Levels of the oxidative stress biomarker malondialdehyde in tears of patients with central serous chorioretinopathy relate to disease activity

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Purpose: Central serous chorioretinopathy (CSCR) has been associated with oxidative stress–related risk factors. The objective of this study was to optimize an analytical method for evaluating the oxidative stress biomarker malondialdehyde (MDA) in human tears and determine its level in the tears of patients with CSCR.

Methods: In this pilot study, tear samples were obtained from 34 healthy donors and 31 treatment-naïve CSCR male patients (eight with acute CSCR and 23 with chronic CSCR). Two analytical methods based on high-performance liquid chromatography followed by fluorescence detection were evaluated, with either 2-thiobarbituric derivative (TBA) or 2-aminoacridone (2-AA). Activity of CSCR was defined by the serous retinal detachment (SRD) height, which was measured by two independent observers on spectral-domain optical coherence tomography.

Results: The 2-AA method showed higher sensitivity and precision compared to the TBA method. When the 2-AA method was applied to tears from healthy donors, the levels of MDA were statistically significantly higher in men compared to women (mean ± standard deviation, SD: 9.914 nM ± 6.126 versus 4.635 nM ± 1.173, p = 0.006). No difference was found in tear MDA levels between male patients with CSCR and age-matched control men (p = 0.17). However, MDA levels were statistically significantly higher in acute compared to chronic CSCR cases (mean ± SD: 12,295 nM ± 8,495 versus 6,790 ± 3,969 nM, p = 0.03). Additionally, there was a correlation between MDA levels and RPE leakage, quantified by the height of the serous retinal detachment (p = 0.02, r = 0.40).

Conclusions: Levels of MDA in tears, measured with an optimized analytical method, correlate with RPE leakage in CSCR.

Oxidative stress is a common driving mechanism in the pathogenesis of major retinal disorders, including age-related macular degeneration [1,2], diabetic retinopathy [3], degenerative myopia [4], inherited retinal dystrophies [5], and retinal detachment [6,7]. As the retina is very rich in membranes containing polyunsaturated fatty acids, lipid peroxidation induced by reactive oxygen species (ROS) has major consequences for retinal functions and may cause retinal cell death [8]. Malondialdehyde (MDA), a reactive aldehyde produced from polyunsaturated lipids degradation by ROS, is a highly toxic compound that forms lipoxidation end-products. MDA has been identified in ocular media and tissues in experimental models for retinal diseases [9] and used as a biomarker of oxidative stress in human ocular media [9,10]. For identifying biomarkers of organ-specific and systemic conditions, there is wide interest in body fluids that can be collected noninvasively, such as saliva, sweat, and tears [11-13]. Tears, despite technical hurdles due to the small volume of samples, offer the advantage of easy access and possible longitudinal sampling. As the tear film is in contact with the atmosphere at the ocular surface, the tear film may reflect individual environmental exposure, as well as endogenous metabolism [14,15]. Tears have been mostly investigated in patients with ocular surface diseases [15], but there is growing evidence that tears could also reflect systemic diseases [14,16,17].

Better characterization of lipid peroxidation markers in tears could provide additional investigative and diagnosis
tools in ocular disorders. To our knowledge, only one previous study has reported a method for assessing MDA levels specifically in tears and related the levels of MDA with age [18]. However, tear MDA levels as a possible oxidative stress biomarker in retinal diseases have not been investigated.

Central serous chorioretinopathy (CSCR) is a choriotinal disorder characterized by serous retinal detachments, secondary to RPE barrier defect, frequently involving the macula. CSCR is associated with choroidal vein dilation, increased choroidal thickness, choroidal vascular hyperpermeability, and pigment epithelial detachments [19]. CSCR is classified as acute or chronic depending on the duration of subretinal fluid or the presence of extensive RPE alterations, or both. This disorder is associated with systemic risk factors, such as hypertension, coronary heart disease, cortisol metabolism deregulation, psychological stress, circadian rhythm disruption and shift work, as well as with genetic predisposition, such as complement factor H polymorphisms [19]. Overactivation of the mineralocorticoid pathway has been involved in the pathophysiology of CSCR [19-23]. Noticeably, oxidative stress is one of the pathogenic consequences of activation of the mineralocorticoid-receptor pathway, particularly in the heart [24], kidneys [25], and vascular system [26]. In addition, disruption of the circadian rhythm and sleep disorders are associated with oxidative stress [3,27]. To our knowledge, only one recent study [28] has investigated the link between oxidative stress and oxygen end-products, and a decrease in biologic antioxidant potential in patients with CSCR compared to controls. The aims of this pilot study were to optimize methods for measuring free and protein-bound MDA in human tear fluid and to measure MDA in the tears of patients with CSCR and investigate possible correlations with disease activity.

METHODS

Subjects and tear collection: This study adhered to the ARVO statement on human subjects and was designed in accordance with the tenets of the Declaration of Helsinki. Collection of clinical data and biologic samples and their analysis were approved by the Ethics Committee of the Swiss Federal Department of Health (CER-VD n°19/15 and CER-91 VD n°340/15). Collection of clinical data and biologic samples and their analysis were approved by the Ethics Committee of the Swiss Federal Department of Health (CER-VD n°19/15 and CER-VD n°340/15). All patients and control subjects gave written informed consent before enrolling in the study. A total of 34 healthy volunteers were recruited, and tears from 15 of the participants were pooled and used for the development and validation of the method for measuring MDA levels (Appendix 1). The 19 samples left (11 men and eight women) were subsequently analyzed individually (Table 1, Appendix 1). For the CSCR study, 31 untreated male patients with CSCR (eight acute and 23 chronic CSCR cases, Table 1) were included. The inclusion criteria were 1) age between 25 and 70 years old; 2) absence of any acute disease, notably infectious diseases; 3) absence of any non-controlled chronic disease, including cardiovascular diseases, endocrinological disease, inflammatory diseases, and malignancy; 4) absence of other associated ocular pathology; and 5) absence of regular contact lens use. Smoking was not considered an exclusion criterion, or controlled diabetes and treated hypertension.

The diagnosis of CSCR was confirmed with fluorescein and indocyanine green angiography, fundus autofluorescence, and spectral-domain optical coherence tomography (SD-OCT), including evaluation of choroidal thickness. Subjects underwent multimodal retinal imaging on Spectralis (Heidelberg Engineering, Heidelberg, Germany). A 20 × 20° 97-section raster scan (approximately 6 × 6 mm) was acquired using the enhanced-depth imaging (EDI) mode.

Acute CSCR was defined as the first episode of serous retinal detachment lasting for less than 6 months. Chronic CSCR was defined by the presence of RPE alterations on fundus autofluorescence and at least one current or previous CSCR episode lasting more than 6 months (with or without serous retinal detachment at the moment of tear collection).

| Table 1. Characteristics of the healthy controls and the CSCR population. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| **Clinical characteristics** | **Controls**                | **CSCR (only men)**         |
|                             | **Men**                     | **Women**                   | **Active**                  | **Chronic** |
| Number of patients, (samples) | 11 (11)*                    | 8 (8)*                      | 8 (8)                       | 23 (23)     |
| Age, years (mean ± SD)      | 44±10                       | 38±12                       | 46±12                       | 49±10       |
| Smokers, n (%)              | 4 (36)                      | 2 (25)                      | 3 (37)                      | 6 (26)      |
| Treated Hypertension, n (%) | 0                           | 1 (12.5)                    | 0                           | 3 (13.0)    |
| Controlled Diabetes, n (%)  | 0                           | 0                           | 0                           | 2 (8.7)     |

CSCR=central serous chorioretinopathy; SD=standard deviation * right eye only was chosen for all healthy controls
Tear samples were collected between March 2014 and June 2016 at Jules-Gonin Eye Hospital (Lausanne, Switzerland).

Tear samples were collected with Schirmer paper strips (Biotech Vision Care Pvt Ltd., Gujarat, India) inserted in the lower conjunctival fornix of both eyes for a maximum of 3 min, or less if the strip was filled earlier to reduce patient discomfort. No tear stimulation, topical anesthetic, or other eye drops were used before sample collection. External factors such as harsh lighting, background noise, and extreme room temperature, all known to affect the content of tear samples, were strictly supervised to ensure reproducibility. Care was taken to avoid damage to the ocular surface. Applanation tonometry was not performed before tear sampling. The trained technician who collected the tear samples wore gloves, and the Schirmer paper was not in contact with patient skin to avoid contamination. The strip was then inserted into a modified 0.5-ml tube with an opening in the bottom, and this assembly was placed in a 1.5-ml tube and centrifuged at 6708 × g for 7 min at 4 °C without any additional buffer to retrieve the tear sample. After centrifugation, the samples were immediately stored at −80 °C until analysis. In some cases, the tears of both eyes were further separately analyzed.

Determination of MDA levels in tears: Two analytical methods (see Appendix 1) were initially considered for the determination of MDA levels in tears. The first method, described by Benlloch-Navarro et al. [18], is based on fluorescence detection of the 2-thiobarbituric (TBA) derivative after high-performance liquid chromatography (HPLC) separation. The drawbacks of this method are a weak specificity for MDA [29] and a possible overestimation of the levels, due to the use of harsh derivatization conditions (temperature and acidic milieu) [30]. To avoid these difficulties, we tested a second derivatization strategy based on the use of 2-aminoacridone (2-AA), allowing mild and selective derivatization of MDA. As MDA can easily bind to amino groups of proteins through the formation of Schiff’s bases [29], the free and bound forms of MDA should ideally be determined. Thus, we adapted to the tear samples the method described by Giera et al. for urine samples [31], to determine the free and bound MDA fraction, the latter obtained after basic hydrolysis. The use of methyl-MDA as internal standard was also assessed.

For validation purposes, the tears from 15 healthy volunteers were collected as described above. Tear samples were diluted ten times in water (v/v) and then mixed together to prepare three pooled samples (one pool for the development and validation of the TBA method, one pool for the development and validation of the 2-AA method, and one pool for the comparison of the two methods). The pooled samples were stored at −20 °C. A stability study for MDA using the 2-AA method indicated an approximate 25% decrease after 1-day storage at −20 °C but stable concentrations for the next 7 days from this point forward (data not shown). The analytical validation of the two methods was performed using the calibration range, limit of detection and quantification, repeatability, and recovery, as detailed in the Appendix. To compare the sensitivity, bias, and precision of the two methods, the same sample of pooled tears was analyzed with both methods. The MDA levels in tears from eight healthy women and 11 age-matched healthy volunteer men (n = 11 samples from the right eye only) and from 31 patients with CSCR (Table 1) were determined with the validated 2-AA method.

**CSCR activity assessment using SD-OCT**: For this study, active CSCR was defined by the presence of serous retinal detachment on SD-OCT. The maximum height of serous retinal detachment was measured on SD-OCT as the axial distance between the inner RPE surface and the outer aspect of the photoreceptor outer segments. Subfoveal choroidal thickness (SFCT) was evaluated manually on enhanced-depth imaging horizontal OCT scans, from the RPE to the interface between the outer choroid and the sclera. All imaging assessments were performed by two independent observers (AD, AM) on anonymized images. The mean between the two measurements was retained for the analysis.

**Statistical analyses**: Agreement between the two observers for the SD-OCT imaging evaluation was calculated using the intraclass correlation coefficient (ICC) with the ‘irr’ package on R software (Version 3.3.0, R Foundation for Statistical Computing, R Core Team, 2016, Vienna, Austria; R-project). Based on the lower end of the 95% confidence interval (CI) of the ICC, agreement was considered poor (<0.50), moderate (0.50–0.75), good (0.75–0.90), or excellent (>0.90). The Spearman coefficient was used to investigate possible correlations between the total MDA levels with the height of the serous retinal detachment, using GraphPad Prism (version 5.0f, GraphPad Software, La Jolla, CA). Comparisons between groups were performed using the non-parametric Mann–Whitney test. Multivariate regression was performed using the lm function on R software. For statistical comparisons, differences with a p value less than or equal to 0.05 were considered statistically significant.

**RESULTS**

The 2-AA assay is more sensible and precise than the TBA assay and allows the determination of free and total MDA levels: From an analytical point of view, the TBA and 2-AA assays were sensitive enough to determine the MDA levels in tears (limits of quantification <25 nM) in a reproducible manner (coefficient of variation <18%). However, the 2-AA
**Table 2. Comparison of the malondialdehyde concentrations obtained with the thiobarbituric acid (TBA) assay and the 2-aminoacridone (2-AA) assay on two samples of pooled tears obtained from 5 (Pool 1) or 6 (Pool 2) healthy subjects.**

| Pool number | Total MDA [nM] | Free MDA [nM] | Total MDA [nM] | TBA/2-AA* | Ratio free MDA/total MDA [%] |
|-------------|----------------|---------------|----------------|-----------|-----------------------------|
| Pool 1      | 337±42 (n=4)   | 34±11 (n=4)   | 5'420±250 (n=3) | 6.2       | 0.63                        |
| Pool 2      | 524±190 (n=4)  | 47±7 (n=5)    | 6'554±840 (n=7) | 8.0       | 0.72                        |

The number of repetitions (n) of the MDA measurement is indicated in brackets. MDA=malondialdehyde; TBA=thiobarbituric acid; 2-AA=2-aminoacridone. *Considering the total MDA measured with the 2-AA technique.

The assay presented additional advantages: It allowed the determination of free and total MDA levels in tears, and it was possible to use an internal standard (methyl-MDA) to correct for possible matrix effects. In addition, the assay was more sensible and precise than the TBA assay (see Appendix 1). As illustrated in Table 2, the two methods provided different results when applied to the same tear sample. The MDA level measured with the TBA assay was about 15 times lower compared to the total MDA levels obtained with the 2-AA assay. In addition, compared to the total MDA levels, the free MDA levels were observed to be negligible (<1%), often at the level of the detection limit (Table 2).

Total MDA levels in the tears of patients with CSCR correlate with disease activity: When the 2-AA method was applied to tears from the right eye of 19 healthy donors (11 men and eight women), the levels of MDA were statistically significantly higher in men compared to women (mean ± SD: 9,914 ± 6,126 nM versus 4,635 ± 1,173 nM, p = 0.006). Therefore, samples from male patients with CSCR (n = 31 samples) were compared to male healthy donors (n = 11 samples). The detailed clinical characteristics of the 31 patients with CSCR are provided in Appendix 2. No difference was found in tear MDA levels between the entire CSCR cohort (7,898 nM ± 6,285) and the healthy donors (9,914 ± 6,126 nM; p = 0.12). However, the MDA levels were statistically significantly higher in the patients with acute CSCR compared to the patients with chronic CSCR (mean ± SD: 12,295 nM ± 8,495 nM versus 6,614 nM ± 4,613 nM, p = 0.02). As nine of 23 patients with CSCR presented without serous retinal detachment at the moment of tear sampling, we repeated the analysis only with patients with serous retinal detachment. The MDA levels remained statistically significantly higher in the patients with acute CSCR compared to the patients with chronic active CSCR (mean ± SD: 12,295 ± 8,495 nM versus 6,790 ± 3,969 nM, p = 0.03). Additionally, the MDA levels measured in the tears of patients with CSCR correlated statistically significantly with the height of serous retinal detachment on SD-OCT at the time of sampling (p = 0.02, r = 0.40, Figure 1 and Figure 2). Agreement between the two observers for the evaluation of the serous retinal detachment height on SD-OCT was excellent (ICC: 0.998, 95% CI:0.994–0.999). Choroidal thickness did not correlate with the tear MDA levels among patients with CSCR (p = 0.6). A multivariate analysis of systemic and ocular factors potentially influencing MDA levels in tears among patients with CSCR identified the height of serous retinal detachment as the only statistically significantly contributing parameter (p = 0.041). Age (p = 0.20), smoking (p = 0.17), or SFCT (p = 0.51) did not influence MDA levels in the tears of patients with CSCR (Table 3).

**DISCUSSION**

In this study, we developed a method for accurately determining the level of MDA in tears, accounting for the technical specificities of this particular body fluid. MDA is commonly measured as a marker of lipid peroxidation in human fluids [9,10], but methods for measuring MDA levels require specific adjustments depending on the matrix. Optimized and sensitive analytic methods are particularly crucial when the sample volume is very small, as is the case for tears (a few microliters per sample). We provide evidence that the selected analytical methodology might have a strong impact on the results. The 15-times lower MDA concentration obtained with the TBA assay compared to the 2-AA assay could be due to differences in hydrolysis efficiency (perchloric versus sodium hydroxide, respectively). The basic hydrolysis of plasma samples is more effective for releasing protein-bound MDA than strong acidic conditions [32]. In biologic matrix, MDA can be found under two forms: free or bound to SH or NH2 groups of macromolecules, such as proteins or nucleosides [33]. By applying the developed 2-AA method to tears, we determined that the free MDA levels were very low and that most of this oxidative biomarker was bound to macromolecules (free MDA/total MDA <1%; Table 1). This result
is in line with other reports showing that levels are underestimated if only free MDA levels were measured, in healthy [34] and disease conditions [35]. Importantly, complement factor H, involved in the pathophysiology of CSCR and other retinal disorders, was shown to directly interact with MDA, neutralizing at least partly its pathogenic effect [36,37].

The MDA levels in the tears of healthy volunteers were two times higher in men, compared to women. The relationship between gender and oxidative stress is important, because oxidative stress is implicated in many diseases that affect men and women differently, such as cardiovascular conditions [38]. For example, plasma biomarkers of oxidative

Figure 1. Height of serous retinal detachment on spectral-domain optical coherence tomography correlates with total MDA levels in tears of patients with CSCR. A: Chronic CSCR (without subretinal fluid). B: Chronic CSCR, extrafoveal subretinal fluid (height = 75 µm) C: Chronic CSCR with foveal subretinal fluid (height = 158 µm). CSCR, central serous chorioretinopathy.
stress are higher in men than in women [39]. Similarly, ROS production is higher in vascular cells from men than in those from women [40]. Overall, women seem to be less susceptible to oxidative stress [38]. These findings are particularly interesting in CSCR, where most cases occur in men, and because risk factors, such as hypertension or coronary heart disease, also have a male predilection, suggesting a possible role of oxidative stress in CSCR pathophysiology.

Although no statistically significant difference in the total MDA tear levels was found between the entire CSCR and control groups, the MDA levels were statistically significantly higher in acute CSCR than in chronic cases. In addition, the total MDA levels in tears were positively correlated with the height of serous retinal detachment, which reflects disease activity in acute and chronic cases. Patients with chronic CSCR with signs of epitheliopathy had lower MDA tear levels than patients with acute CSCR, suggesting that the increase in the total MDA tear level is associated with higher serous retinal detachment, a sign of activity of CSCR. It can be also hypothesized that the presence of a larger amount of subretinal fluid reflects a more extended outer blood barrier breakdown and facilitates passage of compounds from the subretinal space toward the choroid and the pericellular spaces, via transscleral diffusion and venous uptake. This would potentially influence MDA levels measured at the ocular surface. As a result of the blood barrier breakdown,

![Figure 2. Height of serous retinal detachment correlates with total MDA concentrations in tears of patients with CSCR (p = 0.022, r = 0.40, Spearman coefficient). Round point: chronic CSCR, Triangular point: acute CSCR. CSCR, central serous chorioretinopathy.](image)

| Table 3. Multivariate analysis of factors influencing the level of MDA in tears among 31 patients with central serous chorioretinopathy. |
|-----------------------------------------------|
| Factor                                      | Coefficient | P value |
| Age, years (≥50 versus <50 years)           | 30.2         | 0.20    |
| Smoking (yes versus no)                     | 33.4         | 0.17    |
| Serous retinal detachment height, µm (by 10-µm increment) | 0.23         | 0.041   |
| Subfoveal choroidal thickness (by 10-µm increment) | −0.07        | 0.51    |

MDA=malondialdehyde Multiple R²=0.23
plasma proteins can leak in the subretinal space, including free hemoglobin and hemoglobin byproducts, which lead to intraocular iron accumulation and generation of oxygen free radicals. Noticeably, the oxidative stress–mediated retinal toxicity of iron accumulation has been demonstrated in several models of alteration in the blood–retinal barrier [41,42]. Further studies in patients with subretinal fluid from other origins, such as rhegmatogenous retinal detachment or choroidal neovascularization, could provide insights into the specificity and significance of the observed correlation between serous retinal detachment height and MDA tear levels. Markers of lipid peroxidation have been identified in the subretinal fluid of patients with retinal detachment, particularly associated with myopia, and thought to originate from rod outer segments [43]. Lipid peroxidation is also associated with age-related macular degeneration and MDA directly linked to autophagy deregulation in RPE cells and to VEGF production [44].

Regarding the potential interest of MDA as a biomarker for retinal diseases, statistically significant associations were reported among the plasma MDA level, the presence of ARMS2 genetic variants, and imaging phenotypes in neovascular age-related macular degeneration and polypoidal choroidal vasculopathy [45]. However, to our knowledge, the present study is the first to demonstrate a link between the activity of a retinal disease and tear levels of MDA.

This study has several weaknesses, including the following: The number of cases in each group was limited, inherent to an exploratory pilot study. Only male CSCR cases and controls were included, related to the male predominance of CSCR and to the preliminary observation of the influence of gender on MDA levels, which would have biased the results if male and female subjects had been included. The measures on SD-OCT were subjective, compensated by the biobserver evaluation. Longitudinal analysis of the MDA levels over the course of CSCR episodes was lacking, which should be investigated in future studies. Additionally, the choice of the analytical method strongly affected MDA level measures, which emphasizes the need for further research in this field.

In conclusion, these preliminary results suggest that an optimized analytical method with enhanced sensitivity for the measurement of total MDA in tears could provide a novel biomarker of oxidative stress for retinal diseases. This tool could also prove useful for investigating lipid peroxidation in other ocular conditions. Additional confirmation is needed in larger cohorts of patients with CSCR and in other retinal diseases associated with subretinal fluid.

APPENDIX 1. MDA ANALYTICAL METHODS
To access the data, click or select the words “Appendix 1.”

APPENDIX 2. CLINICAL CHARACTERISTICS OF 31 PATIENTS WITH CENTRAL SEROUS CHORIORETINOPATHY ASSESSED FOR TEAR MDA LEVELS.
To access the data, click or select the words “Appendix 2.”

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