

# Systemic Reduction in Glutathione Levels Occurs in Patients with Primary Open-Angle Glaucoma

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**PURPOSE.** To assess the level of plasma glutathione in patients with untreated primary open-angle glaucoma.

**METHODS.** Twenty-one patients with newly diagnosed primary open-angle glaucoma and 34 age- and gender-matched control subjects were subjected to a blood analysis to detect the level of circulating glutathione in its reduced and oxidized forms. The effect of age, gender, and systemic blood pressure on circulating glutathione levels was also analyzed.

**RESULTS.** Age had a negative effect on the level of both reduced and total glutathione ( $P = 0.002$ ,  $r = -0.52$  and  $P = 0.002$ ,  $r = -0.52$ , respectively) in control subjects but not in patients with glaucoma ( $P > 0.05$ ,  $r = 0.27$ , and  $P > 0.05$ ,  $r = 0.22$ , respectively). In the control group, men demonstrated higher levels of both reduced and total glutathione than did women ( $P = 0.024$  and  $P = 0.032$ , respectively). After correction for age and gender influences on blood glutathione levels, patients with glaucoma exhibited significantly lower levels of reduced and total glutathione than did control subjects ( $P = 0.010$ ,  $F = 7.24$  and  $P = 0.006$ ,  $F = 8.38$ , respectively). No differences between study groups were observed in either oxidized glutathione levels or redox index ( $P > 0.05$ ,  $F = 0.50$ ; and  $P > 0.05$ ,  $F = 0.30$ , respectively).

**CONCLUSIONS.** Patients with glaucoma exhibit low levels of circulating glutathione, suggesting a general compromise of the antioxidative defense. (*Invest Ophthalmol Vis Sci.* 2005;46:877-883) DOI:10.1167/iops.04-0777

Primary open-angle glaucoma (POAG) is a chronic, slowly progressive optic neuropathy, characterized by excavation of the optic nerve head (ONH) and a distinctive pattern of visual field (VF) defects.<sup>1</sup> The disease is multifactorial in origin and is associated more closely with elevated intraocular pressure (IOP) resulting in the main from reduced drainage of aqueous humor. Nevertheless, approximately one third of patients with glaucoma exhibit apparently normal IOP. Moreover, a substantial number of POAG cases continue to progress despite therapeutically lowered IOP. All these observations have spurred further research to determine the existence of other possible risk factors for glaucoma.<sup>2</sup>

Besides more extensively investigated factors, such as increased IOP,<sup>3,4</sup> reduced ocular blood flow (OBF),<sup>5-9</sup> ocular

vascular dysregulation,<sup>10-16</sup> and systemic blood pressure (BP) alterations,<sup>17-21</sup> oxidative stress has also been proposed as a contributing factor in the etiology of glaucomatous optic neuropathy.<sup>22-26</sup> Oxidative stress represents a harmful state defined by the presence of pathologic levels of reactive oxygen species (ROS) relative to antioxidant defense. ROS are molecules that oxidize more strongly than oxygen (O<sub>2</sub>) itself, or molecules containing oxygen that generate free radicals. ROS include superoxide (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radical (OH<sup>-</sup>), which is the strongest oxidant produced in biological systems. Other reactive species, reactive nitrogen species (RNS), include nitric oxide and peroxynitrite (ONOO<sup>-</sup>), a product resulting from the reaction between ROS and nitric oxide (NO).

In certain situations, free radicals can be generated in an exaggerated manner and can injure tissues and organs by interacting with lipids, proteins, or DNA.<sup>27</sup> Oxidative stress has been implicated in a large number of human diseases, including different autoimmune diseases,<sup>28</sup> alcoholic liver disease,<sup>29</sup> cancer,<sup>30</sup> and infection with human immunodeficiency virus (HIV)<sup>31</sup> and in ischemia-reperfusion injury.<sup>32</sup>

To survive, the human body has developed a complex, efficient, and highly adaptive antioxidant defense system that includes two categories of antioxidants: (1) enzymatic, such as glutathione peroxidase (GPx), glutathione reductase (GSSGR), superoxide dismutase (SOD), catalase (CAT), and peroxiredoxin,<sup>33</sup> and (2) nonenzymatic, such as reduced glutathione (GSH), antioxidant vitamins, and low-molecular-weight compounds such as urate.<sup>34</sup>

The eye is also protected against oxidative stress by several mechanisms involving antioxidant enzymes such as CAT and SOD, as well as by low-molecular-weight antioxidants such as GSH and ascorbate.<sup>35</sup> Glutathione (GSH, L-γ-glutamyl-L-cysteinylglycine), a tripeptide consisting of glycine, cysteine, and glutamic acid, prevents the effects of ROS either directly as an antioxidant or indirectly, by maintaining other cellular antioxidants in a functional state.<sup>36,37</sup> GSH conjugates with a large variety of products of oxidative stress and carcinogens via reactions facilitated by the enzyme glutathione S-transferase (GST). In living organisms, glutathione exists in two forms: reduced (GSH) and oxidized (GSSG). An optimal GSH:GSSG ratio is critical for survival of the cells and tight regulation of the system is therefore very important.<sup>38</sup>

Abnormal levels of GSH have already been reported in the lens<sup>39-41</sup> and whole blood<sup>42</sup> of patients with age-related cataract, in the vitreous humor, in the blood of patients with proliferative diabetic retinopathy (PDR) or proliferative vitreoretinopathy (PVR),<sup>43</sup> and in the blood of patients with age-related macular degeneration (ARMD).<sup>44,45</sup> Altered GSH and GSH activity has also been reported in the trabecular meshwork and aqueous humor of patients with glaucoma.<sup>23-26</sup> In a recent study, Yang et al.<sup>46</sup> demonstrated the presence of serum antibodies against GST in patients with glaucoma. The results of these studies are of extreme importance. A deficit in the scavenging capacity of GSH could reduce the bioavailability of NO for vasodilation, with consequent disruption of vascular

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**TABLE 1.** Reagents and Equipment Used for the GSH Assay

Reagents	
Sodium phosphate buffer:	125 mM containing 6.3 mM disodium EDTA (pH 7.5)
Daily buffer:	3 mg NADPH into 10 mL sodium phosphate buffer
DTNB solution:	47.56 mg 5.5 DTNB into 20 mL sodium phosphate buffer
SSA solution:	1 g SSA in 1 mL distilled water
GSH solution:	15.35 mg GSH in 0.5 mL distilled water
Enzyme:	41.6 $\mu$ L GSSGR in 5.158 mL sodium phosphate buffer
Equipment	
Pipettes:	1000 $\mu$ L, 100 $\mu$ L, 2 $\mu$ L
Adjustable multichannel pipettes:	5-50 $\mu$ L, 50-250 $\mu$ L
Flat-bottomed 96-well assay plate	
96-Well plate reader	

tone. Although a deficit in NO could result in altered ocular perfusion pressure (OPP) in glaucoma,<sup>47</sup> to date no study has been undertaken to investigate whether altered systemic GSH levels occur in patients who have this disease. Consequently, it is important to determine whether there is a relationship between circulating levels of GSH and the occurrence of POAG. The purpose of the present study was to establish circulating GSH and GSSG levels in patients with newly diagnosed and previously untreated POAG.

## SUBJECTS AND METHODS

### Study Sample

Consecutive patients with newly diagnosed and previously untreated POAG attending the Fast Track Glaucoma Clinic at the Heartlands and Solihull NHS Trust (Birmingham, UK) between February 2002 and December 2003, were considered for inclusion in this prospective study. Ethical approval was obtained from the local medical ethics committee, and written informed consent was received from all subjects before entry into the study. The study was designed and conducted in accordance with the tenets of Declaration of Helsinki.

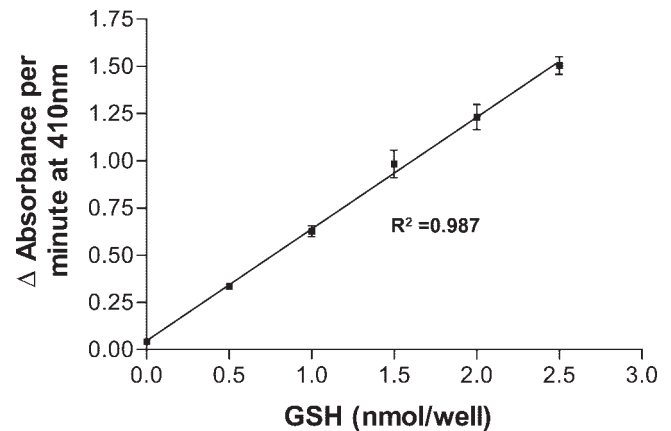
Patients underwent diurnal IOP phasing and were diagnosed as having POAG if IOP measurements were greater than 21 mm Hg by applanation tonometry (with at least two of the measurements being higher than 24 mm Hg), glaucomatous cupping of the optic disc on fundoscopic examination, normal open anterior chamber angles by gonioscopy, and repeatable VF defects consistent with the diagnosis of glaucoma, according to results obtained with program 24-2 of the Humphrey Field Analyzer (HFA: Carl Zeiss Meditec, Dublin, CA). The study's ocular exclusion criteria included narrow iridocorneal angles, evidence of secondary open-angle glaucoma, history of previous intraocular surgery, and other nonglaucomatous ocular diseases including cataract, diabetic retinopathy (DR) and ARMD. Forty-six patients with previously untreated glaucoma were screened during the study period. However, only 33 patients met these first criteria.

The control group was recruited from patients' spouses and from other volunteers, and it was composed of subjects who had never neither glaucoma nor other ocular diseases such as cataract, DR, or ARMD.

Other exclusion criteria for both groups were smoking and a history of any chronic systemic disease with presumed low GSH levels, including autoimmune diseases,<sup>28</sup> alcoholic liver disease,<sup>29</sup> cancer,<sup>30</sup> and diabetes mellitus.<sup>48</sup> After this selection, the experimental group was narrowed to 21 patients with glaucoma and 34 control subjects.

### BP Measurement

BP was measured in each patient and control subject in the morning between 8 and 9 AM, with a BP monitor (UA-779; A&D Instruments, Ltd., Oxford, UK). In preparation for this measurement, each subject rested in a sitting position for approximately 10 minutes in a quiet

**FIGURE 1.** The standard curve for the GSH assay.

room to achieve sufficient mental and physical calm. The systolic BP (SBP) and diastolic BP (DBP) were measured three times (1 minute apart). The average readings for SBP and DBP were then used to calculate the mean BP (MBP) using the formula:  $MBP = \frac{2}{3}DBP + \frac{1}{3}SBP$ .

### Blood Sampling

Subjects were instructed to fast between 9 PM and retiring on the evenings before the blood drawing. On the morning of the test, subjects were requested to have only a light breakfast, such as simple toast. They were asked to avoid any cooked breakfast, meat, cereal, fresh fruits, fruit juice, and any nutritional supplements.<sup>49</sup> Subjects were also asked to abstain from caffeinated beverages and chocolate and from alcohol for at least 2 hours before the visit.

All blood samples were obtained by a qualified phlebotomist in the morning, between 9 and 10 AM. Seven milliliters of blood was collected in EDTA-treated tubes (to prevent oxidation<sup>50</sup>) by venipuncture to the antecubital vein. Thirty microliters of blood was then transferred into centrifuge tubes for initial processing. The GSH was released from the blood cells by protein precipitation and cellular disruption achieved by addition of 33.3  $\mu$ L of 5-sulfosalicylic acid (SSA), 100 mg/mL within 10 minutes from the blood collection.<sup>51</sup> Each sample was then diluted with 936.7  $\mu$ L sodium phosphate buffer (pH 7.5), and the content of each tube was mixed rapidly in a centrifuge at 13,000 rpm for 5 minutes. A portion of supernatant (150  $\mu$ L) was then collected into clean centrifuge tubes and immediately cooled at  $-70^{\circ}\text{C}$ . Samples stored at this temperature are stable for at least 2 months and can be transported on dry ice without deterioration.<sup>52</sup>

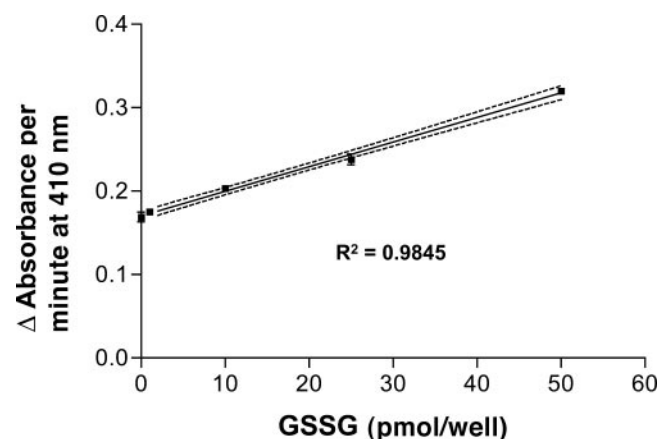
**FIGURE 2.** The standard curve for the GSSG assay.

TABLE 2. Characteristics of the Study Groups

Parameter	Glaucoma Patients (n = 21)	Control Subjects (n = 34)	P
Age (y)	72.40 ± 11.00	68.45 ± 10.11	>0.05
IOP (mm Hg)	24.04 ± 5.47	18.68 ± 3.59	<0.001
MD (dB)	-5.03 ± 7.47	-1.05 ± 2.00	=0.020
PSD (dB)	5.74 ± 3.62	1.70 ± 0.42	<0.001
SBP (mm Hg)	142.72 ± 20.95	142.24 ± 23.66	>0.05
DBP (mm Hg)	79.99 ± 12.34	82.76 ± 12.36	>0.05
MBP (mm Hg)	100.90 ± 13.58	102.58 ± 15.24	>0.05

## Blood Analysis

**GSH Assay.** The total GSH levels (t-GSH) were assessed by the GSSGR-DTNB (5,5 dithiobis-2-nitrobenzoic acid) recycling procedure, as described in previous studies.<sup>50,53</sup> The reagents and equipment used for this assay are listed in Table 1.

A standard curve from 0 to 2 nanomoles in 0.5-nanomole increments using GSH solution (0.2–0.8  $\mu$ L) was prepared. The standards contained the same final concentrations of SSA as for the samples (1%). To each well of a 96-well plate, 150  $\mu$ L of daily buffer (see Table 1), 50  $\mu$ L of DTNB solution, and 25  $\mu$ L of standards or samples were added in quadruplicate, and the plate was incubated at 37°C for 3 minutes. Finally, 25  $\mu$ L GSSGR was added to the previous mixture, and the plate was read at 410 nm using a 96-well plate reader.<sup>54</sup> A standard curve was then generated by linear regression (Excel; Microsoft, Redmond, WA; Fig. 1).

**GSSG Assay.** The GSSG levels were assessed with a DTNB-GSSGR recycling assay.<sup>50</sup> The reagents used in this assay were those described in Table 1 and, in addition, triethanolamine (TEA) and 2-vinyl pyridine (2-VP). TEA prevents a high local pH and oxidation, whereas 2-VP is used to stabilize GSH.

A GSSG standard curve from 0 to 0.25 nanomole, in 0.025-nanomole increments was prepared. Standards and samples (100  $\mu$ L) were transferred into separate centrifuge tubes and 2  $\mu$ L 2-VP was added to each tube. TEA was then used to adjust the pH of the standards/samples to 7.5.

To each well of the 96-well plate, 150  $\mu$ L of daily buffer, 50  $\mu$ L of DTNB solution, and 25  $\mu$ L of standards or samples were added in quadruplicate, and the plate was incubated at 37°C for 3 minutes. Finally, 25  $\mu$ L of GSSGR was added to the previous mixture, and the plate was read at 410 nm with a 96-well plate reader. A standard curve was then generated by linear regression (Excel Microsoft; Fig. 2).

Finally, the GSH levels (GSH = t-GSH – GSSG) and the redox index (defined as the GSH to GSSG ratio) were calculated.

## Statistical Analysis

The statistical analysis was performed on computer (Statistica for Windows; StatSoft, Tulsa, OK). Data are expressed as the mean  $\pm$  SD. Differences between groups at baseline for age, gender, and systemic BP parameters were calculated using Student's *t*-test for unpaired variables. A multivariate analysis was performed to test the influence of age, gender, and systemic BP on blood GSH levels. Differences between the two study groups in blood GSH levels were computed by analysis of covariance (ANCOVA).  $P < 0.05$  was considered statistically significant.

## RESULTS

Twenty-one patients with POAG (7 men and 14 women) and 34 control subjects (15 men and 19 women) years were included in the study. The characteristics of the study groups are given in Table 2. There was no significant difference in age and systemic BP between patients with glaucoma and control subjects ( $P > 0.05$ ); however, there were significant differences in IOP ( $P < 0.001$ ), and the measured VF parameters (mean deviation [MD] $P = 0.020$  and pattern standard deviation [PSD] $P < 0.001$ , respectively).

### The Effect of Age and SBP

In the control group, age had a negative effect on the level of both GSH ( $P = 0.002$ ,  $r = -0.52$ ; Fig. 3) and t-GSH ( $P = 0.002$ ,  $r = -0.52$ ; Fig. 4) but not on the level of GSSG and redox index ( $P > 0.05$ ,  $r = -0.17$ ; and  $P > 0.05$ ,  $r = -0.25$ , respectively). In patients with glaucoma, however, blood GSH

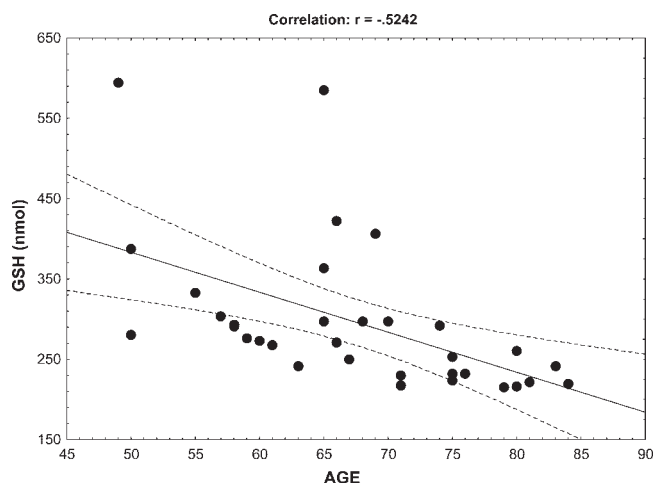


FIGURE 3. The influence of age on plasma GSH levels.

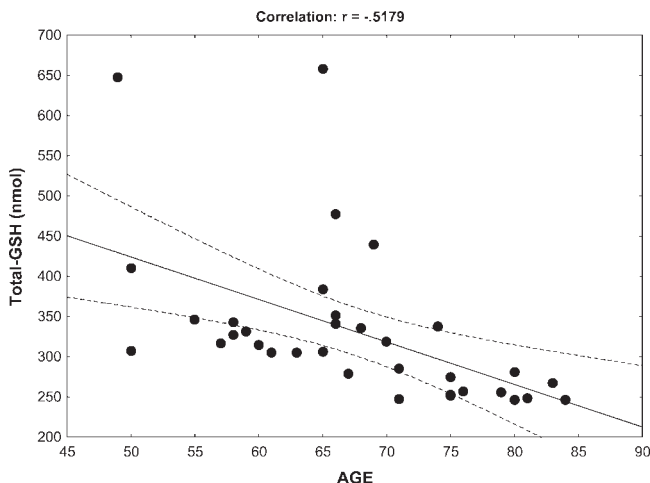


FIGURE 4. The influence of age on t-GSH levels.

TABLE 3. Gender Influence on Blood GSH Levels

Parameter	Glaucoma Patients (n = 21)			Control Subjects (n = 34)		
	Men (n = 7)	Women (n = 14)	P	Men (n = 15)	Women (n = 19)	P
GSH (nmol)	225.42 ± 111.50	224.85 ± 87.36	>0.05	338.47 ± 120.32	265.62 ± 50.68	=0.024
GSSG (nmol)	34.13 ± 8.94	25.22 ± 20.80	>0.05	36.10 ± 16.98	34.94 ± 18.09	>0.05
Redox index (GSH/GSSG)	10.29 ± 7.13	9.13 ± 6.27	>0.05	10.90 ± 5.24	10.24 ± 7.72	>0.05
t-GSH (nmol)	249.93 ± 96.45	258.98 ± 80.44	>0.05	372.35 ± 126.16	300.55 ± 54.85	=0.032

levels were not significantly influenced by age (GSH:  $P > 0.05$ ,  $r = 0.27$ ; t-GSH:  $P > 0.05$ ,  $r = 0.22$ ; GSSG:  $P > 0.05$ ,  $r = -0.27$ ; and redox index:  $P > 0.05$ ,  $r = 0.25$ ). There was no significant association between blood GSH levels and systemic BP in either of the study groups ( $P > 0.05$ ).

### Gender Effect

There were no differences in the proportion of men and women included in the study groups ( $P > 0.05$ ,  $\chi^2$  test). The relationship between gender and blood GSH levels is illustrated in Table 3. In the control group, men demonstrated higher levels of both GSH ( $P = 0.024$ ; Fig. 5) and t-GSH ( $P = 0.032$ ; Fig. 6). GSSG and redox index levels were similar in both men and women ( $P > 0.05$ ). In the glaucoma group, men and women had similar levels of blood GSH ( $P > 0.05$ ).

### Intergroup Differences in Blood GSH

Table 4 shows blood GSH and GSSG levels in both study groups. After correcting for age and gender influence on blood GSH levels, patients with glaucoma exhibited significantly lower levels of GSH and t-GSH than control subjects ( $P = 0.010$ ,  $F = 7.24$ ; and  $P = 0.006$ ,  $F = 8.38$ , respectively; Figs. 7-8). There were no significant differences between study groups in GSSG levels and redox index ( $P > 0.05$ ,  $F = 0.50$ ; and  $P > 0.05$ ,  $F = 0.33$ , respectively).

### DISCUSSION

The present study assessed the circulating GSH and GSSG levels in patients with newly diagnosed and previously untreated POAG. Our results disclosed that, independent of age and gender, patients with glaucoma demonstrated lower GSH

and t-GSH levels than age-matched control subjects. GSSG levels and redox index, however, were similar in both study groups.

GSH is among the most efficient substances that cells and tissues use in their defense against oxidative stress.<sup>37</sup> Age and disease, however, act to decrease the amount of GSH available in the organism. It has been demonstrated that at least half of apparently healthy elderly individuals show low blood GSH levels.<sup>55-57</sup> Indeed, our results show a negative correlation between age and both GSH and t-GSH levels in control subjects. This effect was lost in the glaucoma group. In the general population, age is strongly associated with increased oxidative stress and reduced antioxidant status.<sup>58</sup> The loss of this correlation between age and GSH loss in patients with POAG demonstrated in the present study could indicate that a more important, independent factor than age influences GSH levels in this cohort.

It has been shown that blood GSH levels are influenced by gender, with men demonstrating higher levels than women.<sup>59</sup> Our results also show that in control subjects, men had higher levels of GSH and t-GSH than did women. Nevertheless, in patients in the glaucoma group both genders demonstrated similar blood GSH levels, which again suggests an overriding modulation of GSH in POAG that exceeds that afforded by either gender or age.

Clinical and laboratory studies performed so far have demonstrated a significant decrease in blood GSH concentration in both patients and animals with various systemic<sup>28-31</sup> and ocular<sup>42-45</sup> diseases. Although recent studies have shown that there are altered levels of GSH and GSH activity in the trabecular meshwork and aqueous humor of patients with glaucoma,<sup>23-26</sup> to date no study has demonstrated an abnormal GSH level in the blood of patients with POAG.

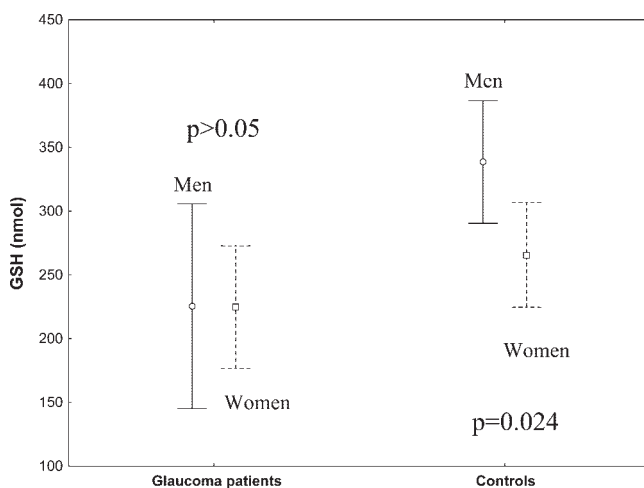


FIGURE 5. Men demonstrated higher levels of GSH than did women in the control group, but not the POAG group.

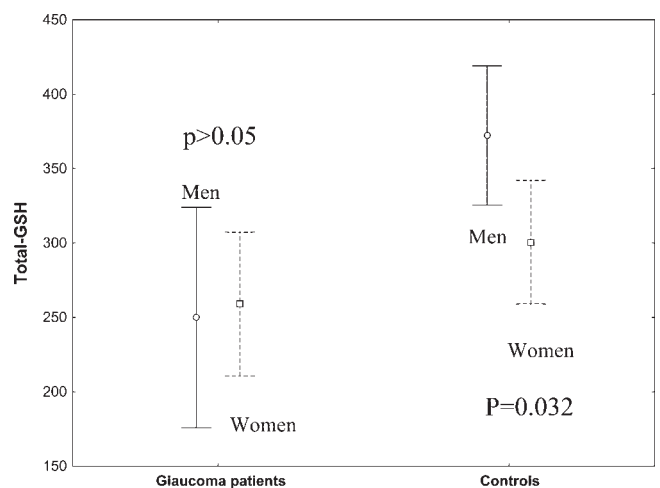


FIGURE 6. Men demonstrated higher levels of t-GSH than did women in the control group but not in the POAG group.

TABLE 4. Blood Glutathione Levels in the Study Groups

Parameter	Glaucoma Patients (n = 21)	Control Subjects (n = 34)	P
GSH (nmol)	225.27 ± 83.03	332.23 ± 98.50	0.010
GSSG (nmol)	31.79 ± 18.61	35.34 ± 17.64	>0.05
Redox index (GSH/GSSG)	9.44 ± 6.34	10.52 ± 6.69	>0.05
t-GSH (nmol)	256.28 ± 83.03	333.04 ± 99.92	0.006

Circulating GSH can be depleted either by subjecting cells to oxidative stress, or by inhibition of synthesis. In patients with glaucoma, due to a high level of oxidative stress,<sup>22-26</sup> GSH could be overused in reactions that result in GSSG production.<sup>51</sup> Nevertheless, our study groups demonstrated similar GSSG levels. One explanation could be that the GSSG resulted from the reaction between GSH, and ROS is rapidly reduced back to GSH, thus completing the normal redox cycle.<sup>60</sup> In normal conditions, GSSG is quite effective in maintaining GSH in its reduced state.<sup>60</sup> Our patients with glaucoma, however, demonstrated a significantly lower GSH level than control subjects, indicating that this mechanism played a role in our model and implying that the protection against ROS afforded by GSH could be reduced in patients with this disease, possibly due to a defective redox cycle.

Another possible reason for low GSH levels in our patients with glaucoma could be defective intracellular synthesis. The liver is the major site for GSH synthesis.<sup>61</sup> The precursors necessary for this synthesis are L-glutamate, L-cysteine, and L-glycine and the first step of the reaction, catalyzed by GSC, is controlled by a negative feedback from its end product, GSH.<sup>62</sup> In the present study, however, the level of GSH in patients with glaucoma was found to be low, therefore suggesting that the limiting factors in this case could be the availability of precursors of GSH.<sup>60</sup> Although both glutamate and glycine are important, it seems that the major determinant of the rate of GSH synthesis is the availability of the amino acid cysteine (Cys).<sup>63</sup> Cysteine results from the metabolism of homocysteine (Hcy) in the presence of cofactors such as vitamins B6, B12, and folate. Any interruption in the Hcy-Cys pathway could result, not only in the accumulation of the former but also in less available quantities from the second amino acid. In high concentrations, Hcy has been implicated in the etiology of various cardio- and cerebrovascular diseases.<sup>64-66</sup> Because vascular risk factors are implicated in the etiology of POAG,<sup>5-9,12-21</sup> one could hypothesize that abnormal levels of

circulating Hcy are to be found in patients with this disease. The results of recent research published on this subject have been contradictory. Bleich et al.<sup>67</sup> reported higher plasma Hcy levels in patients with POAG than in control subjects whereas, in a more recent study, Wang et al.<sup>69</sup> did not detect any significant difference in plasma Hcy between patients with POAG and control subjects. In the present study, we did not seek to quantify the plasma Hcy in our patients and control subjects. We suggest that the abnormal low level of GSH in patients with glaucoma could be at least partially explained by a lower availability of Cys due to an abnormal Hcy-Cys metabolic pathway.

A low level of circulating GSH may also result in a higher rate of oxidative reactions that reduce the bioavailability of NO. Several studies have shown that NO is also involved in the regulation of systemic hemodynamics,<sup>69,70</sup> with disturbed NO homeostasis contributing to alterations in systemic vascular diseases such as hypertension or diabetes, in which basal formation of NO is impaired.<sup>71,72</sup> It has also been hypothesized that a low NO production has a major effect on the equilibrium between the endothelial vasoconstrictive and vasodilatory factors at the ocular level that could also result in a decreased OBF in susceptible patients with glaucoma.<sup>73</sup> In addition to the hemodynamic role, NO has also been shown to induce relaxation of the trabecular meshwork and the ciliary muscle, resulting in a decrease in IOP.<sup>74</sup> Any disturbance in the NO balance could therefore act both at the ocular and systemic levels<sup>47</sup> and have dramatic consequences on the progression of glaucoma. It can be suggested that in some patients with glaucoma, not only the high level of IOP but also the occurrence of vasospasm<sup>75</sup> and thrombosis<sup>76</sup> can be the result of reduced NO bioavailability due to low levels of circulating GSH.

In conclusion, our study demonstrated for the first time that untreated patients with POAG exhibit low levels of circulating

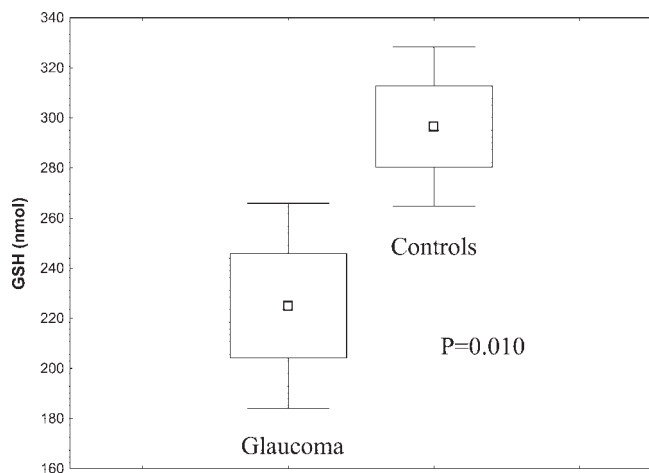


FIGURE 7. Patients with glaucoma exhibited lower levels of GSH than did control subjects.

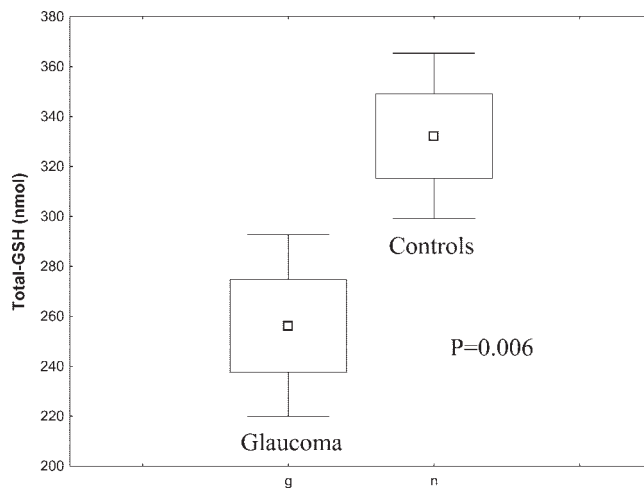


FIGURE 8. Patients with glaucoma exhibited lower levels of t-GSH than did control subjects.

GSH, suggesting a general compromise to antioxidative defense. Further investigations are needed, to identify the cause of this biochemical deficiency.

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