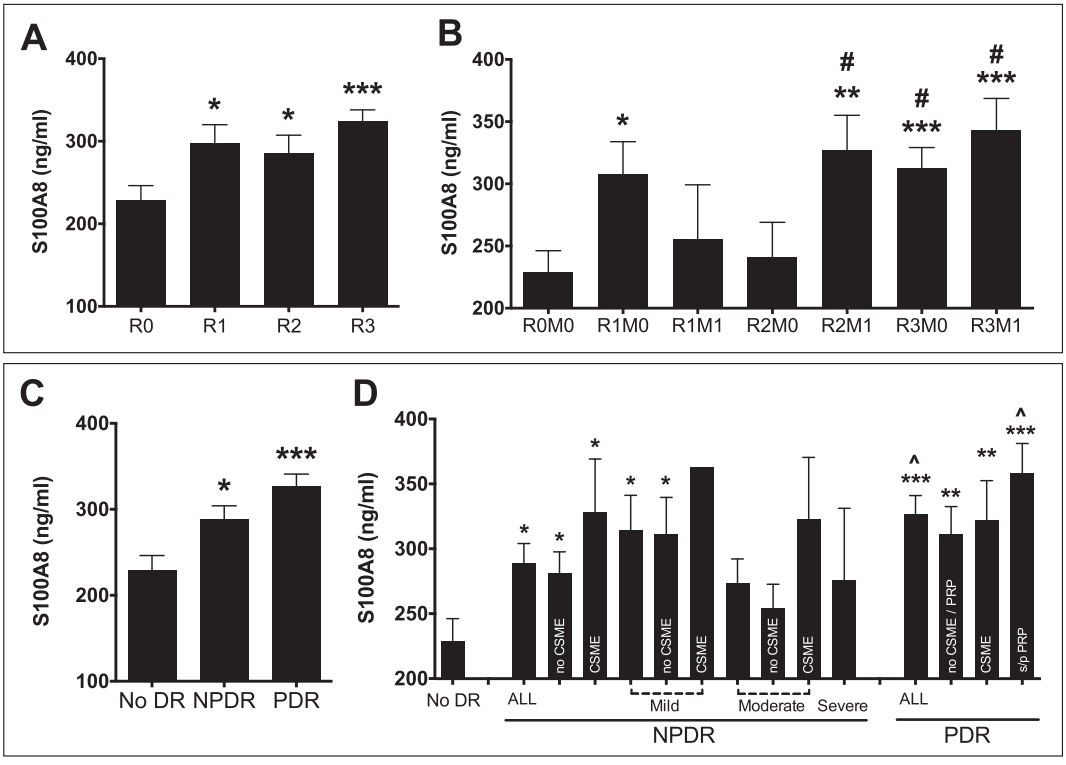


**Fig. 3.** S100A8 concentration was elevated in mild to severe DR. Plasma S100A8 in study cohort classified by (**A, B**) Gloucestershire and (**C, D**) ETDRS grading system. S100A8 was consistently higher in moderate NPDR to PDR patients. Unpaired two tailed *t*-test. Gloucestershire: *, $p < 0.05$ & ***, $p < 0.001$ compared to R0M0; #, $p < 0.05$ compared to R2M0. ETDRS: *, $p < 0.05$, **, $p < 0.01$ & ***, $p < 0.001$ compared to no DR; ^, $p < 0.05$ compared to NPDR. Controls (n = 28), R1 (n = 20), R2 (n = 23), R3 (n = 46), Controls/R0M0 (n = 28), R1M0 (n = 16), R1M1 (n = 4), R2M0 (n = 11), R2M1 (n = 12), R3M0 (n = 29), R3M1 (n = 17), Controls/No DR (n = 28), NPDR (n = 44), PDR (n = 45), NPDR-All (n = 44), NPDR No CSME (n = 37), NPDR + CSME (n = 7), mild NPDR (n = 16), moderate NPDR (n = 22), severe NPDR (n = 6), mild NPDR no CSME (n = 15), mild NPDR + CSME (n = 1), moderate NPDR no CSME (n = 16), moderate NPDR + CSME (n = 6), PDR all (n = 45), PDR no CSME/PRP (n = 21), PDR + CSME (n = 12), PDR + s/p PRP (n = 12). Bar graphs represent mean ± SEM.

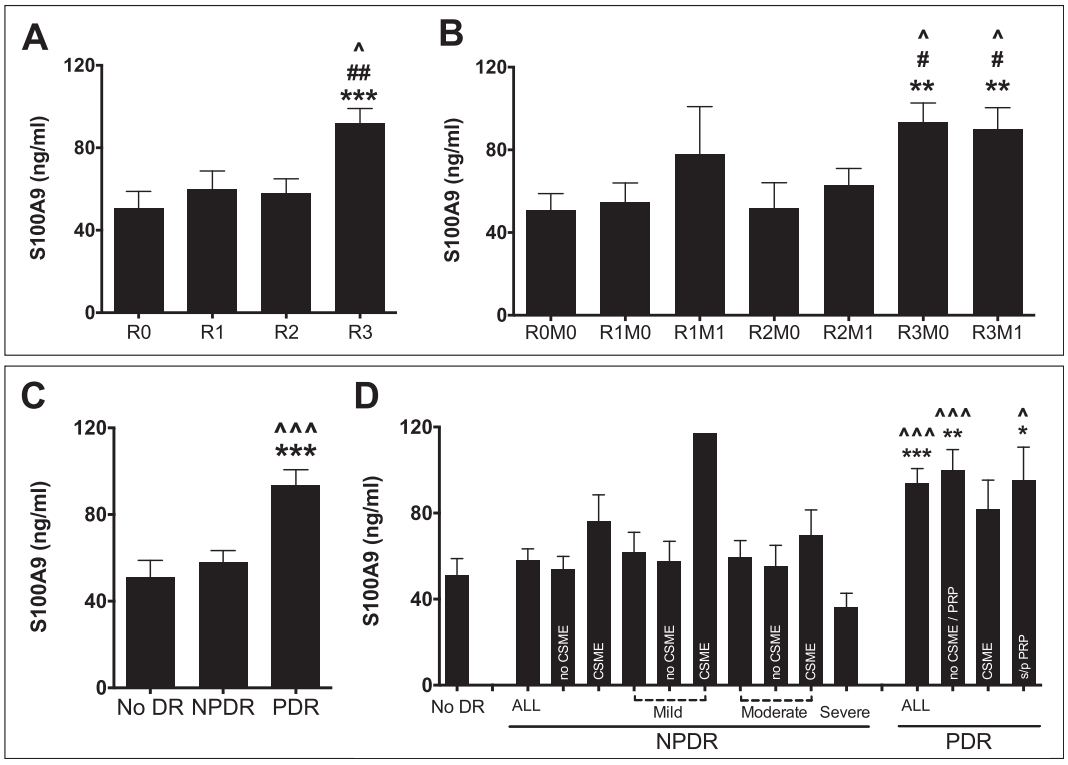


**Fig. 4.** PDR patients showed increased S100A9 concentrations in plasma. Plasma S100A9 in study cohort classified by (**A, B**) Gloucestershire and (**C, D**) ETDRS grading system. PDR patients were found to have elevated S100A9 protein in the plasma. Mann Whitney test. Gloucestershire: **, $p < 0.01$ & ***, $p < 0.001$ compared to R0M0; ^, $p < 0.05$ compared to R1M0; #, $p < 0.05$ & ##, $p < 0.01$ compared to R2M0. ETDRS: *, $p < 0.05$, **, $p < 0.01$ & ***, $p < 0.001$ compared to no DR; ^, $p < 0.05$ & ^^^, $p < 0.001$ compared to NPDR. Controls (n = 28), R1 (n = 18), R2 (n = 20), R3 (n = 42), Controls/R0M0 (n = 28), R1M0 (n = 14), R1M1 (n = 4), R2M0 (n = 9), R2M1 (n = 11), R3M0 (n = 27), R3M1 (n = 15), Controls/No DR (n = 28), NPDR (n = 39), PDR (n = 41), NPDR-All (n = 39), NPDR No CSME (n = 32), NPDR + CSME (n = 7), mild NPDR (n = 15), moderate NPDR (n = 20), severe NPDR (n = 4), mild NPDR no CSME (n = 14), mild NPDR + CSME (n = 1), moderate NPDR no CSME (n = 14), moderate NPDR + CSME (n = 6), PDR all (n = 41), PDR no CSME/PRP (n = 19), PDR + CSME (n = 11), PDR + s/p PRP (n = 11). Bar graphs represent mean ± SEM.

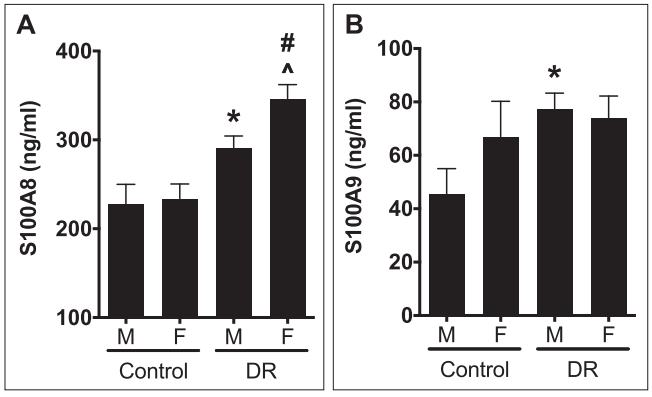


**Fig. 5.** Gender differences in S100 protein levels. Both male and female DR subjects had increased (**A**) S100A8 protein (unpaired two-tailed *t*-test), however, only males had increased (**B**) S100A9 protein in their blood plasma (Mann Whitney test). *, $p < 0.05$ control male vs DR male; ^, $p < 0.05$ control female vs DR female; #, $p < 0.05$ DR male DR vs DR female. S100A8: Control males (n = 21), Control females (n = 7), DR males (n = 62), DR females (n = 27). S100A9: Control males (n = 21), Control females (n = 7), DR males (n = 56), DR females (n = 24). Bar graphs represent mean ± SEM.

S100A8 levels (Fig. 5A) and not S100A9 (Fig. 5B). Furthermore, female DR patients showed elevated production of S100A8 as compared to the male DR patients (Fig. 5A).

### *3.4. Plasma S100A8 and S100A9 levels increase with age and DM duration in DR patients*

In the current cohort, subjects over 50 years of age showed significantly higher S100A8 (Fig. 6A) and S100A9 (Fig. 6B) expression after DR presentation. DM onset after 50 years of age also appeared to increase S100A8 and S100A9 protein expression as DR develops (Fig. 6C and D). S100A8 was also found to be elevated in DR patients with DM onset before 50 years old (Fig. 6C). Moreover, S100A8 (Fig. 7A) levels were elevated in DR patients with >10 years of diabetes duration, which is not surprising since DR symptoms often manifest after similar periods of time. Likewise, S100A9 showed an increased expression in DR patients with <10 years of diabetes duration. (Fig. 7B).

## 4. Discussion

Chronic hyperglycemia and inflammation are acutely related to diabetic patients [48]. Likewise, the elevation of inflammatory and other factors was reported in the circulation of patients with DR [25]. In addition, DAMPs such as HSPs [49] and HMGB1 [50] have also been implicated in diabetes complications such as DR. In the present study, we show for the first time elevated levels of circulatory S100A8 and S100A9 in DR subjects (Fig. 2) as compared to T2DM controls. We further showed the correlation of increased protein presence with the severity of DR (Figs. 3 and 4). These findings indicate a likely role of S100A8 and S100A9 proteins as potential contributors to further aggravate the existing inflammatory milieu involved in the pathogenesis of DR.

S100A8 and S100A9 play decisive roles in the development of inflammation [33]. They are constitutively expressed at high levels in neutrophils and low levels in monocytes and are also produced by epithelial and stromal tissues at the site of injury [51]. A recent

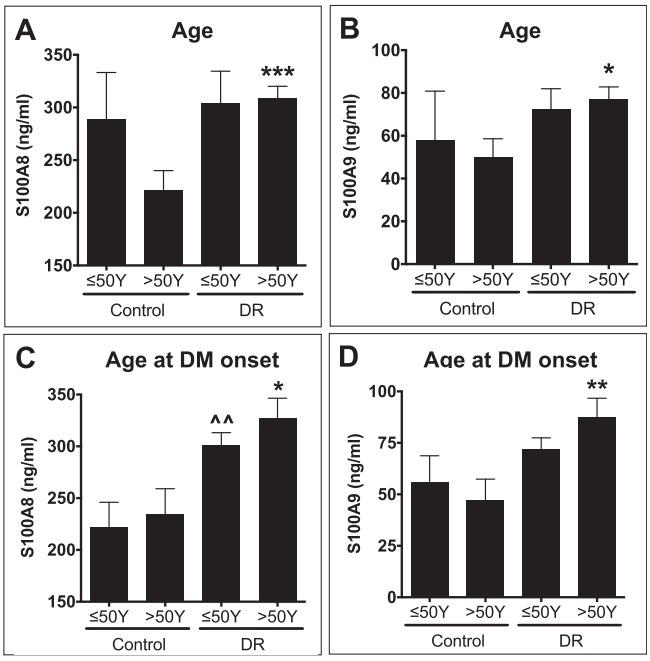


**Fig. 6.** Patient age and late diabetes onset increased S100 protein expression. (**A**) S100A8 (***, $p < 0.001$ Controls >50Y vs DR > 50Y, unpaired two tailed *t*-test) and (**B**) S100A9 (*, $p < 0.05$ Controls >50Y vs DR > 50Y, Mann Whitney test) were significantly elevated in DR patients aged 50 years and above. Age at diabetes onset did not affect (**C**) S100A8 expression in DR patients (*, $p < 0.05$ Controls >50Y vs DR > 50Y; ^^, $p < 0.01$ Controls ≤50Y vs DR ≤ 50Y, unpaired two tailed *t*-test) while (**D**) S100A9 expression was not significant in patients diagnosed with T2DM < 50 years old but was significantly elevated in subjects diagnosed after 50 years of age (**, $p < 0.01$, unpaired two tailed *t*-test). A) S100A8: Controls ≤50Y (n = 3), Controls >50Y (n = 25), DR ≤ 50Y (n = 16), DR > 50Y (n = 73). B) S100A9: Controls ≤50Y (n = 3), Controls >50Y (n = 25), DR ≤ 50Y (n = 15), DR > 50Y (n = 65). C) S100A8: Controls ≤50Y (n = 12), Controls >50Y (n = 16), DR ≤ 50Y (n = 64), DR > 50Y (n = 25). D) S100A9: Controls ≤50Y (n = 12), Controls >50Y (n = 16), DR ≤ 50Y (n = 57), DR > 50Y (n = 23). Bar graphs represent mean ± SEM.

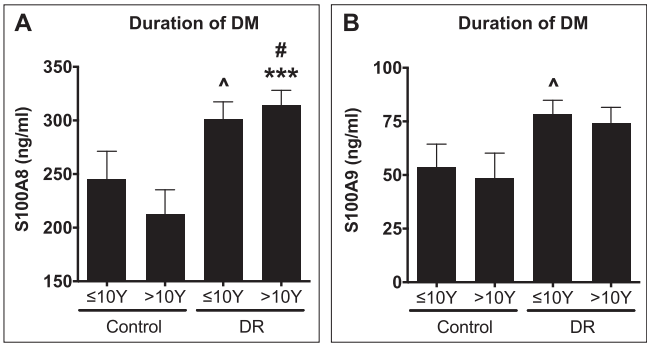


**Fig. 7.** S100A8 and S100A9 levels increased regardless of the duration of diabetes mellitus. (**A**) Plasma levels of S100A8 protein show significantly high levels in DR patients regardless of the duration of diabetes (***, $p < 0.05$ Controls >10Y vs DR > 10Y; ^, $p < 0.05$ Controls >10Y vs DR ≤ 10Y; #, $p < 0.05$ Controls ≤10Y vs DR > 10Y, Mann Whitney test). (**B**) S100A9 concentrations show significant upregulation only in the patients with less than 10 years of diabetes (^, $p < 0.05$ Controls >10Y vs DR ≤ 10Y, Mann Whitney test). S100A8: Controls ≤10Y (n = 14), Controls >10Y (n = 14), DR ≤ 10Y (n = 42), DR > 10Y (n = 47). S100A9: Controls ≤10Y (n = 14), Controls >10Y (n = 14), DR ≤ 10Y (n = 40), DR > 10Y (n = 40). Bar graphs represent mean ± SEM.

publication described S100A9 presence in the epiretinal fibrovascular membrane and vitreous fluid of PDR patients. These subjects had either insulin-dependent or insulin-independent diabetes. In addition, they showed S100A9 to be expressed by vascular endothelial cells, stromal cells and CD45 positive leukocytes in the epiretinal membranes. However, the authors were undetermined on whether S100A9 caused inflammation in the PDR retina [45]. Intense upregulation of proinflammatory cytokines such as TNFα and IL-1β is often seen in response to S100A8 and A9 proteins, which results in the perpetuation of the inflammatory response [33,52]. As the members of the DAMPs family of proteins, S100A8 and S100A9 are heavily involved in the modulation of innate immune response to cellular injury, via intracellular functions in the cytoskeletal organization, and extracellular roles in leukocyte migration and recruitment [33]. This has led to their establishment as a marker for differential diagnosis, monitoring of treatment response and disease relapse in many inflammatory diseases [33], including type 1 diabetes [53]. Hence it should be noted that S100A8 and S100A9 are not disease-specific. Thus, we have excluded patients with co-existing systemic disorders such as IBD, sclerosis, arthritis, nephropathy, and autoimmune diseases in this study. Nevertheless, an increase in circulatory S100A8 and S100A9 suggest an additive effect accompanying the deterioration of the retina in diabetic conditions.

Disruption of the retinal inner blood-retina barrier (iBRB) is pertinent to the progression of DR. S100A8 and S100A9 are known to reduce cell junctional proteins – Occludin, ZO-1, ZO-2 and β-catenin in human umbilical vein endothelial cells [54], as well as activate dermal endothelial cells by upregulation of ICAM-1 on microvascular endothelial cells for binding to Mac-1 on phagocytes [55]. Furthermore, a clinical report showed a positive correlation of S100A8/S100A9 and larger retinal arteriolar lumen caliber in patients with diabetic nephropathy, which the authors discussed to have resulted from the failure of blood flow regulation [56]. However, an increased retinal vessel caliber is a pathological sign often seen in DME, a condition in NPDR and PDR sub-classified by fluid accumulation in the macula. Plasma S100A8 and S100A9 levels in this study strongly correlated with maculopathy or clinically significant DME, hence further indicating an association of DAMPs with disrupted BRB in DR subjects.

The two S100 proteins form homodimer and heterodimers which are biologically active. Heterodimer configurations are more stable; however, proinflammatory stimulus or S100A8 appears to stabilize the S100A9 homodimer [57], making it proteolytically resistant. The complexes bind to PRR toll-like receptor 4 (TLR4) in both mono- and homodimeric form, and receptor for advanced glycation end products (RAGE) in heterodimeric form. The binding to PRR initiates MAP kinase and NF-kB signaling [58] that increases production of proinflammatory cytokines and chemokines such as TNFα, thereby amplifying the inflammatory response. However, S100A8 and S100A9 also have anti-inflammatory functions [33]. Interestingly, we observed a sharp increase in S100A9 in PDR patients as compared to controls and NPDR subjects, which could be indicative of protective functions in NPDR while iBRB is still intact. It would be interesting to investigate further if the S100A8 and S100A9 homodimers or heterodimers have different roles during the early- and advanced-stages of DR.

Gender-associated differences in diabetes complications are influenced by multiple processes ranging from biological, sociocultural to environmental factors [59]. Similarly, the association between gender and diabetic retinopathy varies depending on the study population [60]. In this study, female DR subjects showed significantly high S100A8 presentation compared to their male counterparts (Fig. 5), which is in line with reports on higher inflammatory stress in women [61,62]. On the other hand, there is a downward trend of S1008 and S100A9 in T2DM subjects after the age of 50, which was absent in patients with DR (Fig. 6A and B). This suggests continual production of the inflammatory factors with ongoing diabetic complications, which could be noteworthy to examine the source and contributing factors leading to increased

S100A8 and S100A9 expression with DR. Patients with T2DM onset at ages <45 have been reported to be at higher risk for developing DR, though the duration of diabetes is associated with both younger- and older-onset diabetes [63,64]. Hence it is not surprising to find elevated S100A8 and S100A9 in DR subjects regardless of DM duration (Fig. 7), but interesting to note that the upregulation of S100A9 only occurs in DR patients with older-onset diabetes (Fig. 6D). This could indicate a relationship between S100 proteins and age-related degenerative changes in older patients.

### 4.1. Limitations

Our study had some limitations that should be taken into consideration. Firstly, the sample size while sufficient in totality, was insufficient when divided into classifications of DR severity, especially on the EDTRS system. Therefore several trending patterns were noted but not discussed due to statistical insignificance. This also limited the power to analyze systemic co-morbidities or associations with anti-diabetic drugs if any in the patient cohort. Secondly, all subjects were recruited from the same hospital within a short period, which might contribute to regional and temporal bias. Thirdly, the study cohort was not evenly divided in gender, hence could limit interpretation of results.

Nonetheless, our study show strength as the first to reveal associations between S100A8 and S100A9 and varying severity of DR. The continued production of S100 proteins with advancing stages of retinopathy suggests the systemic influence on retinal disease aggravation after the loss of iBRB integrity. This is also the first study to report S100A8 and S100A9 among the diabetic Indian population.

## 5. Conclusions

In summary, our study suggests S100A8 and S100A9 proteins have a direct correlation with DR severity, contributed by possible roles in aggravation of the inflammatory milieu during DR progression. The ubiquitous presence of S100A8 and S100A9 in diabetic subjects and subsequent elevation upon DR detection warrants further investigation of their potential use as prognosis markers, while examination of their effects on the retina could reveal their relevance as therapeutic targets for the treatment of DR.

## Author contributions

SSC, DPH and, AG designed study; TV, SGG, NKY and SS collected patient information and performed experiments; RRL, RRM, TV, AG and SSC interpreted and analyzed data; RRL and TV wrote the manuscript with input from AG and SSC; all authors reviewed the manuscript.

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## Disclosure statement

Authors declare no conflicts of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dsx.2019.03.014.

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