Cone Degeneration in Aging and Age-Related Macular Degeneration

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Objective: To examine the morphological features of macular photoreceptors in histologically normal retina from normal donor eyes and eyes with age-related macular degeneration (AMD).

Methods: The macular region was excised from 18 donor eyes (aged 22-96 years) and cryosectioned. Sections were stained with hematoxylin-eosin or double immunolabeled using opsins antibodies or synaptic markers.

Results: Three of 8 retinas studied in detail had AMD lesions; the remainder were histologically normal. Immunoreactivity to cone opsin was abnormal in parts of all retinas (3.5%-95.0% of each sample) and was associated with swelling of and altered immunoreactivity in the cone distal axon. In non-AMD retinas, the anomalies were mainly in nonfoveal macular locations. The nature of the anomalies was identical in non-AMD retinas and in parts of AMD retinas adjacent to overt degeneration.

Conclusion: Redistribution of opsin and anomalies in the distal cone axon are common in the aging human macula and may indicate susceptibility to AMD.

Clinical Relevance: The findings are consistent with tests of cone function in aging and early AMD, which suggests that integrated cone functions—including contrast sensitivity, color matching, and short wavelength-sensitive cone sensitivity—are the most reliable prognostic indicators of progression in AMD.


Evidence that cone dysfunction is predictive of (and a reliable measure of severity in) age-related macular degeneration (AMD) has been widely reported (reviewed by Hogg and Chakravarthy). Surface color and color-matching tests, measures of cone sensitivity, isoluminance color-contrast sensitivity, cone-driven multifocal electretinography responses, and cone-adaptation kinetics each suggest a higher degree of pathological manifestation in the cone population than is clinically apparent in early AMD. Despite these data, analyses of photoreceptor topography in donor eyes with AMD indicate that rods in the parafovea show the earliest signs of degeneration in early nonexudative AMD, whereas the foveal cone mosaic superficially appears to be well preserved. In patients with early AMD, defects in rod function correlate with the histological evidence, including observations of an increase in the dark adaptation time constant and a reduction of scotopic sensitivity. To our knowledge, no reports of morphological and immunohistochemical changes in cones correlate with the many reports of cone dysfunction associated with the early stages of AMD.

In this study, we have identified morphological features of photoreceptors, particularly cones, that correlate with the visual dysfunction reported in early AMD. In the absence of traditional histopathological features of photoreceptor degeneration, we observed a number of features of macular cones indicative of pathological changes in non-AMD retinas and in the normal regions of the retina surrounding AMD lesions.

Methods

HUMAN SPECIMENS

Human eyes were collected with informed consent through the Lions Sydney Eye Bank, Sydney, Australia, with ethical approval from the ethics committees of the University of Sydney and the Australian National University. Eighteen eyes were fixed overnight in 4% paraformaldehyde and then stored in 2% buffered paraformaldehyde for 1 to 12 months at 4°C.

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Donor eye cups were rinsed in 0.1M phosphate-buffered saline solution, and the fundus was photographed using a dissecting stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) and a digital camera (C10plus; Jenoptick, Jena, Germany). These images were used to initially classify normal retinas vs those with possible AMD. The complex consisting of the retina, retinal pigment epithelium (RPE), and choroid was dissected from the eye cup, rinsed in 0.1M phosphate-buffered saline solution, incubated until saturated in a solution consisting of 30% sucrose and phosphate-buffered saline at 4°C, and flattened with radial cuts. The flattened retina was incubated in a solution consisting of 14% gelatin and 30% saturated sucrose at 37°C for 1 hour, then cooled to room temperature and set at 4°C. A block approximately 8 × 10 mm, including the macula, was excised and cryosectioned at 10 or 14 µm parallel to the horizontal meridian. One in every 60 (10-µm) or 43 (14-µm) sections was stained using hematoxylin–eosin (H&E) for histopathological analysis. These sections were graded for a range of histopathological features by 3 experienced graders (M.C.M., P.L.P., and J.M.P.), using published criteria and classified by consensus as normal or AMD affected. The criteria include total basal deposits, number of drusen, RPE morphological features (eg, nonuniform or atrophied), detachment choroidal neovascularization, and inner/outer segment loss.

### IMMUNOHISTOCHEMISTRY

A series of sections immediately adjacent to the H&E-stained sections was double immunolabeled with antibodies against long/medium wavelength–sensitive opsin (L/M opsin) and rhodopsin, followed antigen retrieval (Reveal Ag; available at http://www.immunosolution.com) using standard procedures. Details of the antibodies used are given in Table 1. Immunofluorescence was viewed using a laser scanning microscope (Carl Zeiss, Inc, Jena), acquired using software from the manufacturer (PASCAL, version 4.0; Carl Zeiss, Inc) and prepared for publication using image-editing software (Adobe Photoshop CS2; Adobe Systems, Inc, San Jose, California).

### CONSTRUCTING THE VIRTUAL FLAT MOUNT

Two-dimensional maps of the macula were created by first plotting on graph paper the identified histological landmarks in a full set of H&E-stained sections from each specimen (eg, vessel cross-sections and the edge of an AMD lesion), using microscope stage coordinates as a guide for position in the x-axis and the distance between plotted sections as a guide for location in the y-axis. Then, the distributions of defined grades of L/M opsin and rhodopsin labeling (described in the “Results” section) from the adjacent series of sections were plotted on the same axes, with reference to the histological features identified in the H&E-stained sections. In this way, a virtual flat mount was reconstructed representing data sampled from every 0.6-mm strip of each macula.

### CONTROLLING FOR POSTMORTEM DECAY

Adult Sprague-Dawley rats were humanely killed using an intraperitoneal injection of an overdose of pentobarbital sodium (>60 mg/kg [Valabarb; Jurox Pty Ltd, Silverwater, Australia]) with ethical approval from the animal ethics committee of The Australian National University. The left eyes of 4 rats (the same age and same sex) were removed and immediately fixed in 4% paraformaldehyde. Protocols for the right eyes simulated the handling conditions of the human donor tissue. After left eye enucleation, rats were left at room temperature for 1 hour, then stored at 4°C. Right eyes were collected at 6-hour intervals up to 24 hours after death and fixed in 4% paraformaldehyde. All eyes were stored in fixative for 2 weeks before further processing. The retina-RPE-choroid complex was then dissected from the eye cup, embedded in gelatin, and cryosectioned at 14 µm. Sections were stained using H&E, and adjacent sections were double immunolabeled with antibodies to L/M opsin, rhodopsin, and vesicular glutamate transporter 1 (vGluT1) to mimic processing of the human material.

### RESULTS

#### RAT CONTROL RETINAS

Rat retinas with fixation delays of 6 hours or more showed organizational changes in the photoreceptor inner and outer segments and outer nuclear layer and some modification of immunoreactivity compared with the controls; these changes were identified as resulting from postmortem decay (Figure 1C-H). Three specific features attributable to postmortem decay were identified. First, we noted photoreceptor sloughing, in which the layer of inner and outer segments is thrown into folds and appears detached at the level of the external limiting membrane (Figure 1D, E, G, and H). Second, “beading” of the cone axonal process—suggestive of breakdown of the axonal membrane—was evident in L/M opsin–labeled sections (Figure 1F and F). Third, intense rhodopsin immunoreactivity was seen in the membrane of the rod somata and inner segments (Figure 1E). Immunoreactivity along the whole cone cell membrane without axon beading (seen in some of the human retinas) was not observed in any of the rat retinas. Immunoreactivity for vGluT1 was not altered by fixation being delayed up to 24 hours (Figure 1C, F, and I).

### HISTOPATHOLOGICAL FEATURES AND SPECIMEN EXCLUSION

Ten of the 18 human retinas examined had features resembling artifacts identified in the delay-fixed rat retinas, including photoreceptor sloughing (Figure 1D-H) and beading of the cone axonal process (Figure 1E, arrow). Data from these eyes are not included in this analysis.
Figure 1. Images from rat retinas used to control for changes due to postmortem artifact. The left column shows the histological appearance of the retinas; the middle and right columns show immunohistochemical findings for long/medium wavelength-sensitive opsin (L/M opsin) and rhodopsin and for L/M opsin and anti-vesicular glutamate transporter 1 (vGluT1). A through C, Normal rat retina. The arrow in A points to the external limiting membrane. D, After a 6-hour delay, the retinal layers appear disorganized and the external limiting membrane is disrupted (arrow). E and F, Opsin immunoreactivity has spread to the somata of rods (E) and cones (E and F). Cone axons are L/M-opsin immunoreactive and appear beaded (white arrows in part E). G and H, After 24 hours, the inner (IS) and outer (OS) segments are highly disorganized. The arrow in G points to the external limiting membrane. I, The somata of cones appear hypertrophied (arrow). Labeling of the outer plexiform layer (OPL) synapses with vGluT1 appears unchanged (C, F, and J). Scale bars represent 50 µm. GCL indicates ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; and ONL, outer nuclear layer.
Intra-abdominal hemorrhage, diabetes mellitus, hypertension, osteoarthritis, splenectomy, mitral and aortic valve replacement, and photoreceptors lacking inner and outer segments had rhodopsin or L/M opsin–immunoreactive cell membranes (Figure 3E-G).

We defined 3 grades of opsin immunoreactivity (0-2), in which grade 0 is the expected/normal pattern (Figure 3A) and grade 2 characterizes degenerating cells that lack inner/outer segments and are immunoreactive throughout their membranes (Figure 3D-G). The intermediate grade (grade 1) describes cells with moderate to intense (L/M opsin) or low to moderate (rhodopsin) immunoreactivity of the inner segment and soma, sometimes involving the axon and synaptic terminal. The differences in intensity of labeling of the cell membranes of rods and cones in the intermediate grade reflects our observation that subtle changes in cone immunoreactivity, but not in the rods, were widespread in histologically normal retina.

The graded immunolabeling for L/M opsin and rhodopsin are shown in 3 representative virtual flat mounts in Figure 4, a quantitative assessment of the proportions of the different grades of labeling in 8 retinas is shown in Figure 5. Retina specimen 0605 had a large disciform scar (Figure 4A) covering about 50% of the sample area (Figure 5B). In H&E-stained sections, photoreceptors in the central portion of the scar were absent or had lost their photoreceptor morphological features (hatched area), whereas those on the margin were obviously degenerating (gray zone). Grade 2 opsin immunoreactivity of photoreceptors (Figures 4B and C) is consistent with the degenerative condition observable in H&E-stained sections. However, photoreceptors in the remaining histologically normal retina (white area, Figure 4A) were grade 1 for L/M opsin (Figure 4B) and rhodopsin (Figure 4C). Although 50% of the retina was histologically normal, less than 10% of the sample area had photoreceptors with normal (grade 0) opsin immu-

### Table 2. Specimens Analyzed

<table>
<thead>
<tr>
<th>Specimen No./Sex/Age, y</th>
<th>Time From Death to Fixation, h</th>
<th>Cause of Death</th>
<th>Clinical History</th>
<th>Histopathological Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0605/F/75</td>
<td>&lt;12</td>
<td>End-stage chronic airways limitation</td>
<td>Type 2 diabetes mellitus, hypertension, osteoarthritis</td>
<td>Basal deposits, RPE clumping, disciform scar</td>
</tr>
<tr>
<td>0606/M/78</td>
<td>8</td>
<td>Intra-abdominal hemorrhage</td>
<td>Splenectomy, mitral and aortic valve replacement</td>
<td>Normal retina</td>
</tr>
<tr>
<td>0608/M/83</td>
<td>6</td>
<td>Ischemic stroke, cardiovascular arrest</td>
<td>Hypertension</td>
<td>Basal deposits, normal retina</td>
</tr>
<tr>
<td>0609/F/79</td>
<td>13.5</td>
<td>Cerebral bleeding</td>
<td>Cataract surgery, emphysema</td>
<td>Basal deposits, large drusen, normal retina</td>
</tr>
<tr>
<td>0611/M/84</td>
<td>21</td>
<td>Prostate cancer</td>
<td>AMD, high blood pressure, high cholesterol level</td>
<td>Basal deposits, CNV, GA</td>
</tr>
<tr>
<td>0701/F/97</td>
<td>6</td>
<td>Myocardial ischemia</td>
<td>AMD</td>
<td>Drusen, basal deposits, disciform scar</td>
</tr>
<tr>
<td>0703/F/22</td>
<td>8</td>
<td>Spontaneous subarachnoid hemorrhage</td>
<td>NA</td>
<td>Normal</td>
</tr>
<tr>
<td>0705/F/96</td>
<td>6</td>
<td>Myocardial infarction</td>
<td>Ischemic heart disease, hypertension, depression</td>
<td>Small drusen, basal deposits, pigmented disturbance, normal retina</td>
</tr>
</tbody>
</table>

Abbreviations: AMD, age-related macular degeneration; CNV, choroidal neovascularization; GA, geographic atrophy; NA, not available; RPE, retinal pigment epithelium.

*Indicates linear/laminar deposits between the RPE and Bruch’s membrane.

**Classified as AMD retinas.

***Indicates normal RPE and retina.

Redistribution of rhodopsin into the membrane of the soma was seen in some of the remaining human samples, but was not considered an artifact because (1) immunoreactivity of the rod soma and axon was very low compared with the artifactual immunoreactivity in delayed fixed rat retinas; (2) this feature was regional and not found throughout the specimen; and (3) low immunoreactivity for rhodopsin in the inner segment and soma was detected in human retinas in the absence of other artifacts (described by Léveillard et al18).

Details of the 8 eyes further analyzed are shown in Table 2, and examples of their histological appearances are shown in Figure 2. Three retinas had histopathological features consistent with advanced AMD12 (mean age, 85 years), 4 were aged non-AMD eyes (mean age, 84 years), and 1 was from a healthy young adult (aged 22 years). The histopathological features of all eyes are shown in Table 2.

**DISTRIBUTION OF OPSIN PROTEINS: VIRTUAL FLAT MOUNTS AND QUANTITATIVE ANALYSES**

Varying patterns of opsin immunoreactivity were observed at different locations in each retina, including the young adult retina (specimen 0703). In normal retinas, L/M opsin was confined to the outer segment, as expected (Figure 3A [green]), or distributed throughout the photoreceptor membrane (Figure 3B and C [green]). Similarly, aberrant rhodopsin labeling was identified throughout the cell membrane at levels of immunoreactivity lower than in the outer segment (Figure 3C [red, arrows]). In AMD retinas, L/M opsin or rhodopsin immunoreactivity of the entire photoreceptor membrane (as shown in Figure 3B and C) was commonly detected contiguous with areas of degeneration (Figure 3E and F),
Noreactivity (Figure 5B). Specimen 0609 was histologically normal throughout the sample area (Figure 4D). However, approximately 37% of the sample area was grade 1 with respect to opsin immunoreactivity, with sparing of the foveal region (Figure 4E). A much smaller area (approximately 2%) showed grade 1 rhodopsin labeling (Figure 4F). The young adult retina was histologically normal, with normal rod labeling throughout (Figure 4G), but a narrow region of anomalous cone opsin immunolabeling (approximately 3.5% of the sample area) was detected in the vicinity of the optic disc (Figure 4H).

All 5 non-AMD retinas included regions of anomalous cone labeling (grade 1, 3.5%-75.0% of the sample areas). In contrast, only 3 of the 5 non-AMD retinas had anomalous rhodopsin immunolabeling (<10% of the sample area; Figure 5A). In each of the 3 AMD retinas, the proportion of the samples with anomalous cone immunolabeling (90%-100%) is much larger than that of the histological lesions (30%-55%) (Figure 5B).

**DISTAL AXON ENLARGEMENT AND vGluT1 IMMUNOREACTIVITY**

Where L/M opsin immunoreactivity was noted in the axon and pedicle, abnormal enlargement or swelling of the distal axon was also detected (Figure 6A-C) and was associated with aberrant distribution of immunoreactivity for vGluT1 (Figure 6B and C). This transporter is normally confined to the presynaptic terminal19 (Figure 6E and F). However, in regions of grade 1 L/M opsin labeling, moderate to intense immunoreactive vGluT1 was present in the pedicle and appeared to accumulate in axonal swellings (Figure 6B).

Regional changes in L/M opsin immunoreactivity and the intracellular distribution of vGluT1 are shown in
Figure 3. Normal and anomalous photoreceptor immunolabeling defined in this study. A, Expected or normal (grade 0) labeling of the outer segments using the study antibodies. B, Grade 1 long/medium wavelength-sensitive opsin (LM opsin) labeling shows immunoreactivity also present in the cell membrane of the inner segment, soma, and an axon (arrows), although the outer and inner segments are intact. Rod immunoreactivity is normal in this field. C, Cones are labeled as in part B, but grade 1 rhodopsin immunoreactivity includes labeling of the somata and axon of rods (arrows). D, Grade 2 labeling shows opsin-immunoreactive rod and cone remnants, lacking inner and outer segments, and autofluorescent retinal pigment epithelial (RPE)–derived material (arrowheads) in degenerating retina. E, Rod and cone opsin immunoreactivity on the edge of an age-related macular degeneration (AMD) lesion. The most central rods are indicated by the thick arrow; arrowheads indicate rhodopsin-immunoreactive somata. F, The boxed region from part E shows remnant cones (green), some with pedicles (thin arrow), and remnant rods (thick arrow) immediately adjacent to the lesion. G, Remnant cones (green) sandwiched between the inner nuclear layer (INL) and scar tissue of the AMD lesion. There are no inner or outer segments, and some cones have neurites extending into the INL (arrow). The green line at the bottom of the image is Bruch’s membrane. Scale bars represent 50 µm. GCL indicates ganglion cell layer; ONL, outer nuclear layer.

Our analyses indicate that (1) broad areas of histologically normal retina include regions of anomalous photoreceptor immunoreactivity (Figures 4 and 5) and (2) regions of anomalous LM opsin immunoreactivity are more extensive than regions of anomalous rhodopsin immunoreactivity (Figure 5). Redistribution of opsin from the outer segment to the soma and cell membrane has been described in several studies as part of the retinal response to injury and in disease.20-23 We found a nongr uniform redistribution of photoreceptor opsins, observed in broad regions of retina often involving the optic nerve head, in regions surrounding AMD lesions and in association with morphological changes in cones detected using other immunohistochemical markers.

Our findings are consistent with reports of cone dysfunction in the literature.17 We observed that the cone anomalies identified in the histologically normal retina adjacent to AMD lesions are identical to those identified in the macula of aged retinas, commonly in the context of histopathological correlates of early AMD. We suggest that cone dysfunction in aging and early AMD is associated with the cellular changes described herein.

### CONE ANOMALIES IN THE MACULA

We detected cone anomalies in every specimen and conclude that such anomalies are common, although no as-
 sessment of prevalence can be made because of the small number of specimens in the final cohort. These anomalies consistently involved the optic disc region, including a specimen from a 22-year-old patient, in which grade 1 cone anomalies surrounded the optic disc, suggesting retinal changes associated with peripapillary chorioretinal atrophy. Our findings are consistent with a previous study that identified peripapillary chorioretinal atrophy in 100% of eyes from donors aged 39 to 93 years and that found the histopathological features to closely resemble AMD.24 Similar abnormalities have been reported in cone dystrophies20,21 and in aged and AMD retinas,26 although in those studies the macular region was not specifically investigated. Studies of cone dystrophies20,21 emphasize cone pedicles rather than the distal axon as the location of the swelling. However, comparison of the enlarged structures described in cone dystrophies with those described herein in the axon indicates strong morphological correspondence. Comparison of our findings with those of Pow and Sullivan26 suggests that enlargements of the distal axon correspond with regions identified as the origin of sprouting neurites. We did not specifically investigate neurite sprouting, although evidence of axonal branching can be detected in some of our sections. Accumulation of vGluT1 in the distal axon may indicate redirection of vesicular glutamate into neurites or more general dysfunctional trafficking of intracellular proteins, including opsin. Abnormalities in the immunoreactivities of histologically normal cones has also been described in retinas from donors who had retinitis pigmentosa.21

PROTECTION OF THE Fovea

Our data suggest that, in the absence of AMD, foveal cones may be protected from the damaging changes described herein. In 3 of 5 non-AMD retinas (specimens 0606, 0608, and 0609), the foveal region appeared to be inured from the cone anomalies (eg, Figure 4E), including specimen 0606, in which 75% of the sample area had grade 1 cones. This observation is consistent with physiological evidence because several studies have concluded that visual acuity is not the best predictor or measure of progression in AMD.2,27,28 Despite this, acuity continues to be used as a principal outcome measure for major clinical trials. Visual acuity is mediated in the central fovea by intraretinal circuits that originate from individual cones and is conveyed to the brain through nonconverging circuits via midget ganglion cells.29 Other measures of visual function, including contrast sensitivity and sensitivity to blue light, rely on integrated cone functions and convey information from many cones that converges on other ganglion cell types (parasol and bistratified ganglion cells, respectively) for relay to the brain. These measures are sensitive to changes in macular function and are good predictors of progression in AMD.3,6,30-34 Sig-

Figure 4. Virtual flat mounts of 3 human retinas, including 1 with age-related macular degeneration (AMD) (A-C) and 2 normal retinas (D-F) and 1 young adult (G-I). The maps describe the histological appearance (top row) and the grades of opsin immunoreactivity in cones (middle row) and rods (bottom row). A. The histological lesion is indicated by the patterned regions and occupies about 50% of the mapped area; the remaining 50% of the area is histologically normal (white). B. The lesion area includes many degenerated cones (grade 2, dark gray); most of the histologically normal retina surrounding the lesion consists of abnormal cones (grade 1). C. Degenerating rods are present in the center of the lesion (grade 2, dark gray), surrounded by an extensive area of abnormal rods (grade 1) overlapping with the histologically normal region. D. This aged retina appears completely normal according to histological criteria. E. An extensive region of grade 1 cone labeling surrounds the optic disc and is superior and inferior to the fovea. F. A narrow band of grade 1 rod labeling is evident around the optic nerve head. G. Rod photoreceptors appear normal throughout the entire sample area. H&E indicates hematoxylin-eosin; L/M opsin, long/medium wavelength-sensitive opsin; ONL, outer nuclear layer; and RPE, retinal pigment epithelium.
significantly, both originate predominantly from cones in nonfoveal locations, 20, 35, 36.

CONE AND RODS: DIFFERENT RESPONSES TO AGING

In our non-AMD retinas, a higher proportion of the sample areas show anomalies in the cone population compared with the rod population (Figure 3A). This finding appears to contradict reports that rods are preferentially vulnerable to aging and degeneration because cone density at the fovea does not change significantly with age, whereas rod density decreases by 30% by the ninth decade of life. 37 Furthermore, in AMD retinas, the photoreceptors surviving in and around disciform scars tend to be cones rather than rods, and cones also appear more likely to survive in nonexudative AMD. 8 We also observed in this study that very few rods survive in the retina bordering an AMD lesion, whereas cones are numerous in the surrounding retina and islands of remnant cones persist even within old scars (Figure 3E-G), confirming that rod death precedes cone death.

However, our data also suggest that, in non-AMD retinas, cones show signs of damage ahead of rods but, as the conditions generating the response progress, damaged cones linger in the retina longer than do rods. It appears that, although signs of incipient photoreceptor degeneration have onset in cones earlier than in rods, rods succumb to cell death signals more readily than do cones. The abundance of prolapsed cones detected, but not rods, also supports this contention. Displacement of photoreceptor nuclei into the outer plexiform layer and subretinal space has been described previously. 36 From the morphological features and immunoreactivity to L/M opsin, we identified most of these displaced or prolapsed photoreceptors as cones. We did not detect prolapsed rhodopsin-immunoreactive photoreceptors in the subretinal space. Our observations suggest that cones can be displaced, presumably losing their synaptic contacts 36 without immediately succumbing to cell death; on the other hand, if prolapsed rods occur in equal proportions, they appear to die more rapidly.

In conclusion, cone death subsequent to rod loss has been described in a number of retinal disorders, including AMD and retinal dystrophy, 19, 42 and several authors have suggested targeting rod survival factors to facilitate cone survival. 18, 41, 43 Our findings show that anoma-
lies in the distal cone axon are common in the aging human macula and may indicate susceptibility to AMD. If cell death is considered the main indicator of morbidity in the retina and if indicators of incipient degeneration in cones are not recognized, attempts to rescue truly functional cones by using survival factors are likely to be unsuccessful. This and other recent studies\(^{26,44,45}\) indicate a need for further investigation into the processes of normal aging in the human retina and the role of aging in the emergence and progression of AMD.

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