Trace Element Status and Free Radical Defense in Elderly Rhesus Macaques (*Macaca mulatta*) with Macular Drusen (43864)

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Abstract. **Research into the mechanisms underlying the development of age-related macular degeneration (AMD), the leading cause of visual loss in the United States and Europe in people over 60 years old, has been limited in part by the lack of animal models for this disease. In the current study, we examined 62 elderly (≥20 years old) rhesus macaques (*Macaca mulatta*) for the presence and severity of macular drusen. Drusen were observed in 47% of the macaques; they were similar histologically and in clinical appearance to the drusen observed in humans with AMD. It has been proposed that excessive tissue free radical damage may contribute to the development of AMD. Thus, circulating levels of select components of the free radical defense system and plasma thiobarbituric acid reactive substances (TBARS), an estimate of lipid peroxides, were measured in the above animals. Macaques diagnosed with drusen were characterized by alterations in concentrations and activities of several components of the free radical defense system. Alterations were most evident with respect to those enzymes associated with copper. The concept that excessive oxidative lipid damage might be a factor contributing to the occurrence of this disease is suggested by the findings of higher plasma TBARS concentrations in animals with >10 drusen compared with animals without drusen.** [P.S.E.B.M. 1995, Vol 208]

Age-related macular degeneration (AMD) is the leading cause of visual loss in the United States and Europe in people over 60 years old, affecting up to 30% of those over the age of 75 (1, 2). It is currently thought that the disease is an advanced stage of an age-related deteriorative process that occurs in all eyes to some degree (3, 4); however, the mechanisms underlying the development of the disease are poorly understood. One hypothesis for the increased susceptibility of some individuals to develop AMD is that these individuals may have membrane damage resulting from their inability to detoxify free radicals generated via photooxidation in the retina (5–7).

Characteristic of AMD is atrophy of the retinal pigment epithelium (RPE) that is most pronounced in the central macular region of the eye, the area of greatest visual acuity (8, 9). The RPE cells have many functions, including the transportation of nutrients to the neurosensory retina and disposal of waste products from the photoreceptor cells (rods and cones), through an extensive lysosomal system (10). Early stages of the disease are characterized by the presence of drusen, which are yellowish-white, elevated nodules of lipofuscin deposited within and between Bruch's membrane and the RPE (11, 12). Evidence suggests that the density, size, and lipid composition of drusen determine the magnitude of vision loss associated with AMD (13, 14). Although there are numerous theories regarding the pathogenesis of drusen, there is no general agreement on how they are formed. They appear to occur due to alterations in the cells' digestive mech-

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anisms, probably resulting from a failure of several different metabolic processes (11). As the size and number of drusen increase, so do the chances that the RPE cells will die (8) and that AMD will progress (13, 14).

A major impediment in studying AMD has been the lack of available animal models. However, the presence of macular subretinal hypopigmented lesions, which resemble drusen found in humans with AMD, has been reported in rhesus macaques (11, 12, 15, 16). The reported frequencies of affected animals in the colonies surveyed varied from 6% to 76%, and, when combined, the results from these studies indicate that the prevalence of the disease increases with age (12, 15). Given the above, we sought to determine if a significant frequency of macular drusen was present in elderly populations of rhesus macaques maintained at the California Regional Primate Research Center (CRPRC) at the University of California, Davis (Davis, CA) and at the Wisconsin Regional Primate Research Center (WRPRC) at the University of Wisconsin (Madison, WI), and if the clinical presentation of the drusen was similar to that observed in humans. In addition, plasma and erythrocyte levels of select components of the antioxidant defense system, and plasma thiobarbituric acid reactive substances (TBARS), an estimate of lipid peroxides, were measured to determine if there was evidence of an altered circulating oxidative defense system in macaques with drusen.

Materials and Methods

Subjects. Colonies of elderly rhesus macaques (*Macaca mulatta*) housed in indoor animal facilities at the CRPRC and WRPRC were included in the present investigation. All macaques were maintained according to AAALAC standards, housed in raised stainless steel cages with automatic watering systems, and fed a commercial, complete primate diet (Purina Monkey Chow; Purina Mills Inc., St. Louis, MO). All procedures used conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. Protocols were approved by each campus's veterinarian and Animal Care and Resources Committee.

From the CRPRC, a total of 16 female and 13 male macaques were included, with ages ranging from 20 to 33 years old; ages of females and males were 23.6 ± 0.9 and 23.4 ± 0.6 years, respectively. From the WRPRC, a total of 23 female and 10 male macaques were included, with ages ranging from 21 to 36 years old; ages of females and males were 24.2 ± 0.6 and 28.4 ± 1.7 years, respectively.

Clinical Observations. Ocular examinations were conducted under ketamine hydrochloride anesthesia (10 mg/kg body wt). Mydriacil (1%) was applied to the cornea for pupillary dilation. Indirect ophthalmoscopy, with either a 20D or 14D lens, was used to confirm the presence or absence of drusen. Fundus photographs were taken with either a KOWA RC2 or Zeiss fundus camera. Photographs were reviewed by two independent, experienced readers. Each CRPRC animal's health status was evaluated using physical examination, hemograms, and serum chemistries. Indirect blood pressure measurements were taken on California macaques using a Dinamap vital signs monitor (Model 1846SX; Critikon Inc., Tampa, FL), while the animals were under ketamine anesthesia.

Fundus fluoroscein angiograms were performed on four macaques from the CRPRC and one macaque from WRPRC, under anesthesia as described above. Two milliliters of 25% sodium fluoroscein were injected intravenously into the antecubital vein. Photographs were taken at intervals using a fundus camera.

Histopathology. During the course of the study an elderly macaque at the CRPRC with paralyzing spinal cord compression was euthanized with an intravenous overdose of pentobarbital (1 cc/kg body wt). The animal was enucleated immediately following death and the eyes processed for histopathology. The eyes were fixed by immersion in 10% neutral buffered formalin. After fixation the globes were opened in a parasagittal plane and a central portion containing the macula was processed routinely and embedded in paraffin. Five micron sections were cut and stained with periodic acid-Schiff (PAS) and examined by light microscopy to identify drusen.

Collection and Processing of Blood Samples. Peripheral venous blood samples (5–7 ml) were collected into heparinized tubes. Aliquots of whole blood were stored at −80°C for subsequent analysis of glutathione peroxidase (GSHPx) and glutathione reductase (GSHRed) activities. Diluted plasma was analyzed for TBARS as an estimate of plasma lipid peroxide levels. One hundred microliters of whole blood was added to 1 ml of physiological saline and centrifuged at 1500*g* for 15 min; the supernate was stored at −80°C until analyzed for TBARS (17). The remaining blood was centrifuged at 1500*g* for 20 min, and plasma fractions were stored at −80°C until analyzed for copper and zinc concentrations, and ceruloplasmin (Cp) activity. To prepare lysates for red blood cell superoxide dismutase (RBC SOD) activity determination, packed red blood cells were diluted with an equal volume of distilled, deionized water, mixed, and centrifuged at 1500*g*. Plasma samples from WRPRC macaques were collected and stored at −80°C until analyzed for the above parameters; these samples were also analyzed for vitamins A and E, extracellular (EC) SOD and GSHPx activities, and Cp concentrations. Seven milliliters of venous blood obtained from

the CRPRC macaques was collected into tubes with EDTA for complete blood counts, and into tubes free of anticlotting agents for chemistry panels.

Enzyme and Trace Element Analysis of Blood Fractions. Analytical grade chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Blood and plasma GSHPx activities were assayed according to the method of Lawrence and Burk (18). Selenium-dependent GSHPx activity was specifically measured by utilizing 5 m*M* hydrogen peroxide in the assay system. Glutathione peroxidase activity is presented as units per milligram hemoglobin (for blood) or units per milliliter (for plasma), where one unit is defined as one nanomole NADPH oxidized per minute.

Blood GSHRed activity was measured as described by Rogers and Augusteyn (19); data are expressed as units per milligram hemoglobin, where one unit is defined as one nanomole of NADPH oxidized per minute.

Following hemoglobin extraction from the red cell lysates (20), RBC SOD activity was determined according to Marklund and Marklund (21). One unit of SOD activity is defined as the amount of enzyme needed to inhibit the autooxidation of pyrogallol by 50%. Results are expressed as units of activity per milligram hemoglobin. Extracellular SOD was determined in plasma using a modification of the pyrogallol autooxidation method (22); results are expressed as units of activity per milliliter plasma.

Hemoglobin concentrations were determined by the cyanomethemoglobin colorimetric procedure (Diagnostic Kit #525).

Plasma TBARS were measured as described by Yagi (17); results are expressed as nanomoles malondialdehyde (MDA), an end product of lipid peroxidation, per milliliter blood. The analytical standard used was 22 μ*M* 1,1,3,3-tetraethoxypropane.

Plasma zinc and copper concentrations were determined by flame atomic absorption spectrophotometry (23) (Model 551; Thermo Jarrell Ash, Wilmington, MA). Plasma was diluted with 0.1 *N* Baker's Instra-Analyzed nitric acid (J. T. Baker, Phillipsburg, NJ) prior to analysis.

Plasma Cp activity was measured following the oxidation of o-dianisidine dihydrochloride as described by Schosinsky *et al.* (24). Units of activity are defined as the difference in absorption at 540 nm at 5 and 15 min, and are expressed per liter plasma. Plasma Cp protein concentrations were determined using a commercial radial immunodiffusion kit (The Binding Site, Birmingham, England); results are expressed as milligrams per liter plasma.

Plasma concentrations of vitamins A and E were measured simultaneously by HPLC according to the method of Driskell *et al.* (25). Concentrations were quantitated by comparing heights of retinol or tocopherol peaks to the height of the retinyl acetate internal standard peak.

Chemistry panels and complete blood counts were determined by the University of California Veterinary Medicine Teaching Hospital diagnostic laboratory (Davis, CA).

Data Analysis. Results are presented as mean ± SEM. One-way analysis of variance (ANOVA) was used for statistical analysis (Statview II; Abacus Concept, Berkeley, CA). A P value ≤ 0.05, using Fisher's least significant difference test, was considered statistically significant.

Results

Data for control monkeys from the two colonies (CRPRC and WRPRC) were not significantly different from each other, and therefore, to ease presentation, the data from the two sets of animals have been combined.

Clinical Observations. Ophthalmic examination and photographs of the fundi revealed that 14 of the 29 animals at the CRPRC and 15 of the 33 animals at the WRPRC examined had subretinal yellowish-white lesions, considered to represent drusen. When further categorized according to the number of drusen per animal, a total of 18 animals had 1–10 drusen and 11 had >10 drusen. Ages of the animals were similar among the groups (24.1 ± 0.7, 24.8 ± 0.7, and 25.5 ± 1.1 years old for no drusen, 1–10 drusen, and >10 drusen groups, respectively). Males and females were evenly distributed among the groups.

The drusen were confirmed by fluoroscein angiography in five animals and by histopathology in one animal (Fig. 1–3). The drusen were clinically distinct from the previously described intra-RPE flecks (26), and were of both the soft and hard type. All animals had bilateral involvement, but there was no evidence of subretinal neovascular hemorrhages or disciform scars in any of the animals observed.

There were no differences in systolic or diastolic blood pressure among the three groups (data not shown).

Histopathology. Figure 3 shows an example of several sub-RPE deposits that are PAS positive; the histopathology was consistent with the description of drusen in human eyes (27).

Enzyme and Trace Element Analysis of Blood Fractions. Plasma Cp activity was significantly higher in macaques with 1–10 drusen compared with macaques without drusen and those with >10 drusen (Table I). Cp protein concentrations were significantly higher in macaques with 1–10 drusen than in macaques with >10 drusen; macaques without drusen had levels

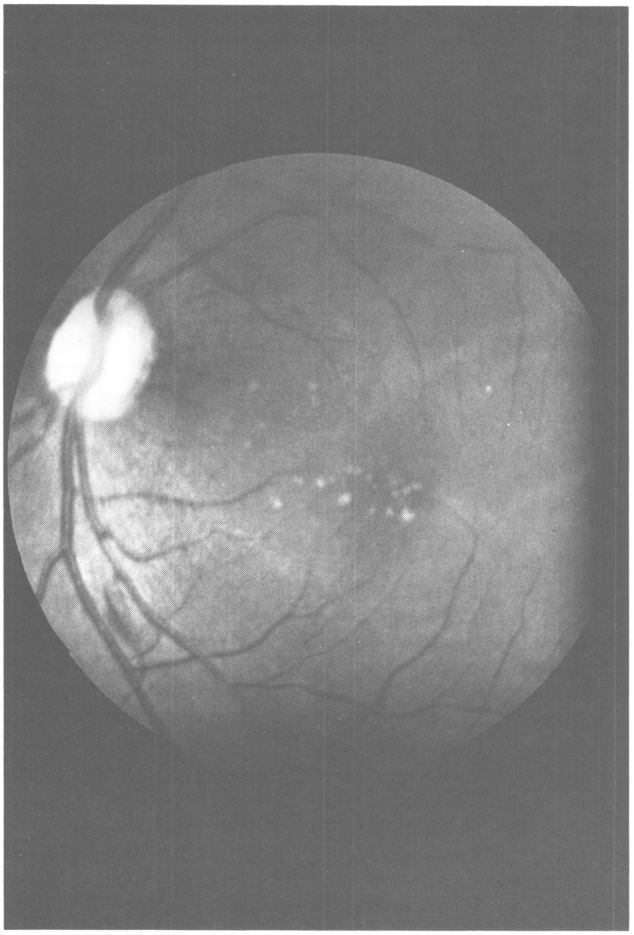

Figure 1. A fundus photograph of a rhesus macaque with macular drusen.

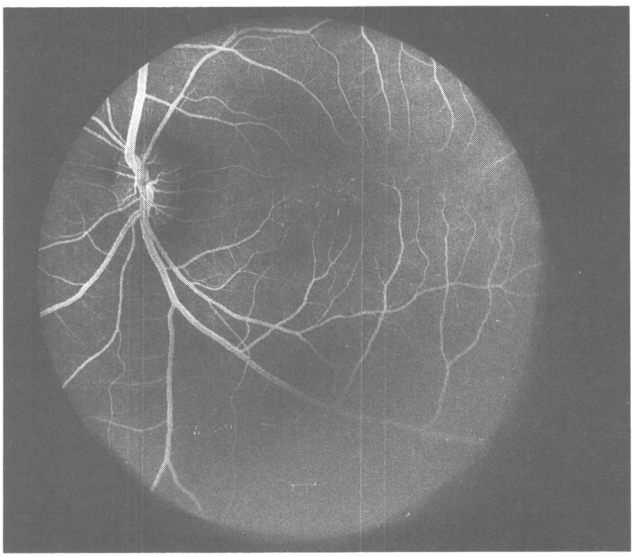

Figure 2. A fundus fluorescein angiogram of a rhesus macaque showing hyperfluorescent window defects characteristic of drusen.

that were similar to values obtained from animals diagnosed with drusen (Table I).

Macaques with >10 drusen had significantly lower RBC SOD activities than macaques diagnosed with 1–10 drusen, and tended to have lower activities than those free of ocular lesions ($P = 0.14$) (Table I). Blood GSHPx and GSHRed activities tended to be higher in macaques with drusen compared with those without: those with 1–10 drusen had higher blood GSHRed activities than controls, and macaques with >10 drusen had higher blood GSHPx activities compared with macaques without drusen (Table I). Activities of plasma GSHPx and EC SOD, and concentrations of vitamins A and E were similar among the groups (Table I).

Consistent with plasma Cp activity, plasma copper concentrations were highest in the animals with 1–10 drusen ($P = 0.07$ vs macaques with no drusen) (Table II). When copper concentrations were regressed against Cp activity, macaques without drusen and those with 1–10 drusen had r^2 values typically observed for this analysis ($r^2 = 0.63$ and 0.61, respectively; $P < 0.0001$), whereas macaques with >10 drusen had a very poor regression value ($r^2 = 0.08$).

Plasma zinc concentrations tended to be lower in macaques with >10 drusen compared with those diagnosed without drusen ($P = 0.10$) and those with 1–10 drusen ($P = 0.12$) (Table II).

Plasma TBARS concentrations were higher in macaques as the number of drusen increased; those macaques with >10 drusen had significantly higher circulating TBARS than macaques without drusen (Table II).

Enzyme activities measured to evaluate liver function (ALT and AST) in the CRPRC animals, were similar among the groups (data not shown). There were no differences among the groups with regard to other CBC and chemistry panel parameters.

Discussion

The present investigation was undertaken to achieve two major goals: (i) to determine if macular drusen was evident in colonies of elderly rhesus macaques at a high frequency, and if the clinical presentation of the drusen was similar to that observed in humans, and (ii) to determine if there was evidence of an altered circulating oxidative defense system in the macaques with drusen.

With respect to the first goal, the occurrence of drusen was observed in 47% of the macaques examined aged 20 years and older ($n = 62$). This finding is consistent with that of Ulshafer *et al.* (12) who observed a frequency of drusenoid lesions in 76% of 29 aged (mean age, 20 years) rhesus macaques examined. In a survey of 574 rhesus macaques located at 12 different primate research centers, Stafford *et al.* (16) reported that only 5.9% of the macaques had drusen, however, the ages of the macaques ranged from 10 to 31 years. This suggests that similar to humans, drusen

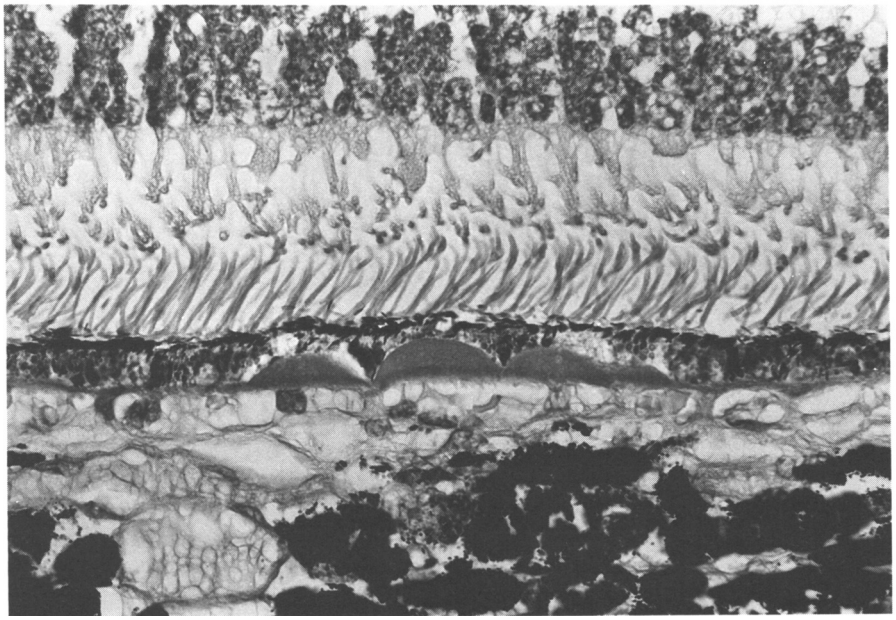

Figure 3. A photomicrograph of macular drusen in a rhesus macaque showing PAS-stained subretinal pigment epithelium deposits.

are more frequently observed in older animals. Consistent with this, El-Mofty *et al.* (27) and Bellhorn *et al.* (15) have reported a general increased incidence of macular pigmented anomalies in old compared with young macaques. The observation of soft drusen in the current investigation support the use of the macaque model in studying AMD, as it is the soft drusen, more so than hard drusen, that are associated with the progression of AMD in humans (13, 14).

In addition to the clinical and histopathologic analyses, the current study evaluated circulating concentrations of select trace elements and activities of several antioxidant enzymes to test the hypothesis that macaques with macular drusen have an altered free radical defense system. It has been suggested that the antioxidant activity of the RPE is reduced in AMD (9, 28) and that this reduction may be linked to the RPE deterioration observed with AMD (8, 29). Oxygen turnover in the retina is very high, in part as a consequence of the intensive energy production associated with the large numbers of mitochondria in the photoreceptor inner segments; this high oxygen turnover contributes to the retina's exposure to high concentrations of oxygen free radicals (30). The combination of the RPE's extremely high proportion of polyunsaturated, and, thus, peroxidizable fatty acids and a high generation of reactive oxygen species may induce the degenerative changes and damage seen in the aging macula (9, 30). In the current study, plasma zinc and copper concentrations were measured in part because the RPE has one of the highest concentrations of zinc and copper in the body (31, 32). In addition, a signif-

Table I. Circulating Antioxidant Levels and TBARS Concentrations in Elderly Rhesus Macaques

	No drusen	1–10 drusen	>10 drusen	*P* value
Plasma Cp activity (U/liter)[a]	128 ± 7[b]	162 ± 10[c]	123 ± 15[b]	0.012
Plasma Cp protein (mg/liter)[d]	450 ± 18[b,c]	491 ± 28[b]	418 ± 21[c]	0.049
Plasma vitamin A (μg/dl)[d]	54.3 ± 3.2	56.0 ± 10.5	69.0 ± 13.0	0.360
Plasma vitamin E (μg/dl)[d]	1070 ± 56	1103 ± 77	1120 ± 93	0.873
Plasma EC SOD activity (U/ml)[d]	31.0 ± 3.0	28.1 ± 3.1	27.8 ± 3.4	0.740
Plasma GSHPx activity (U/ml)[d]	98.9 ± 5.5	94.9 ± 9.7	109.1 ± 8.0	0.469
Blood GSHPx activity (U/mg Hb)[a]	17.4 ± 0.7	18.6 ± 0.7	20.1 ± 1.2	0.127
Blood GSHRed activity (U/mg Hb)[a]	2.26 ± 0.09	2.55 ± 0.13	2.53 ± 0.21	0.141
RBC SOD activity (U/mg Hb)[a]	0.525 ± 0.019[b]	0.634 ± 0.030[c]	0.469 ± 0.038[b]	0.001
Plasma TBARS (nmoles MDA/ml)[a]	5.82 ± 0.16[b]	6.08 ± 0.36[b,c]	7.05 ± 0.55[c]	0.034

Note. Values are the mean ± SEM. Cp = ceruloplasmin; EC = extracellular; SOD = superoxide dismutase; GSHPx = glutathione peroxidase; GSHRed = glutathione reductase; TBARS = thiobarbituric acid reactive substances.
[a] n = 33, 18, 11 for monkeys without drusen, 1–10 drusen, and >10 drusen groups, respectively.
[b,c] Values within a row not sharing a common superscript are significantly different from each other ($P < 0.05$).
[d] n = 18, 7, 8 for monkeys without drusen, 1–10 drusen, and >10 drusen groups, respectively.

Table II. Plasma Copper and Zinc Concentrations in Elderly Rhesus Macaques

	No drusen	1–10 drusen	>10 drusen	*P* value
Plasma copper (μM)	17.9 ± 0.8	20.3 ± 1.2	18.7 ± 0.9	0.16
Plasma zinc (μM)	11.6 ± 0.6	11.8 ± 0.8	9.6 ± 1.1	0.19

Note. Values are the mean ± SEM (n = 33, 18, and 11 for no drusen, 1–10 drusen, and >10 drusen groups, respectively).

icant proportion of the zinc and copper is associated with the antioxidant enzymes, CuZn SOD (33) and EC SOD (34). Zinc is also involved in oxidative defense via its ability to stabilize biomembranes against peroxidative damage (35) and via its association with metallothionein, which has putative free radical quenching properties (36, 37).

Recently, there has been considerable interest in the idea that zinc metabolism may be altered in individuals with AMD. While the functions of zinc in the retina are still poorly defined, zinc deficiency can lead to visual impairment, which is reversible with administration of zinc (38–40). With regard to AMD the interest in zinc is primarily based on two observations; first, Wyszynski *et al.* (41) reported that the activity of the zinc-dependent lysosomal enzyme, α-mannosidase, in human RPE cells cultured *in vitro* decreased as donor age increased. These investigators reported that $ZnSO_4$ added to the cell supernates stimulated enzyme activity, suggesting that low α-mannosidase activity in the old RPE cells was due in part to a lack of the metal. Second, Newsome *et al.* (42) reported that the use of oral zinc supplements reduced the rate of visual loss in humans with AMD. The latter study was conducted based on the concept that zinc plays an important metabolic role in the retina and that the elderly are at risk for zinc deficiency. It is important to note that in the study by Newsome *et al.*, neither prenor postsupplementation plasma zinc values were significantly different from those of an ocular-normal group. In the present study, plasma zinc concentrations tended to be lowest in macaques with the highest incidence of drusen, suggesting that zinc metabolism may be altered in macaques with a high number of drusenoid lesions. Table III summarizes the results for plasma zinc and copper concentrations and plasma Cp activity found in the literature in humans with AMD compared with control populations.

Our data regarding plasma copper distribution among elderly macaques with and without drusen are similar to the findings of Newsome *et al.* for humans with AMD (43). Macaques with 1–10 drusen had higher activities of plasma Cp, a copper-binding protein. The higher Cp activity observed in these animals may be indicative of a chronic acute phase reaction in response to chronic physiological stress and/or tissue injury. Given that one putative function of Cp is to scavenge reactive oxygen species (44), the finding of high plasma Cp activity in macaques with drusen may reflect a protective response to an increased rate of oxidant production in these animals. These findings are thus consistent with the general hypothesis that one factor underlying the initiation and/or progression of AMD is excessive oxidative damage.

As anticipated, copper-dependent parameters (e.g., plasma copper and Cp protein concentrations; Cp and RBC SOD activities) were altered in macaques with drusen compared with those without. An unanticipated observation was that while plasma Cp activity was elevated in animals with 1–10 drusen, it was not elevated in animals with >10 drusen. A possible explanation for this finding is that animals in the earlier stages of the disease process (1–10 drusen) are characterized by alterations in copper metabolism, which are secondary in part to a chronic acute phase response. One of the hallmarks of an acute phase response is an increased production of Cp in the liver. The addition of copper to Cp is a post-translational modification of the protein, and both copper-containing Cp and apo Cp can be secreted from the liver. It can be postulated that with time, in the face of chronic acute protein synthesis, the liver copper stores needed for activation of Cp may become reduced to the point that significant amounts of apo Cp are released. Since the turnover of apo Cp is faster than that of holo Cp (45), the above would result in both a reduction in Cp protein and Cp activity in the animals with >10 drusen. Consistent with the above is the observation that plasma Cp activity and copper concentrations are well correlated in monkeys with 0–9 drusen, while plasma Cp activity and copper concentrations in macaques with >10 drusen are poorly correlated. This would be the situation if, as the disease progresses, there is less copper associated with Cp.

Table III. Plasma Copper and Zinc Concentrations and Ceruloplasmin (Cp) Activity in Humans and Rhesus Monkeys with Drusen Relative to Controls Without Drusen

	Plasma		
	Copper	Zinc	Cp activity
Humans:			
Newsome *et al.* (42, 43)	—	—	↑
Silverstone *et al.* (50)	↓	↑	NR
Wu (51)	—	↓	NR
Rhesus monkeys:			
Present study	—	—	↑[a]

Note. NR = not reported; ↑, ↓ = subjects with AMD reported to have significantly higher or lower values than control subjects; — = no significant difference detected between AMD and control subjects.

[a] Significantly higher in macaques with 1–10 drusen.

An additional putative function of Cp is to transfer copper to extrahepatic sites (46). However, despite elevated plasma Cp activity, extrahepatic tissue copper concentrations can be low in vascular disease (47, 48). Thus, the finding of high plasma Cp activity in macaques with drusen does not necessarily translate into an adequate supply of copper to the eye for synthesis of CuZn SOD and other critical cuproenzymes. Consistent with the above, among the three groups' macaques with >10 drusen had the lowest activity of the superoxide scavenging RBC SOD. The observation that higher plasma TBARS concentrations were highest in macaques with >10 drusen further supports the idea that these animals were compromised with respect to their ability to metabolize reactive oxygen species. This is critical given the fact that the retina contains the highest proportion of peroxidizable lipids in the body (49).

In summary, histopathologic, clinical, and biochemical observations indicate that the age-related changes in rhesus macaques with drusen are similar to those seen in the humans with AMD. Although it is unknown if concentrations and activities of the circulating parameters investigated reflect ocular levels, the data presented in the current study support the hypothesis that oxidative damage plays a role in the pathogenesis of drusen. The macaque model should provide an opportunity to investigate in detail the comparative metabolism of copper and zinc in animals with and without drusen, as well as the association between altered oxidant defense systems and the development of macular drusen.

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