Title:

**Serum glial fibrillary acidic protein and neurofilament light chain as biomarkers of retinal neurodysfunction in early diabetic retinopathy: results of the EUROCONDOR study**

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**ABSTRACT**

**Aims:** Neurodegeneration and glial activation are primary events in the pathogenesis of diabetic retinopathy (DR). Serum glial fibrillary acidic protein (GFAP) and neurofilament light chain (NfL) are biomarkers of underlying neuroinflammatory and neurodegenerative disease processes. The aim of the present study was to assess the usefulness of these serum biomarkers for the identification and monitoring of retinal neurodysfunction in subjects with type 2 diabetes.

**Methods:** a case-control study was designed including 38 patients from the placebo arm of the EUROCONDOR clinical trial: 19 with and 19 without retinal neurodysfunction assessed by multifocal electroretinography. GFAP and NfL were measured by Simoa.

**Results:** Serum levels of GFAP and NfL directly correlated with age (r=0.37, p=0.023 and r=0.54, p <0.001, respectively). In addition, a direct correlation between GFAP (pg/mL) and NfL was observed (r=0.495, p=0.002). Serum levels of GFAP were significantly higher at baseline in those subjects in whom neurodysfunction progressed after the 2 years of follow-up (139.1±52.5 vs. 100.2±54.6; p=0.04).

**Conclusions:** GFAP could be a useful serum biomarker for retinal neurodysfunction. Monitoring retinal neurodysfunction using blood samples would be of benefit in clinical decision-making. However, further research is needed to validate this result as well as to establish the best cutoff values.

**Keywords:** retinal neurodysfunction**;** diabetic retinopathy; serum biomarkers; glial fibrillary acidic protein; neurofilament light chain.

INTRODUCTION

The concept of diabetic retinopathy (DR) as a microvascular disease has evolved, and rather than a merely microvascular disease it is now considered a more complex diabetic complication in which neurodegeneration plays a significant role [1-4]. Neurodegeneration and glial activation are primary events in the pathogenesis of DR and have been observed to occur before overt microangiopathy in experimental models of DR and in the retina of diabetic donors [5-7]. Given the lack of autonomic innervation in the retinal vasculature, local factors released from endothelial cells and the other components of the neurovascular unit (i.e. neurons and glial cells) play a key role in regulating retinal blood flow [8, 9]. Thus, the neurodegenerative process (glial activation and neuron death) contributes significantly to microvascular damage through several mechanisms that have been reviewed elsewhere [2]. In addition, neuroprotection itself is important to prevent or halt deficiencies in sensory capacity, including decreased hue discrimination, contrast sensitivity, delayed dark adaptation, and abnormal visual fields, all resulting in reduced vision-related quality of life [10-12]. Therefore, the assessment of neurodegeneration/neurodysfunction is now emerging as an essential determinant, besides microvascular impairment, of the final visual outcome of patients with diabetes [13].

There are few clinical trials aimed at treating retinal neurodysfunction induced by diabetes [14-16]. The largest published study on this issue is the EUROCONDOR clinical trial in which 449 type 2 patients with diabetes were included. This phase II-III randomized placebo-controlled clinical trial was aimed at evaluating the effects of topically administered (eye drops) neuroprotective drugs (brimonidine and somatostatin) in halting or preventing early retinal neurodegeneration in early DR [16]. These neuroprotective agents appeared capable of halting the worsening of preexisting retinal neurodysfunction after two years of follow-up. Although further clinical trials with longer follow-up are needed to demonstrate the effectiveness of this approach in reducing microvascular progression, the results observed in the EUROCONDOR study point to screening for retinal neurodysfunction as a critical issue to identify a subset of patients in whom neuroprotective treatment might be of benefit.

Serum glial fibrillary acidic protein (GFAP) and neurofilament light (NfL) are biomarkers of underlying neuroinflammatory and neurodegenerative disease processes such as mild traumatic brain injury [17], multiple sclerosis [18], and neuromyelitis optica spectrum disorder [19].

On this basis, the aim of the present study was to assess the usefulness of GFAP and NfL as serum biomarkers for the identification and monitoring of retinal neurodysfunction in the EUROCONDOR cohort.

**SUBJECTS, MATERIAL AND METHODS**

**Subjects**

EUROCONDOR [NCT01726075] was a European multicenter, 96-week prospective, interventional, phase II to III, randomized controlled clinical trial aimed at evaluating the effect of topical neuroprotective agents to arrest or prevent retinal neurodegeneration in early DR [16, 20]. Briefly, 449 patients were recruited in 11 European centers and randomized 1:1:1 to topical treatment twice daily with placebo, brimonidine tartrate 0.2%, or somatostatin 0.1%.

The inclusion criteria were: type 2 diabetes with no, minimal, or mild DR as determined from retinal photographs (Early Treatment Diabetic Retinopathy Study [ETDRS] levels of 10, 20, or 35), at least five years of known diabetes, age between 45 and 75 years, and absence of other diseases which may induce retinal neurodegeneration (e.g. glaucoma). One eye per patient was included in the study. If both eyes met the inclusion criteria, one eye was randomly chosen. Microvascular changes were assessed by standard seven-field color fundus photography.

Retinal neurodysfunction was assessed by multifocal electroretinopathy (mfERG), with implicit time (IT) being the primary end-point. The mfERGs were recorded in the study eye using the RETIport/scan 21 (Roland Consult, Berlin, Germany) visual electrophysiology system. Stimulation and recording were performed according to the International Society for Clinical Electrophysiology of Vision guidelines [21, 22]. The stimuli array consisted of 103 hexagons displayed at a 60-Hz frame rate centered on the fovea covering a visual field of 30°.

Retinal neurodysfunction was defined as the presence of six or more altered hexagons for implicit time (IT) [23]. An altered hexagon was defined as a hexagon with a z-score of 2 or higher for IT compared with a normative database that had been created previously [24].

The study was approved and funded by the European Commission Seventh Framework Program (Grant Agreement No. FP7–278040). At all centers, the study was conducted in accordance with the tenets of the Declaration of Helsinki with approvals of the local scientific ethnical committees, and written informed consent was obtained from all patients.

In the present sub-study, only patients treated with placebo were included, to avoid any interference of neuroprotective drugs on the natural history of retinal neurodysfunction. In this subset of the population, a case-control cross-sectional study was designed to compare the circulating levels of GFAP and NfL between patients with and without retinal neurodysfunction at baseline adjusted by age and HbA1c levels.

**Serum biomarkers**

Serum samples for blood biomarkers were collected by venipuncture at baseline and at 12 months of follow-up. GFAP and NfL were assessed simultaneously in a Simoa human Neurology 4-Plex assay “A” (Quanterix, Lexington, MA). The assay was run on the fully automated ultrasensitive Simoa HD-1 Analyzer (Quanterix, Lexington, MA), with samples analyzed in duplicate in accordance with manufacturers' instructions with appropriate standards and internal controls. Sera were autodiluted 4X. Lower limits of detection were 0,22 pg/mL for GFAP and 0,10 pg/mL for NfL. The intra-assay and interassay coefficients of variation were 4.4% and 3.6% for GFAP and 3% and 3.9% for NfL, respectively.

**Statistical analyses**

For comparisons between continuous variables, Student’s and ANOVA t-tests were used. Results are expressed as the mean ± standard deviation (SD). For comparisons between categorical variables, the Fisher’s exact test was used. To evaluate correlations, the Spearman’s correlation test was performed. All p values are based on a two-sided test of statistical significance. Significance was accepted at the level of p < 0.05. Statistical analyses were performed with the SPSS Statistics version 15.

**RESULTS**

Clinical characteristics of type 2 diabetic subjects included in the study according the presence or not of retinal neurodysfunction at baseline are detailed in **Table 1**.

Serum levels of GFAP and NfL directly correlated with age (r=0.37, p=0.023 and r=0.54, p <0.001, respectively). In addition, a direct correlation between GFAP and NfL was observed (r=0.495, p=0.002). Furthermore, the differences of GFAP and NfL levels between baseline and 12 months of follow-up were also correlated (r=0.34, p=0.03).

Notably, serum levels at baseline of both GFAP and NfL strongly correlated with levels obtained at 12 months of follow-up (r=0.88; p<0.001, and r=0.83; p<0.001; respectively), thus indicating a low biological variability of these parameters (**Figure 1**).

At baseline, levels of GFAP and NfL were higher in type 2 diabetic patients with neurodysfunction in comparison with those without neurodysfunction, but the differences did not reach statistical significance (Table 2). Notably, serum levels of GFAP were significantly higher at baseline in those subjects in whom neurodysfunction progressed after 2 years of follow-up (increase of IT abnormal hexagons) in comparison with non-progressors (**Table 2**). In addition, baseline GFAP levels were associated with neurodysfunction progression after adjusting for age and HbA1c (p=0.044). Furthermore, the GFAP levels at 12 months of follow-up were also significantly higher in those patients in whom neurodysfunction progressed in comparison with non-progressors (156.5±61.5 pg/mL vs. 97.6±58.8 pg/mL; p=0.007).

DISCUSSION

The present study suggests that GFAP serum levels could be useful for identifying and monitoring retinal neurodysfunction in patients with type 2 diabetes and early diabetic retinopathy.

Previously, we found that circulating levels of recognized factors involved in microvascular damage such us AGEs (i.e. carboxy-methyl-lysine) and a specific component of basement membrane, Laminin P1 could help us identify subjects with type 2 diabetes and early stages of DR in the well-characterized EUROCONDOR cohort [25]. One of the main lessons of the EUROCONDOR clinical trial was that retinal neurodysfunction (as assessed by mfERG) was not identified in a large proportion (35%) of patients with early microvascular lessions (ETDRS 20-35) [16, 20], suggesting that neurodegeneration could be the herald of DR in only a specific subset of subjects with diabetes. This finding points to screening for retinal neurodysfunction as a critical issue to identify those patients in whom neuroprotective treatment might be of benefit [16]. In this regard, the assessment of serum GFAP levels could be useful to improve the characterization of those patients with retinal neurodysfunction and for monitoring neuroprotective interventions.

GFAP is located in the macroglia and is released after cell injury and death [26]. GFAP exhibits clinical prognostic potential in adult brain injury, including traumatic injury and stroke [27, 28], and in neurodegenerative disorders [29, 30]. Serum GFAP level increases with age and is an emerging biomarker of cognitive decline [31, 32]. In the setting of DR, reactive gliosis is the general response of glial cells to the diabetic milieu. It is characterized by upregulation of proinflammatory cytokines and various kinds of molecules, one of the most characteristic is GFAP. This intermediate filament protein is expressed in the normal retina mostly by astrocytes and minimally by Müller cells. In diabetes, Müller cells acquire prominent GFAP immunoreactivity, whereas astrocytes progressively lose GFAP immunoreactivity and may also decrease in number [33]. Thus, the aberrant overexpression of GFAP in Müller cells is a useful biomarker of the presence and degree of glial activation in both experimental and post-mortem studies of human retinas [5-7]. Since Müller cells produce factors capable of modulating blood flow, vascular permeability and cell survival, and their processes surround all the blood vessels in the retina, it seems that these cells play a key role in the pathogenesis of retinal microangiopathy in the diabetic eye [34]. Since reactive gliosis runs in parallel or even anticipates retinal neural death, GFAP can be considered a potential biomarker and predictor of retinal neurodegeneration.

Neurofilaments (NfPs) are classified as a type IV class of intermediate filaments specific to neurons [35]. Mature mammalian neurons usually express five different NfPs: neurofilament light chain (NfL), neurofilament medium chain (NfM), neurofilament heavy chain (NfH), alpha-internexin (INA) and peripherin (PRPH). In mature neurons of CNS, Nfs are generally composed of NfL, NfM, NfH, and INA [36], whereas in the peripheral nervous system they mainly consist of NfL, NfM, NfH and PRPH [37]. Much interest in the field has been recently focused on the detection of NfPs and degradation fragments released from neurons into blood as surrogate markers of neuronal damage [38]. NfL shows promise in evaluating acute and chronic severity and the course of neurodegenerative conditions, such as Alzheimer’s disease, Parkinson’s disease, and multiple sclerosis [39-41], as well as hypoxic and traumatic brain injuries [42, 43]. Recently, it has been reported that increased serum NfL concentration is associated with covert MRI findings of vascular brain injury, especially the burden of white matter hyperintensities, predict cognitive decline among older adults without a history of stroke [44]. In addition, NfL and GFAP added predictive value beyond Aβ and p-tau to the progression of cognitive decline in individuals with subjective cognitive decline [45]. Therefore, the retina and brain not only share common mediators involved in neurodegeneration [46], but also related biomarkers such as GFAP and NfL.

In the present study, we observed a non-significant trend to higher levels of NfL in patients with neurodysfunction at baseline and in progressors. A possible reason could be that, whereas GFAP reflects glial activation or reactive gliosis, NfL is a marker of structural damage of neurons (neuroaxonal injury), an event that takes place in a substantial manner after important glial activation has already occurred [47]. In this regard, it seems reasonable to postulate that GFAP is already increased when only neurodysfunction is present, whereas NfL enhancement may depend on a critical amount of neuronal death. Supporting this concept, the association between increased serum NfL levels and neuroaxonal retinal damage in subjects with multiple sclerosis is more pronounced in advanced stages of the disease [48]. Moreover, it has been reported that the release of NfL and GFAP into the extracellular space and peripheral blood, depends on the extent of damage [49, 50].

Simoa (Single molecule array) testing is a powerful new technique that is orders of magnitude more sensitive than standard sandwich-based immunoassay techniques. Traditional ELISA measurements are limited to pg/ml levels of detection whereas Simoa can achieve sensitivity as low as femtogram (fg/ml), allowing the detection and quantification of biomarkers at concentrations previously difficult or impossible to measure. In addition, this platform can be used to detect 4 to 10 biomarkers in a single test [51].

Several novel biomarkers are very promising, especially blood-based markers. However, many biomarkers tested have had low reproducibility. It has been reported that serum levels of NfL and GFAP remained unaffected by a comprehensive set of pre-analytical variables [52]. In the present study, to minimize all pre-analytical factors, the protocol of sampling blood collection was identical across participating centers. On the other hand, longitudinal testing of biomarkers with low variability may provide meaningful information. Thus, the repeatability of a biomarker measurement determines its association with disease outcomes in epidemiological studies [53]. In this regard, it should be stressed that we found a high correlation between the two determinations of GFAP and NfL drawn one year apart.

Strengths of this study include its prospective multicenter design with homogeneous collection of centrally-processed samples. On the weakness side we cannot completely rule out the brain as a contributing source to the baseline and follow-up levels of GFAP. However, the exclusion of neurodegenerative diseases makes this possibility unlikely. Secondly, the sample size was small and larger studies are needed.

In conclusion, we suggest that GFAP could be a useful blood-based biomarker for retinal neurodysfunction in patients with type 2 diabetes and early DR. The possibility of detecting and monitoring retinal neurodysfunction in peripheral blood samples could be of benefit in clinical decision-making. However, further research is needed to validate this result as well as to establish the best cutoff values

**Author Contributions:** Conceptualization, C.H. and R.S.; methodology, C.H., O.S., M.P., J.G., S.H., U.F., J.G-A., L.R., P.S., J.C., and R.S; formal analysis, C.H. O.S., and R.S.; investigation, C.H., O.S., M.P., J.G., S.H., U.F., J.G-A., L.R., P.S., J.C., and R.S; formal analysis, C.H. O.S., and R.S; resources, C.H., O.S., M.P., J.G., S.H., U.F., J.G-A., L.R., P.S., J.C., and R.S; writing—original draft preparation, C.H.; writing—review and editing, C.H. and R.S.; project administration, C.H. and R.S.; funding acquisition, EUROCONDOR Consortium (coordinator: R.S.). All authors have reviewed and agreed to the published version of the manuscript.

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**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Conflict of Interest:** None

**Data Availability Statement:** The data presented in this study are available upon request from the corresponding author.

**Conflicts of Interest:** The authors declare no conflict of interest.

Appendix

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**Table 1.** Baseline characteristics of type 2 diabetes mellitus patients according the presence or absence of retinal neurodysfunction at baseline.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Without  neurodysfunction | With  neurodysfunction | p |
| n | 19 | 19 |  |
| Age (years) | 63.9±6.7 | 64.7±5.0 | n.s |
| BMI (Kg/m2) | 30.0±4.8 | 31.0±7.2 | n.s |
| Diabetes duration (years) | 12.22±5.17 | 9.8±3.1 | n.s |
| HbA1c (%) | 7.14±1.0 | 7.17±1.0 | n.s |
| Hypertension (yes, %) | 68.4% | 78.9% | n.s |
| Dyslipidemia (yes, %) | 68.4% | 68.4% | n.s |
| Cardiovascular disease (yes, %) | 26.3% | 15.8% | n.s |
| ETDRS level 10/20-35(%) | 26.3/73.1 | 33.3/66.6 | n.s |
| BCVA letter score | 86.1±2.9 | 85.56±3.4 | n.s |

BMI: Body mass index; HbA1c: Hemoglobin A1c. ETDRS: Early Treatment Diabetic Retinopathy Study. BCVA: Best corrected visual acuity. Data are expressed as mean±SD or %.

**Table 2.** Baseline levels of GFAP and NfL according the presence of neurodysfunction or its progression after 2 years of follow-up.

|  |  |  |
| --- | --- | --- |
|  | **GFAP**  (pg/mL) | **NfL**  (pg/mL) |
| **Neurodysfunction at baseline**  Yes (n=19)  No (n=19)  p  **Neurodysfunction progression**  Yes (n=13)  No (n=25)  p |  |  |
| 122.4±64.6 | 16.6±7.9 |
| 105.7±47.6 | 15.1±6.1 |
| 0.12 | 0.68 |
|  |  |
| 139.1±52.5 | 18.5±9.7 |
| 100.2±54.6 | 14.8±5.6 |
| 0.04 | 0.15 |

**Figure 1.** A) Correlation between GFAP levels (pg/mL) at baseline and 1-year follow-up. (r=0.88; p<0.001). B) Correlation between NfL levels (pg/mL) at baseline and 1-year follow-up (r=0.83; p<0.001).

