Serum Iba-1, GLUT5, and TSPO in Patients With Diabetic Retinopathy: New Biomarkers for Early Retinal Neurovascular Alterations? A Pilot Study

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Purpose: This study explored the possibility of highlighting early retinal neurovascular alterations of diabetic retinopathy (DR) by monitoring in DR patients the serum levels of microglial biomarkers ionized calcium-binding adapter molecule 1 (Iba-1), glucose transporter 5 (GLUT5), and translocator protein (TSPO), along with serum changes of the endothelial dysfunction marker arginase-1.

Methods: Serum markers were determined by enzyme-linked immunosorbent assay in 50 patients: 12 non-diabetic subjects, 14 diabetic patients without DR, 13 patients with non-proliferative DR (NPDR), and 11 patients with proliferative DR (PDR). The results were correlated with hyperreflective retinal spots (HRS), observed with optical coherence tomography (OCT).

Results: Although HRS were absent in diabetic patients without DR, NPDR patients showed an average of 4 ± 1 HRS, whereas the highest presence was detected in PDR patients, with 8 ± 2 HRS (P < 0.01 vs. NPDR). HRS were positively correlated (P < 0.01) with serum levels of arginase-1 (r = 0.91), Iba-1 (r = 0.96), GLUT5 (r = 0.94), and TSPO (r = 0.88). Moreover, serum proinflammatory cytokines and chemokines showed a positive correlation (P < 0.01) with HRS number and the serum markers analyzed.

Conclusions: Serum markers of microglial activation positively correlate with retinal HRS in NPDR and PDR patients.

Translational Relevance: These data corroborate the possibility of highlighting early retinal neurovascular changes due to diabetes by monitoring circulating microglial markers.

Introduction

Neurodegeneration during diabetic retinopathy (DR) is an early phenomenon occurring together with inflammation and is strictly dependent on structural and functional modifications of microglia and macroglia (astrocytes and Müller cells), likely caused by persistent high glucose.¹,² These modifications, sometimes evident in diabetic patients before any clinical signs of DR, may be identified as hyperreflective intraretinal spots (HRS), visible by optical coherence tomography (OCT).³,⁴ Spots usually corresponding to aggregates of microglial cells rush to the site as early as high glucose concentrations begin, resulting in oxidative stress and hypoxia in the retina and leading to late neurovascular alterations typical of DR and progressive states.⁴–⁶ Therefore, the evaluation of microglial activation during hyperglycemia/diabetes may be a tool to monitor the sequelae leading to...
the appearance and progression of DR damage. Particularly, monitoring the serum biomarkers of activated microglia may correlate with early HRS insurgence and, subsequently, with clinical neurovascular alterations. A simple and non-invasive way to monitor this process in humans could be evaluation of the circulating M1 microglial marker ionized calcium-binding adapter molecule 1 (Iba-1) in serum. This suggestion is based on a study by Lafrenaye et al., which reported that changes in the levels of serum Iba-1 and glial fibrillary acidic protein, a marker of activated astrocytes, reflect the pathological neurovascular sequelae following a traumatic brain injury. They claimed that changes in circulating markers of microglia mirrored changes due to brain injury.

Due to the availability of specific commercial enzyme-linked immunosorbent assays (ELISAs), the aim of the present study was to investigate the time of appearance and serum levels of the microglial biomarkers Iba-1, glucose transporter 5 (GLUT5), and translocator protein (TSPO) in patients with DR, and then to correlate them with the number of HRS observed by OCT in patients with either non-proliferative DR (NPDR) or proliferative DR (PDR). This study was designed to establish whether or not changes in these serum markers reflect initiation or evolution of DR and thus can serve as prognostic markers in humans.

Furthermore, it is important to consider that the typical DR vascular signs, due to increased permeability and increased extravasation of monocytes, are only visible with fluorescein angiography. Because these retinal alterations are preceded by macro- and microcirculation endothelial dysfunction, we also analyzed the serum levels and activity of arginase-1, a marker of endothelial dysfunction found to be related to retinal endothelial damage in animal models.

### Methods

#### Clinical Study Design

The study was approved by the local ethics committee of the AOU University of Campania “Luigi Vanvitelli” (protocol number 42 DEC, 30-1-2019) and was performed at the Eye Clinic of the University of Campania “Luigi Vanvitelli” (Naples, Italy), where 50 patients were recruited between January 1, 2019, and December 31, 2019. All participants read and signed informed consents after receiving an explanation of the nature and possible consequences of the study. All of the procedures during the study adhered to the tenets of the Declaration of Helsinki and Good Clinical Practice guidelines. For all of the subjects, a baseline examination was performed, during which the informed consent was obtained and the inclusion and exclusion criteria were evaluated (Table 1).

#### Evaluation of DR Stages

Medical histories were obtained, the ocular fundus was examined, best-corrected visual acuity (BCVA) was determined, and spectral-domain OCT (SD-OCT) analyses were performed in order to evaluate DR stages. According to the American Academy of Ophthalmology Guidelines, DR was staged based on the number of hemorrhages, microaneurysms, intraretinal microvascular abnormalities, and retinal neovascularization.

#### Detection of HRS Number

An OCT horizontal scan (Cirrus SD-OCT system; ZEISS, Jena, Germany) was used to analyze the number of HRS. Two retinal ophthalmologists identified HRS as well-circumscribed dots of equal or higher intensity.

### Table 1. Inclusion and Exclusion Criteria for Study Participants

| Inclusion Criteria                                                                 | Exclusion Criteria                                                                 |
|-----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| Age of at least 45 years                                                          | Uncontrolled diabetes                                                               |
| Diabetes duration of at least 5 years                                             | Vitrectomy and argon laser coagulation in the last 6 months                         |
| NPDR or PDR clinically diagnosed by medical history, examination of ocular fundus | Anti-vascular endothelial growth factors or steroid intravitreal injections in the last 12 months |
| BCVA, and spectral domain OCT                                                      | Use of lipid-lowering agents, non-steroidal anti-inflammatory drugs, steroids, or immunosuppressive drugs |
|                                                                                 | Systemic or recent infections                                                       |
|                                                                                 | Recent cardiovascular or cerebrovascular events                                      |
|                                                                                 | Other severe diabetic complications (nephropathy/ketoacidosis)                      |
reflectivity as the retinal pigment epithelium band, with a diameter within 20 to 40 μm. HRS were then counted within an area of analysis that had a radius of 1500 μm, with the origin in the fovea. The two operators (GC, FP) were unaware of DR staging.

**Collection of Serum Samples**

Fasting venous blood samples were obtained from all enrolled subjects through the use of dry and sterile vacutainer tubes. Within 2 hours from blood sampling, whole blood was incubated for 30 minutes at 20°C and then centrifuged at 3000 rpm for 15 minutes at 4°C. Sera were aliquoted and stored at −80°C for the subsequent analysis of serum markers. This was performed at the Pharmacology Section of the University of Campania “Luigi Vanvitelli” (Naples, Italy) by ELISA.

**Enzyme-Linked Immunosorbent Assays**

Arginase-1 activity and levels, as well as Iba1 (or allograft inflammatory factor 1), GLUT5, TSPO, and cytokine/chemokine levels, were assessed in sera of patients with different DR stages using commercial ELISAs according to the manufacturers’ protocols: KA1609 Arginase 1/ARG1/Liver Arginase Assay Kit (Colorimetric) (Novus Biologicals, Littleton, CO); EH1274 Human Arginase-1 ELISA Kit (FineTest, Wuhan, China); EH1425 Human AIF1 ELISA Kit (FineTest); MBS9304003 Human GLUT5 Elisa Kit (MyBioSource, San Diego, CA); CSB-EL025168HU Human TPSO Elisa Kit (Cusabio Technology, Houston, TX); SEJ628Hu ELISA Kit for Translocator Protein (TSPO) (Cloud-Clone Corporation, Wuhan, China); and ARY005B Proteome Profiler Human Cytokine Array Kit (R&D Systems, Minneapolis, MN).

**Statistical Analysis**

Differences between groups were analyzed by using a one-way analysis of variance (ANOVA) followed by the Tukey–Kramer post hoc test for multiple group comparison. The strength of association between serum markers and HRS number was evaluated by Pearson correlation analysis. P < 0.05 was considered significant. Statistical analysis and graph design were performed with Prism 6.0 (GraphPad, San Diego, CA).

**Results**

**Subjects**

A total of 50 patients (31 males and 19 females) were recruited for the study. Based on the ocular evaluations, these were divided into the following groups:

1. CTRL group—non-diabetic subjects without ocular pathologies (n = 12)
2. Diabetic group—diabetic patients without signs of DR (n = 14)
3. NPDR group—diabetic patients with non-proliferative DR (n = 13)
4. PDR group—diabetic patients with proliferative DR (n = 11)

Among the three disease groups, no significant differences in age or diabetes duration were observed (Table 2).

| Table 2. Clinical Characteristics of Control Subjects (n = 12), Diabetic Subjects Without Signs of DR (n = 14), Patients With NPDR (n = 13), and Patients With PDR (n = 11) |
|----------------------------------------|---------|---------|---------|---------|
| Age (y), range                        | 48–66   | 49–68   | 46–65   | 47–69   |
| Age (y), mean ± SD                    | 56.5 ± 6.5 | 55.7 ± 5.9 | 55.6 ± 6.6 | 56.8 ± 7.1 |
| Female, n                             | 5       | 5       | 5       | 4       |
| Male, n                               | 7       | 9       | 8       | 7       |
| Type of diabetes (%)                  |         |         |         |         |
| Type I                                | NA      | 27      | 40      | 58      |
| Type II                               | NA      | 73      | 60      | 42      |
| Diabetes duration (y), mean ± SD      | NA      | 6.5 ± 0.5 | 7.0 ± 0.7 | 8.0 ± 1.0 |
| Time from DR diagnosis (y), mean ± SD | NA      | NA      | 2.5 ± 0.3 | 2.9 ± 0.1 |

NA, not applicable.
Figure 1. HRS determination by OCT in non-diabetic subjects (n = 12), diabetic subjects without signs of DR (n = 14), patients with NPDR (n = 13), and patients with PDR (n = 11). The numbers of HRS (white circles) are reported as mean ± SD. Statistical significance was calculated with one-way ANOVA followed by Tukey’s comparison test. **P < 0.01 versus diabetic; ***P < 0.01 versus NPDR. Scale bar: 100 μm.

HRS Number

HRS were absent in both the CTRL and diabetic groups. NPDR patients showed an increase in number of HRS compared with diabetic patients (4 ± 1 HRS; P < 0.01 vs. diabetic), whereas the highest presence of HRS was detected in PDR patients (8 ± 1 HRS; P < 0.01 vs. NPDR) (Fig. 1).

Serum Iba-1, GLUT5, and TSPO Levels

The diabetic group exhibited low serum Iba-1 levels (29 ± 10 pg/mL), similar to those of the CTRL subjects (33 ± 7 pg/mL). Interestingly, NPDR patients showed a significant increase in serum Iba-1 levels compared with the diabetic group (741 ± 111 pg/mL; P < 0.01 vs. diabetic), whereas the highest serum Iba-1 content was detected in PDR patients (1392 ± 200 pg/mL; P < 0.01 vs. NPDR) (Fig. 2). Likewise, the CTRL and diabetic subjects showed similar serum levels of GLUT5 (CTRL, 2.1 ± 0.9 ng/mL; diabetic, 2.2 ± 0.8 ng/mL) and TSPO (CTRL, 2.2 ± 0.7 ng/mL; diabetic, 2.0 ± 0.8 ng/mL). These levels significantly increased in patients with NPDR (GLUT5, 5.4 ± 0.8 ng/mL; TSPO, 4.6 ± 1.0 ng/mL; both P < 0.01 vs. diabetic) and in those with PDR (GLUT5, 8.5 ± 1.0 ng/mL; TSPO, 8.0 ± 1.3 ng/mL; both P < 0.01 vs. NPDR) (Fig. 2).

HRS Number Correlates With Microglial Activation

Among the group of subjects, HRS number showed a significant positive correlation (P < 0.01) with all the
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Figure 2. ELISA evaluations of Iba-1, GLUT5, and TSPO levels in the serum of non-diabetic subjects (n = 12), diabetic subjects without signs of DR (n = 14), patients with NPDR (n = 13), and patients with PDR (n = 11). Serum Iba-1 levels are reported as pg/mL ± SD; serum GLUT5 and TSPO levels are reported as ng/mL ± SD. Statistical significance was calculated with one-way ANOVA followed by Tukey’s comparison test. **P < 0.01 versus diabetic; ***P < 0.01 versus NPDR.

Microglial serum markers analyzed (Iba-1, r = 0.96; GLUT5, r = 0.93; TSPO, r = 0.88). This indicated a significant association between the increasing number of HRS in NPDR and PDR patients and the higher levels of the serum markers (Fig. 3).

Serum Arginase-1 Detection and Correlation With HRS Number

Diabetic patients exhibited serum arginase-1 levels (35 ± 6.8 ng/mL; P < 0.01 vs. CTRL) and activity (5.8 ± 1.3 U/L; P < 0.01 vs. CTRL) that were significantly higher compared with those of the CTRL subjects (levels, 2.4 ± 1.2 ng/mL; activity, 2.0 ± 0.4 U/L). Serum arginase-1 levels progressively increased in patients with NPDR, as well as arginase-1 activity (65.5 ± 12.6 ng/mL and 8.8 ± 0.8 U/L, respectively; both P < 0.01 vs. diabetic). A similar increase was detected when comparing patients with PDR to those with NPDR (123.0 ± 20 ng/mL and 13.9 ± 1.5 U/L, respectively; both P < 0.01 vs. NPDR), with PDR patients showing the highest values of serum arginase-1 levels and activity. This increase was positively correlated with number of HRS (serum arginase-1 levels, r = 0.91; activity, r = 0.89; both P < 0.01) (Fig. 4).

Serum Cytokines and Chemokines

Diabetic patients showed a significant increase in the levels of serum C-X-C motif chemokine ligand
Correlation of Serum Cytokines/Chemokines With HRS and Other Serum Markers

The serum levels of proinflammatory cytokines modulated during the different DR stages showed a significant positive correlation with number of HRS, serum arginase-1 (both levels and activity), Iba-1, GLUT5, and TSPO (all $P < 0.01$) (Table 3).
Serum arginase-1 levels, activity, and correlation with number of HRS detected in non-diabetic subjects ($n=12$), diabetic subjects without signs of DR ($n=14$), patients with NPDR ($n=13$), and patients with PDR ($n=11$). Serum arginase-1 levels are reported as ng/mL ± SD; serum arginase-1 activity is expressed as U/L ± SD. Statistical significance was calculated with one-way ANOVA followed by Tukey’s comparison test. The relationship between number of HRS and arginase-1 serum levels and activity is reported with Pearson correlation coefficients ($r$) and significance levels ($P$). **$P<0.01$ versus CTRL; ***$P<0.01$ versus diabetic; °°$P<0.01$ versus NPDR.

M1 microglia and vascular endothelial impairment, events that trigger alterations of the retina at vascular and neuronal levels. We found for the first time that biomarkers of activated microglia can be assayed in sera of diabetic patients with different signs of neurovascular retinal alterations. In diabetic patients without signs of DR, the levels of Iba-1, GLUT5, TSPO, and arginase-1 increase in their sera, along with an increase in HRS formation and progression from NPDR to PDR.

Until now, several preclinical DR models have shown only locally higher retinal levels of Iba-1, and no study has reported systemic M1 polarized microglia as DR first develops. Worthy of note, activation of M1 microglia has been reported in diabetic patients from retinal tissues only postmortem or after surgical enucleation. Therefore, the correlation we found between the changes in serum Iba-1, GLUT5, TSPO, and number of HRS with DR stages represents a clinically relevant insight and provides an innovative and useful tool in the prevention and possible therapy of the disease.

Among their several actions, microglial cells are also recruited for reparative purposes; however, due to the persistent hyperglycemia that occurs in diabetes, they remain in an activated state, clusterize, form HRS, and continuously release cytokines and damaging factors into the retina, leading to degeneration of several components, including retinal ganglion cell axons, somas, and synapses. Following persistent activation, perivascular microglia can further damage endothelial cells of microcirculation by phagocytosis, cause rupture of blood vessels, and participate in breaking the blood–retinal barrier (BRB).
Consequently, substances circulating in the blood and cells can enter the retina, activate, and recruit further microglial cells by amplifying their damaging power. As a consequence of the breakdown of the BRB, inflammatory cells from the retina can infiltrate systemic circulation. In this context, it is tempting to speculate that the observed systemic changes in microglial biomarkers occur just because activated microglial cells are directly transduced from retina into the blood through the wall of the vasculature, as hypothesized by Lafrenaye et al. for Iba-1 in pigs, which is worthy of further study for the development of novel DR therapies.

In addition to Iba-1, two other important M1 microglial markers are GLUT5 and TSPO. Particularly, GLUT5 is an energy metabolism–related microglial marker that increases when the microglia shifts from quiescent to the activated M1 phenotype, moving from oxidative phosphorylation for adenosine triphosphate (ATP) production toward faster adaptive energy metabolism through aerobic glycolysis and increased cell energy production. TSPO is a protein that is expressed in the mitochondrial outer membrane of microglia in the central nervous system, and its expression is greatly increased when microglial cells are activated. Studies found that TSPO deficiency significantly inhibited microglial activation and impaired mitochondrial function, suggesting that it could serve as a useful biomarker of activated microglia and biomarker of neuroinflammation-related diseases such as neuroalterations of age-related macular degeneration and DR.

Here, we report that changes in serum Iba-1, GLUT5, and TSPO levels are accompanied by increased serum arginase-1 activity, which marks vascular endothelium dysfunction in diabetes. This enzyme is commonly upregulated by hyperglycemia and hyperglycemia-derived oxidative stress,
Table 3. Correlation of Serum Cytokines With HRS Number, Serum Arginase-1, Iba-1, GLUT5, and TSPO

| Cytokine | HRS Number | Arginase-1 Levels | Arginase-1 Activity | Iba-1 Levels | GLUT5 Levels | TSPO Levels |
|----------|------------|-------------------|---------------------|--------------|--------------|-------------|
| CXCL10   | $r = 0.85$ | $r = 0.88$        | $r = 0.88$          | $r = 0.84$   | $r = 0.82$   | $r = 0.81$  |
| CCL4     | $r = 0.91$ | $r = 0.93$        | $r = 0.88$          | $r = 0.88$   | $r = 0.89$   | $r = 0.85$  |
| IL-1β    | $r = 0.92$ | $r = 0.94$        | $r = 0.90$          | $r = 0.92$   | $r = 0.91$   | $r = 0.89$  |
| IL-6     | $r = 0.89$ | $r = 0.94$        | $r = 0.94$          | $r = 0.90$   | $r = 0.86$   | $r = 0.92$  |
| TNF-α    | $r = 0.94$ | $r = 0.95$        | $r = 0.93$          | $r = 0.96$   | $r = 0.92$   | $r = 0.96$  |
| MCP-1    | $r = 0.93$ | $r = 0.96$        | $r = 0.92$          | $r = 0.94$   | $r = 0.91$   | $r = 0.89$  |
| IL-8     | $r = 0.93$ | $r = 0.94$        | $r = 0.93$          | $r = 0.94$   | $r = 0.88$   | $r = 0.90$  |
| IL-2     | $r = 0.89$ | $r = 0.88$        | $r = 0.90$          | $r = 0.88$   | $r = 0.87$   | $r = 0.87$  |
| CD40     | $r = 0.89$ | $r = 0.89$        | $r = 0.89$          | $r = 0.91$   | $r = 0.88$   | $r = 0.87$  |
| RANTES   | $r = 0.89$ | $r = 0.87$        | $r = 0.89$          | $r = 0.88$   | $r = 0.82$   | $r = 0.84$  |

Pearson correlation coefficient ($r$) values were determined for the relation of serum cytokines (mean pixel density ± SD) with HRS number, serum arginase-1 (levels as ng/mL ± SD and activity as U/L ± SD), Iba-1 (pg/mL ± SD), GLUT5 (ng/mL ± SD), and TSPO (ng/mL ± SD). All of the correlations reported were significant ($P < 0.01$).

and its increased activity underlies nitric oxide depletion in the endothelium and severe dysfunction.\textsuperscript{19,33} It is our opinion that, following persistent glucose disturbance as in diabetes, an increase in arginase-1 activity drives retinal vascular leakage, which promotes microvascular permeability and thereby allows microglia to migrate in the circulation, with the endpoint being assayable.

DR development is strictly related to local and systemic inflammation,\textsuperscript{34} which leads to neurovascular retinal alterations by increasing oxidative stress, VEGF, and advanced glycation end-products,\textsuperscript{1,33–37} along with activation of retinal M1 microglia.\textsuperscript{2,35–39} This causes ganglion cell apoptosis through M1-driven local overproduction of TNF-α, IL-6, IL-1β, MCP-1, IL-18, IL-8, and fractalkine (CX3CL1).\textsuperscript{40–44} Here, we found that the changes of serum microglial markers were paralleled by changes in the pattern of inflammatory mediators, such as IL-6, TNF-α, IL-8, IL-2, CXCL10, CCL4, CD40, RANTES (all $P < 0.01$ vs. CTRL) and MCP-1 ($P < 0.05$ vs. CTRL), typical of DR development.\textsuperscript{34–47} Linear regression confirmed that modifications of Iba-1, GLUT5, and TSPO reflected modifications of the levels of inflammatory mediators and the passage of a diabetic retina from a healthy state to a proliferating pathological state.

We therefore suggest that a condition such as DR, characterized by a mixture of events including activation of M1 microglia, retinal vessel alteration with hyperpermeability, and BRB breakdown could be followed by monitoring the peripheral changes in microglial biomarkers in the sera, even in different stages of retinopathy.

This pilot study, although presenting limitations essentially linked to the low number of patients analyzed and the lack of follow-up, describes for the first time, to our knowledge, the significant association between the activation of retinal microglia and various serum markers in diabetic subjects. These biomarkers could be useful both in the early diagnosis and in the monitoring of DR, allowing the identification of subjects more at risk of developing severe forms of DR and should start personalized preventive therapy early.

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