Dynamic and Quantitative Analysis of Choroidal Neovascularization by Fluorescein Angiography

Syed Mabmood Shab, Sinan Tatlipinar, Edward Quinlan, Jennifer U. Sung, Homayoun Tabandeh, Quan Dong Nguyen, Ahmed S. Fahmy, Ingrid Zimmer-Galler, R. C. Andrew Symons, Jesse M. Cedarbaum, and Peter A. Campochiaro

PURPOSE. In this study, the authors sought to develop and characterize techniques for measuring changes in choroidal neovascularization (CNV) lesion size and fluorescence over time for quantitative analysis of fluorescein angiograms.

METHODS. Initial assessment of the quantitative technique was made by retrospectively analyzing digital fluorescein angiograms taken before and 3 months after photodynamic therapy (PDT) for CNV (6 patients, group 1). The method was then applied prospectively to digital fluorescein angiograms (baseline and day 71) obtained on 12 patients taking part in a clinical trial investigating the effect of vascular endothelial growth factor (VEGF) Trap in CNV (group 2). Two masked observers, with different threshold settings, measured the area of hyperfluorescence and fluorescence intensity above background. Values for each image were plotted against time after dye injection to generate curves, and each area under the curve (AUC) was calculated.

RESULTS. The physician who treated the patients in group 1 judged the condition of three patients to be improved and of three to be worse 3 months after PDT. Masked retrospective grading of fluorescein angiograms showed an 11% decrease in AUC for fluorescence area and a 52% decrease in AUC for fluorescence intensity in the three patients whose conditions clinically improved but increases of 131% and 292% in the three patients whose conditions clinically worsened. In group 2, a 38% decrease in AUC for fluorescence intensity and a 19% decrease in AUC for fluorescence area were observed in patients who received VEGF Trap compared with increases of 66% (P = 0.004, Mann-Whitney U test) and 21% (P = 0.07) for patients who received placebo. Macular volume decreased by 11% in VEGF Trap–treated patients and increased by 10% in placebo-treated patients (P = 0.03).

CONCLUSIONS. This study reports a technique for analysis of change in fluorescence area and intensity over time during fluorescein angiography (FA) using a continuous scale and its application in a clinical setting and a clinical trial. Compared with previous techniques making use of categorical scales, this approach provides an advantage for evaluating responses to treatment that may improve the value of FA as an outcome measure in clinical trials. (Invest Ophthalmol Vis Sci. 2006;47: 5460–5468) DOI:10.1167/iovs.06-0012

Fluorescein angiography (FA) is valuable for visualizing choroidal neovascularization (CNV) and is used to assess the response to treatment during clinical care and in clinical trials. In clinical trials, trained observers evaluated FA in masked fashion and either graded lesions using categorical scales or used planimetry to measure lesion size and leakage area. These techniques provide useful information, but FA provides a great deal of additional untapped information.

Recently, optical coherence tomography (OCT), a technique that allows noninvasive cross-sectional imaging of the retina, has become available. It provides measurements of retinal thickness on a continuous scale and is a valuable addition for quantitative comparisons before and after treatment. The usefulness of OCT has to some extent pushed FA into the background. However, FA should not be abandoned because it provides information different from that obtained using OCT. In patients with CNV, OCT provides a quantitative measure of retinal thickness and subretinal fluid. Reduction in subretinal fluid and retinal thickness from one scan to the next suggests that the average amount of leakage over days to weeks before the second scan was less than that before the first, but it does not provide a direct indication of the current amount of leakage. This information is provided by FA, which also demonstrates CNV lesion size, another outcome measure that cannot be assessed by OCT. Thus, FA and OCT provide complementary information. The shift to greater reliance on OCT is not the result of the type of information provided but, rather, the nature of the data; the continuous scale data provided by OCT allows for easier and more powerful quantitative comparisons.

In this study, we sought to develop a means to capture information provided by FA along a continuous scale, something that has become feasible because of recent advances in digital imaging and computer-based image analysis. We initially evaluated the technique by retrospectively grading digital fluorescein angiograms obtained before and after performing photodynamic therapy (PDT) and comparing the results with the previous clinical assessments of the treating physician. We then used the technique prospectively in a clinical trial designed to evaluate the effect of an antagonist of vascular endothelial growth factor (VEGF), VEGF Trap, in patients with neovascular age-related macular degeneration (NVAMD).

MATERIALS AND METHODS

Patient Populations

To explore the correspondence of quantitative FA analysis to clinical status, we retrospectively analyzed fluorescein angiograms taken be-
fore and 3 months after PDT for 6 patients with NVAMD. The patients were treated and evaluated by one of the investigators (IZG). They were given intravenous infusion of 6 mg/m² verteporfin over 10 minutes, and after 5 minutes lesions were exposed to 50 J/cm² of a 689-nm diode laser for 83 seconds.

We then used the quantitative FA technique as an outcome measure in a second group of 12 patients with NVAMD who were participating in a randomized, placebo-controlled clinical trial to assess the effects of intravenous (IV) administration of VEGF Trap, a recombinant fusion protein containing the binding domains of VEGF receptors 1 and 2. This phase 1 preliminary safety and preliminary bioeffect study was conducted in compliance with the Declaration of Helsinki, US Code 21 of Federal Regulations, and the Harmonized Tripartite Guidelines for Good Clinical Practice (1996). The study was reviewed and approved by the Western Institutional Review Board (WIRB) for some centers and by local institutional review boards for other centers. Primary results of the trial are reported elsewhere and do not include the analysis of fluorescein angiograms, which instead is provided as part of this article. The first two cohorts of the trial, consisting of patients randomly assigned to receive placebo (n = 4), 0.3 VEGF Trap (n = 3), and 1 mg VEGF Trap (n = 5), were included. The trial was stopped because hypertension developed in patients after 3-mg doses were administered in the third cohort; thus, enrollment was discontinued and data are unavailable for the 3 mg/kg cohort. The patients in the study received infusions on days 0, 29, 43, and 57, and the primary end point was at day 71, 2 weeks after the last infusion. Results of FA and OCT performed on days 0 and 71 were analyzed independently by two observers masked with respect to treatment group.

**Data Capture by Digital Fluorescein Angiography**

Digital fluorescein angiograms that were analyzed retrospectively in the 6 patients treated with PDT and in those analyzed prospectively as part of the VEGF Trap trial were performed with a fundus camera (Zeiss FF4; Carl Zeiss Meditec, Oberkochen, Germany) integrated with a digital acquisition system (MRP Systems, Boston, MA). Each frame was captured at 2000 × 2000 pixels using an effective camera resolution of 4 million pixels.

**Optical Coherence Tomography**

OCT was performed (StratusOCT; Carl Zeiss Meditec, Dublin, CA) on the 12 patients participating in the VEGF Trap trial. Two standard acquisition protocols (6-mm fast macular thickness map and 6 × 6-mm cross hair) and one customized protocol (3-line, 8-mm papillomacular axis scan) were used. Foveal thickness (FTH, measured in micrometers), defined as the mean distance between the inner and outer reflectivity bands in the central 1-mm diameter circle surrounding fixation and total macular volume (TMV, measured in cubic millimeters), was automatically computed (StratusOCT, version 4.0; Carl Zeiss Meditec). Because of the advanced nature of the disease and the extensive changes in RPE morphology, scan profiling was used to correct any artifacts produced by the automated analysis algorithm.

**Measurement of Hyperfluorescent Area**

Two fellowship-trained retina specialists served as independent graders and reviewed every frame of each fluorescein angiogram. Frames that did not include the entire lesion were discarded. For group 2 patients, the graders were masked with respect to treatment group. A histogram analysis algorithm was written (MATLAB 6; MathWorks, Inc., Natick, MA) to select frames with appropriate exposure for grading (Fig. 1). Images were imported (ImagePro Plus, Media Cybernetics, Inc., Silver Spring, MD), and the border of the entire hyperfluorescent area (classic and occult components plus any collection of dye in surrounding tissue) and the classic component were outlined by each of the graders with a digitizer tablet. Color fundus photographs were used for comparison to distinguish hyperfluorescence caused by CNV from that caused by pigmented changes. Contrast enhancement and image segmentation techniques available with image analysis software, including the one used (Image Pro Plus, Media Cybernetics, Inc.), helped delineate lesion borders. Although these techniques are effective, they are not necessary for the quantitative analysis and will not be described further here.

Once a grader outlined hyperfluorescent lesions on each original frame of a fluorescein angiogram, the image analysis software provided the lesion area in pixels. The grader also outlined the optic disk, and its area was determined. Lesion area was divided by the disk area for normalization to control for differences in magnification among the fluorescein angiograms. The ratio of lesion area/disk area (normalized lesion area) was plotted against time after fluorescein dye injection, the best-fit curve was generated, and the area under the curve (AUC) was calculated. The change in AUC from one fluorescein angiogram to the next provided a quantitative measure of change in hyperfluorescent area during the period between fluorescein angiograms. This analysis was performed at baseline and after PDT FA for each of the six patients in the PDT group and at baseline and day 71 FA for each of the 12 patients in the VEGF Trap clinical trial group.

**Measurement of Net CNV Lesion Fluorescence**

For analysis of change in fluorescence intensity over time, the fluorescein angiogram frame showing the maximum border of the lesion (e.g., all occult and classic CNV and maximum leakage) was determined, and an image overlay mask slightly larger than the lesion was created (Fig. 2A). The overlay mask was saved and applied to all images in sequence. Areas of background fluorescence fairly close to, but outside the boundaries of, the CNV lesion were selected to provide a measure of fluorescence solely...
from normal retinal and choroidal capillaries in areas adjacent to the lesion (background). An image overlay mask was applied to each of the background areas (Fig. 2B). On each frame of the FA, the average background fluorescence intensity was determined (sum of grayscale intensity for each pixel within all the selected regions divided by the total number of pixels). The average background fluorescence intensity per pixel was subtracted from the fluorescence intensity of each pixel within the lesion borders, and the fluorescence intensity above background for each pixel was summed to give the net fluorescence above background for the lesion on that frame of the angiogram. The goal was to create an image overlay mask that encompassed all lesion fluorescence with minimal inclusion of background, but the actual amount of background included was irrelevant because it was subtracted. Net lesion fluorescence obtained from each frame was plotted against time after fluorescein injection, and a best-fit curve was generated. The AUC provided a global numeric index of net lesion fluorescence. This analysis was performed on baseline and posttreatment fluorescein angiograms for each of the patients in this study.

**RESULTS**

**Demonstration of Dynamic and Quantitative Analysis of Fluorescein Angiograms**

Pretreatment and posttreatment digital fluorescein angiograms were analyzed retrospectively for six patients with NVAMD who were treated with PDT. FA was also used prospectively as an outcome measure in a clinical trial aimed at assessing the effect of intravenous VEGF Trap in patients with NVAMD. The analysis is illustrated in detail with one patient from each group. Figure 3 shows early, middle, and late FA frames from the first patient in group 1 at baseline (first column) and 3 months after PDT (second column). At baseline, the frame from the early phase shows a small, predominantly classic CNV lesion with a thin rim of blocked fluorescence and a larger surrounding area of mildly increased fluorescence. The baseline red-free fundus photograph (column 3, top) shows a ring of pigment atrophy corresponding to the surrounding area of mildly increased fluorescence, indicating that it is a window defect. In the middle phase of the baseline FA, the borders of the CNV lesion are blurred. In the late phase, the hyperfluorescent area is larger and very blurred, indicating leakage of dye into the tissue surrounding the lesion. Three months after PDT, the early-phase fluorescein angiogram shows that the CNV has become larger and now has a central classic component and a surrounding occult component. The red-free fundus photograph (column 3, bottom) shows no hypopigmentation corresponding to hyperfluorescence, indicating that none of it can be attributed to a window defect. Middle- and late-phase fluorescein angiograms show that the surrounding area of mild fluorescence increased, consistent with occult CNV. Little change is observed in the classic component between the early and middle phases; in the late phase the central portion is brighter, but the margin of the classic component is less bright (column 2, bottom). This pattern is commonly described as staining rather than leaking.

Figure 4A shows a normalized hyperfluorescent area from each frame plotted against the time from fluorescein injection during which the frame was captured, and it shows the best-fit curve for the baseline and posttreatment fluorescein angiograms for the patient illustrated in Figure 3. The baseline curve (solid line) shows a rapid increase in size during the early phase as the CNV lesion fills with dye and then a gradual increase as dye diffuses beyond the edges of the lesion. Three months after PDT (dotted line), the hyperfluorescent area became much larger, and, because the first gradable frame showed essentially the whole lesion, the curve had no vertical part. Subsequently, the hyperfluorescent area changed little, as shown by the minimal change in slope, which corresponded well with what is shown in Figure 3 (middle column).

Figure 4B shows change in net fluorescence intensity over time for the baseline and post-PDT fluorescein angiograms for the patient illustrated in Figure 3. The curve for the baseline fluorescein angiogram (solid line) shows a gradual increase in net fluorescence intensity over time that closely paralleled the change in hyperfluorescent area over time (Fig. 4A, solid line). The curve for the posttreatment fluorescein angiogram (dotted line) shows a steady increase in net fluorescence intensity. Together the posttreatment curves in Figures 4A and 4B indicate that dye collected within the lesion (staining) but did not spread beyond the borders of the membrane (leakage), as evident by the fluorescein angiogram; these features are usually associated with lesions that contain considerable fibrosis.

Fluorescein angiograms for a patient who received 1.0 mg/kg VEGF Trap are shown in Figure 5. The baseline fluorescein angiogram (first column) shows a predominantly classic...
CNV lesion that leaks profusely on middle and late frames. At the primary end point, day 71 (second column), the CNV lesion was contracted but not substantially different in size; however, the leakage appears almost completely resolved. The fluorescein angiogram from day 99 (column 3), 6 weeks after the last infusion, suggests some recurrent leakage during the late, but not middle, phase.

Figure 6A shows the normalized hyperfluorescent area from each frame plotted against time and the best-fit curve for the baseline, day 71, and day 99 FAs shown in Figure 5. The baseline curve (solid line) shows a rapid rise in hyperfluorescent area in the early phase as the CNV fills with dye and then a gradual increase in hyperfluorescent area caused by leakage. At day 71 (dashes), there is a sharper break point, after which the slope of the curve is nearly 0. At day 99 (dotted), the slope after the break point has slightly increased from that at day 71. Examination of the fluorescein angiograms shows that the break points corresponded to 10 seconds after injection; after that, the increase in hyperfluorescent area was caused by the leakage of dye into surrounding tissue. The increase in AUC after 10 seconds is a measure of leakage. This measure was reduced by 74% between baseline and day 71 and was increased by 38% between day 71 and day 99, which is still a 65% reduction compared with baseline. Good agreement was observed between the categorical assessments of the grader and the measurements, but what was lost in the categorical assessments was the magnitude of improvement or worsening. Even with a more detailed categorical grading scale, it would not have been possible to capture the information provided by the measurements.

Correlation of Numerical Indices Generated from Quantitative Analysis of Fluorescein Angiograms with Other Outcome Measures in a Clinical Trial

Figure 7 shows macular volume and AUC for net lesion fluorescence/time at baseline and the primary end point, day 71, in the 12 patients in the first two cohorts of the VEGF Trap trial. Seven of eight patients who received 0.3 or 1 mg/kg of VEGF Trap (Fig. 7, top 2 rows) had reduced AUCs for net lesion fluorescence over time. This correlated well with total macular volume, which was reduced in all 8 patients, and with visual acuity, which was improved in 7 of the 8 patients. Macular volume increased in 3 of 4 patients who received placebo (Fig. 7, bottom row), and AUC for net lesion fluorescence over time increased in all four patients. Visual acuity worsened in two patients and improved in two patients. Patients who received VEGF Trap had a 38% decrease in AUC for lesion fluorescence/time and a 19% decrease in AUC for hyperfluorescent area/time compared with 66% and 21% increases in patients who received placebo ($P = 0.004$ and $P = 0.07$, respectively, Mann-Whitney $U$ test; Table 2). Treated patients also had an 11% decrease in TMV compared with a 10% increase in placebo patients ($P = 0.05$).
FIGURE 4. Normalized hyperfluorescent area (A) and net lesion fluorescence above background (B) plotted against time after fluorescein injection for the patient illustrated in Figure 3. The normalized hyperfluorescent area (A) and the net lesion fluorescence above background (B) were plotted compared with time after fluorescein injection for each frame of the fluorescein angiogram taken at baseline (solid line) and 3 months after photodynamic therapy (dotted line). Best fit-curves were generated, and the area under each curve was calculated. Large increases were observed in both parameters 3 months after photodynamic therapy.
DISCUSSION

For many years, FA has been a valuable tool for the diagnosis and management of patients with retinal or choroidal diseases. This is true even though much of the information it yields is not used. The development of digital cameras that create image files that can be evaluated with the use of new image analysis software has made it possible to explore much of the untapped information. In this study, we report a new semiautomated approach to measure changes in hyperfluorescent area and intensity over time that may facilitate longitudinal comparisons.

Fluorescein angiography is a dynamic test; dye is injected, and a sequence of pictures is taken showing the collection and movement of dye over time. Assessment of a CNV lesion by FA shows the filling of the lesion with dye, which can be slow or fast, uniform or irregular, and faintly or brightly fluorescent. Lesions that fill quickly and fairly uniformly and that fluoresce brightly are categorized as classic CNV, and those that fill slowly and irregularly and have faint fluorescence are categorized as occult CNV. In fact, most lesions have features of both and are categorized as predominantly or minimally classic. Classic and occult CNV have important biologic differences. Classic CNV is associated with more rapid growth and deterioration of vision than occult CNV. Predominantly, classic CNV lesions also respond more favorably to PDT, but these lesions have not been reported to respond differently to VEGF antagonists. The techniques outlined in this report allow dissection and analysis of the various lesion components. For example, a patient in the 0.3 mg cohort of the VEGF Trap trial had not undergone previous treatment and refused to consider PDT or any intraocular injections. At baseline, the lesion consisted of a small classic component with a larger area of surrounding occult CNV. Posttreatment FAs showed substantial reduction in the occult component that resulted in an overall decrease in lesion size, but the classic component increased. Based on the categorical grading, one would say the patient had a minimally classic lesion that responded well to treatment because lesion size decreased, but this does not tell the whole story. In another patient who received PDT (Fig. 3), not only did the classic component of the CNV lesion grow in the 3 months after treatment, a substantial occult lesion also developed. These observations support the potential importance of measuring the different lesion components and systematically assessing their response to treatment.

Analysis of change in hyperfluorescent area over time also provides quantitative assessment of CNV lesion filling and leakage, as illustrated in Figure 6. Previously, lesion size was analyzed by subjective determination regarding when the lesion was completely filled, but not leaking, so that the borders were revealed and size was measured. Plotting the borders over time avoids the subjective determination of which measurement reveals the true size of the lesion and provides information regarding how the lesion fills and leaks, which may have prognostic significance.

Our technique also avoids subjective assessments of fluorescence intensity, which are crude because they are greatly influenced by the degree of exposure of the frame and because deciding when the determination should be made relative to injection time has not been standardized. This leads to categories such as fluoresces brightly early or fluoresces brightly late. The quantitative assessment reported herein deals with both categories. Subtracting background fluorescence and plotting net fluorescence over time tracks the change in fluorescence above background within the lesion. This controls for
FIGURE 6. Normalized hyperfluorescent area (A) and net lesion fluorescence above background (B) plotted against time after fluorescein injection for the patient illustrated in Figure 5. Plots are shown for fluorescein angiograms taken at baseline, day 71 (2 weeks after the last infusion; primary end point), and day 99 (6 weeks after the last infusion). (A) The normalized hyperfluorescent area for each of the time points increased rapidly until approximately 10 seconds, after which the slopes substantially decreased. Examination of the fluorescein angiograms showed that the increase in hyperfluorescent area after 10 seconds was caused by leakage of dye into the surrounding tissue. The increase in AUC after 10 seconds was a measure of leakage; at baseline it was 1110. At day 71, the slope after 10 seconds was close to 0, indicating that little leakage occurred. The increase in AUC after 10 seconds was 281, a 74% reduction from baseline. At day 99, the increase in AUC after 10 seconds was 389, a 38% increase from day 71 but still a reduction of 65% compared with baseline. This provided a valuable quantitative assessment of the antipermeability effect of VEGF Trap. (B) Net lesion fluorescence above background, which is an indicator of dye filling of the entire lesion and not just leakage, was also reduced after VEGF Trap treatment, but the percentages of reductions were less than the reductions in leakage. Reductions from baseline were 15.4% at day 71 and 19% at day 99.
level of exposure of each frame and allows for better comparisons between fluorescein angiograms taken at different times, which often vary in level of exposure. The amount of fluorescence within a lesion is determined by several factors, including the size and thickness of the lesion, the size of new vessels within the lesion, and the extent to which the lesion is covered by RPE and other light-absorbing cells or materials. One or more of these factors may have prognostic significance. Studies are needed to investigate the prognostic significance of net fluorescence over time.

It should be noted that quantitative assessments must be performed in conjunction with observations of fundus photographs and fluorescein angiograms to avoid misinterpretation. For instance, subretinal hemorrhage reduces fluorescence, and its presence must be taken into account, particularly if a change is observed with earlier or subsequent FA findings included in the analysis. Hyperfluorescence from occult CNV appearing in late frames around classic CNV must be distinguished from leakage and graded accordingly. It was not possible to eliminate all subjective assessments for determination of lesion size because automated identification of CNV borders is not yet sufficiently reliable. Two independent graders, assisted by contrast enhancement and image segmentation techniques, determined lesion size on each frame. The close scrutiny of fluorescein angiograms by the masked graders to make these assessments was necessary, but the semiautomated nature of the technique is still a substantial advance.

Previous studies have reported methods to quantify dye leakage, CNV lesion size, or both using FA images captured on film and then digitized.6,7 These are pioneering studies that point toward the future, but scanning film introduces errors and reduces resolution, making it substantially inferior to primary capture of the data with a digital camera. Other shortcomings in those studies may be partly related to limitations of

<table>
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<th>Patient</th>
<th>Baseline Lesion Size (AUC)</th>
<th>Baseline Net Fluorescence/Time (AUC/1000)</th>
<th>Month 3 Lesion Size (AUC)</th>
<th>Month 3 Net Fluorescence/Time (AUC/1000)</th>
<th>Change from Baseline (%) Lesion Size (AUC)</th>
<th>Change from Baseline (%) Net Fluorescence/Time (AUC/1000)</th>
<th>Subjective Change Lesion Size</th>
<th>Subjective Change Net Fluorescence/Time</th>
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The area of each lesion in pixels and the net fluorescent intensity in relative units were plotted against time after injection of fluorescein, and the best curves were generated. AUC was calculated for each curve, and AUC for lesion size and net fluorescence at baseline and month 3 are shown for each patient. Subjective grading of an observer is shown in the last two columns. Except for the lesion size for patient 6, agreement was observed between the direction of measurement change and the subjective assessments.
technology. For instance, no control was included for differences in exposure on different frames and different angiograms. The investigators relied heavily on automated image registration, which is imperfect because of frame-to-frame image shift. FA is usually performed in stereo. This was achieved by rapid shifting of the optical pathway from one side of the pupil to the other, which offset the image and changed the focus and exposure. In our technique, manual image registration was used to construct image overlay masks. Otherwise, we did not rely on registration because data measurements were made independently on each frame, normalized, and then plotted by curve fitting. Although image shifting can be tolerated with our technique, it makes the analysis more difficult. This difficulty can be overcome with the use of a customized FA acquisition protocol, designed to provide additional standardization and to maximize data points at appropriate time intervals to facilitate analysis. This protocol is under investigation and will be described in a future publication.

Berger and Yoken used digital image processing for CNV area measurements, with normalization to optic disk area to control for magnification differences, which is similar to the approach we used. They also measured relative CNV lesion fluorescence intensity over time. This captured the dynamic aspects of dye accumulation and was more informative than a measure of fluorescence intensity by subtracting the background, but they approached we used. They also measured relative CNV lesion area measurements, with normalization to optic disk area to control for magnification differences, which is similar to the approach we used. They also measured relative CNV lesion fluorescence intensity over time. This captured the dynamic aspects of dye accumulation and was more informative than a measure of fluorescence intensity by subtracting the background, but they also used it to construct image overlay masks. Otherwise, we did not rely on registration because data measurements were made independently on each frame, normalized, and then plotted by curve fitting. Although image shifting can be tolerated with our technique, it makes the analysis more difficult. This difficulty can be overcome with the use of a customized FA acquisition protocol, designed to provide additional standardization and to maximize data points at appropriate time intervals to facilitate analysis. This protocol is under investigation and will be described in a future publication.

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