How Heart Valves Evolve to Adapt to an Extreme-Pressure System: Morphologic and Biomechanical Properties of Giraffe Heart Valves

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Background and aim of the study: Heart valves which exist naturally in an extreme-pressure system must have evolved in a way to resist the stresses of high pressure. Giraffes are interesting as they naturally have a blood pressure twice that of humans. Thus, knowledge regarding giraffe heart valves may aid in developing techniques to design improved pressure-resistant biological heart valves.

Methods: Heart valves from 12 giraffes and 10 calves were explanted and subjected to either biomechanical or morphological examinations. Strips from the heart valves were subjected to cyclic loading tests, followed by failure tests. Thickness measurements and analyses of elastin and collagen content were also made. Valve specimens were stained with hematoxylin and eosin, elastic van Gieson stain, Masson’s trichrome and Fraser-Lendrum stain, as well as immunohistochemical reactions for morphological examinations.

Results: The aortic valve was shown to be 70\% (95\% CI 42-103\%) stronger in the giraffe than in its bovine counterpart (p <0.001). No significant difference was found between mitral or pulmonary valves. After normalization for collagen, no significant differences were found in strength between species. The giraffe aortic valve was found to be significantly stiffer than the bovine aortic valve (p <0.001), with no significant difference between mitral and pulmonary valves. On a dry weight basis, the aortic (10.9\%), pulmonary (4.3\%), and mitral valves (9.6\%) of giraffes contained significantly more collagen than those of calves. The elastin contents of the pulmonary valves (2.5\%) and aortic valves (1.5\%) were also higher in giraffes.

Conclusion: The greater strength of the giraffe aortic valve is most likely due to a compact collagen construction. Both, collagen and elastin contents were higher in giraffes than in calves, which would make giraffe valves more resistant to the high-pressure forces. However, collagen also stiffens and thickens the valves. The mitral leaflets showed similar (but mostly insignificant) trends in strength, stiffness, and collagen content.

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Currently, the most frequent treatment for severe heart valve disease is valve replacement therapy (1). One possibility is to replace the native heart valve with a mechanical prosthesis, but this requires that the patient receives life-long anticoagulant medication. An alternative option is to replace the valve with a biological prosthesis, which does not require anticoagulation therapy, but due to ‘wear and tear’ - particularly in the systemic circulation - the durability of biological valve prostheses is limited and hence they are not offered to younger patients (1,2). Thus, the development and optimization to prolong the durability of biological heart valve prostheses continues to be an important challenge, and tissue engineering provides a promising avenue to achieve this goal (3).

Heart valve failure is predominantly caused by mechanical forces such as shear stress, bending stresses, and also tensile and compressive forces (2,4-6). It has also been shown that a high closing pressure increases the calcification of biological prostheses (5). The closing pressure is related to the blood pressure, which is an additional clinical risk factor for heart valve degeneration (6). It is, therefore, essential to
develop biological heart valves that are resistant to pressure and thereby high tissue stress levels. A biologically relevant high-pressure model is needed for this purpose. Giraffes are endowed with the highest blood pressure of any other mammal, their ‘normal’ blood pressure being twice as high as humans and other mammals (7-10). This enormous pressure secures a normal cerebral perfusion in these long-necked animals (8,9,11), but it does not appear to evoke any obvious signs of early heart valve disease. Exactly how the giraffe heart valves have adapted to these extreme pressures remains unknown, but the target was to determine whether giraffe heart valves have specific design features that confer a unique resistance to mechanical stress, and could serve as a valid scientific inspiration to improve existing biological valve prostheses to create a longer durability. Thus, the aim of the study was to characterize the morphology and biomechanical properties of heart valves in the giraffe, and to compare them to those of bovine species, which have a similar body mass but normal mammalian blood pressure. This may ultimately be of help to heart valve patients, and potentially offer a younger patient group the choice of a biological prosthesis.

Materials and methods

Animal studies

The present study was performed as a part of the Danish Cardiovascular Giraffe Research Programme (DaGiR), where a number of in-vivo experiments and tissue samplings were performed at WildLife Assignments International in Hammanskraal, South Africa. The in-vivo studies included a range of assignments international in Hammanskraal, South Africa. The in-vivo studies included a range of animal studies.

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Heart valves from four giraffes (Giraffa camelopardalis) were used for histological examination, and from eight giraffes for mechanical testing. The median estimated age of the 12 giraffes was 33 months (range: 18 to 48 months), and the median body weight was 456 kg (range: 227 to 654 kg). The control group consisted of 10 Holstein-Frieser calves with a median age of 12 months (range: 11 to 14 months) and a median body weight of 472 kg (range: 430 to 508 kg) that were sampled at a Danish slaughterhouse. Heart valves from four calf hearts were used for histological examination, and from six calves for mechanical testing (including one heart for pilot tests).

The heart valve cusps were harvested from freshly euthanized animals and immediately frozen in liquid nitrogen (within 2 h of death). The specimens were maintained in a frozen state at -18°C in a freezer, or in a container of dry ice during transportation.

The project was conducted in collaboration with an experienced team consisting of wildlife veterinarians, capture specialists and animal technicians under the supervision of a veterinarian from Witwatersrand University, South Africa. Ethical approval to conduct the study was obtained both from the Inspectorate of Animal Experimentation under the Danish Ministry of Justice, Denmark, and from the University of Witwatersrand, South Africa.

Biomechanical measurements

The heart valve tissues were thawed at room temperature and kept moistened with a 50 mM Tris/HCl buffer (pH 7.4). Two or three strips (3 mm wide) were cut in both radial and circumferential directions from the aortic and pulmonary semilunar leaflets, using a multicutter (fitted with parallel-mounted razor blades). A total of 137 aortic and 126 pulmonary strips was sampled from the giraffes, and a total of 89 aortic and 61 pulmonary strips from calves. The thickness of the central part of the strip, which corresponded to the part mounted between the clamps (see below), was measured using an electronic length gauge (MT25/ND 281B; Heidenhain, Traunreut, Germany) under standardized stress (30 kPa) for 10 s. The specimen thickness was measured in both directions for the aortic and pulmonary valves, and with a fixed-width (3 mm) the cross-sectional area (mm²) was proportional to the thickness. One rectangular specimen of the anterior mitral cusp was cut out between the tendinous branching of two chordae tendineae. This specimen comprised the middle part of the cusp between the free margin and the leaflet attachment. Due to the irregularity of the mitral valve from the chordae branches, the mitral specimens were only prepared for testing in the circumferential direction. In total, eight strips were obtained from giraffes, and four from calves. Only the anterior mitral cusp was tested as the posterior cusp was too irregular. The ends of the strips and mitral specimens were gripped in clamps mounted with a 10 mm nominal distance in a material testing machine (Alwetron TCT5; Kista, Sweden) equipped with a load cell (20, 50 or 100 N) as appropriate. To avoid slippage and also cutting of the strips, the clamp jaws were coated with either Tegaderm® (3M; grain size 80) for the pulmonary cusps or emery cloth for the aortic and mitral anterior cusp, and screwed together to an adjusted and standardized moment with a torque wrench. During testing at 50 mm/min the strips were soaked in buffer. Each strip was subjected to five cyclic loadings to 0.5 N (pulmonary strips, radial aortic strips), 1 N (circumferential aortic strips), or 8 N (mitral specimens), followed by a failure test. Afterwards, the ruptured pieces of strips were cut close to the clamps and collected for collagen analysis. The strip materials...
between the jaws of the clamps, as well as all remnants of leaflets near the prepared strips, were pooled for each leaflet and individual animal for the analysis of dry defatted weight, elastin, and collagen (see below). From the fifth cycling test the hysteresis energy (i.e., the area between the loading and unloading curve) was calculated and the ratio of energy lost to energy absorbed (mechanical hysteresis) was derived. These mechanical hysteresis measurements were performed in order to obtain a measure of elastic efficiency (resilience). The energy ratio expresses the relative importance of viscosity to the mechanical response, whereby a lower ratio indicates a higher elastic efficiency of the tissue (12). The load-deformation failure curve was converted to a load-strain curve. The original length of the strip (l0) was the sum of the nominal jaw distance, and the deformation until a small load (0.01 N or, for mitral, 0.015 N) was obtained. Strain is (l - l0)/l0. Maximum stress (s max) is maximum load (F max)/cross-sectional area (MPa), and F max normalized for unit collagen (N/mg/mm) is F max divided by milligrams of collagen per millimeter original length of the strip (l0). Maximum stiffness (N) was calculated as the maximum slope of the load-strain curve.

Collagen and elastin determination

Hydroxyproline was determined according to the procedure of Woessner (13,14); the collagen content was calculated as hydroxyproline content × 7.46 (15).

In order to determine elastin and collagen contents (as a percentage of dry defatted weight), the pooled leaflets remnants were defatted with acetone, freeze-dried, and extracted essentially according to the Lansing procedure to determine the dry weight of the remaining elastin (16). The collagen (hydroxyproline) content was determined in aliquots of the tissue extracts.

Morphological preparation

Heart valve specimens were fixed in 4% buffered formaldehyde, and after one week of fixation were immersed in phosphate-buffered saline. Sections for histological investigation were taken from the anterior pulmonary, non-coronary aortic and anterior mitral valve. Each section consisted of a 2 mm-wide strip from the valve and included the free edge of the valve and its ‘anchoring’ to the fibrous annulus. The valvular strips were paraffin-embedded and sections (3-4 µm thick) were cut on a microtome (Thermo Scientific Microm HMX555S). Serially cut sections were stained with hematoxylin and eosin, elastic van Gieson, Masson’s trichrome and Fraser-Lendrum stains. Immunohistochemical reactions for factor VIII, CD31, CD34 (primarily for endothelial cells), S100 (Schwann cells), smooth muscle cell actin and tyrosine hydroxylase (for sympathetic nerves) were carried out.

Statistical analysis

All data were analyzed using a linear mixed effects regression model, with species (giraffe/calf), valve and direction as fixed effects. Animal and valve (as well as strip and direction) within animal were included as random effects. Standardized residuals were inspected and consequently F max, F max adjusted for collagen, s max and maximum stiffness as well as collagen density were analyzed on a logarithmic scale. Results were provided as estimated species means and differences for outcomes that were not log-transformed. For outcomes analyzed on a logarithmic scale, results were given as estimated species medians (back-transformed means on the log-scale) and ratios of medians (as percentages). All values were given with 95% confidence intervals (CI) in parentheses. All data were analyzed using Stata version 14.1 (Stata Corporation LP, College Station, TX, USA).

Results

Maximum load (F max)

The aortic valve was significantly stronger in the giraffe than in the calf, in both radial and circumferential directions [70% (CI 42 to 103%); p <0.001]. The same was true for the mitral valve, although the difference was not significant [75% (CI -10 to 240%); p = 0.10]. No significant difference was found between the giraffe and calf regarding pulmonary valve strength [7% (CI -12 to 32%); p = 0.50] (see Fig. 1 and Table I).

Maximum stiffness

The maximum slope of the load-strain curve was calculated as a measure of maximum stiffness. The giraffe aortic valve was significantly stiffer than the calf aortic valve in both radial and circumferential directions [34 (CI 14 to 58%); p <0.001]. No significant difference was found between species for either the mitral valve [16% (CI -32 to 98%); p = 0.59] or the pulmonary valve [2% (CI -15 to 22%); p = 0.85] (see Fig. 2 and Table I).

Valve thickness

Specimen thickness (i.e., cross-sectional area with a fixed width) was measured in both directions for aortic and pulmonary valves. The aortic valve in the giraffe was found to be significantly thicker than in the calf [difference of 0.23 mm2 (CI 0.07 to 0.40 mm2); p = 0.006]. However, the pulmonary valve had a larger cross-sectional area in the calf than in the giraffe (difference of 0.23 mm2 (CI 0.05 to 0.41 mm2); p = 0.01) (see Fig. 3).
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Maximum load adjusted for cross-sectional area

After adjusting the maximum load for cross-sectional area (ultimate tensile stress, $s_{\text{max}}$), a significant difference was found between giraffes and calves in aortic and pulmonary valves in both radial and circumferential directions ($p < 0.001$). In general, the ultimate tensile stress in giraffe heart valves was 39% (CI 18 to 64%) larger than in calves. Further details are listed in Table I.

Mechanical hysteresis

To estimate the elastic efficiency the mechanical hysteresis (7) was measured for specimens from the three valves. No significant difference was found between giraffes and calves in mechanical hysteresis (elasticity ratio) in the circumferential direction for the aortic valve [-0.02 (CI -0.05 to 0.01); $p = 0.25$], mitral valve [-0.04 (CI -0.11 to 0.03); $p = 0.26$] or pulmonary valve [-0.02 (CI -0.06 to 0.01); $p = 0.17$]. In the radial

### Table I: Summary of data from mechanical testing.

<table>
<thead>
<tr>
<th>Valve/direction</th>
<th>Maximum load (N)</th>
<th>Ultimate tensile stress (MPa)</th>
<th>Maximum stiffness (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Giraffe</td>
<td>Calf</td>
<td>Giraffe</td>
</tr>
<tr>
<td>Aortic/Circumferential</td>
<td>13.29* (11.52-15.33)</td>
<td>7.83 (6.63-9.25)</td>
<td>6.52* (5.61-7.58)</td>
</tr>
<tr>
<td>Aortic/Radial</td>
<td>1.88* (1.63-2.18)</td>
<td>1.11 (0.94-1.31)</td>
<td>1.10** (0.94-1.28)</td>
</tr>
<tr>
<td>Mitral/Circumferential</td>
<td>42.69 (29.05-62.72)</td>
<td>24.43 (14.18-42.09)</td>
<td>Not performed</td>
</tr>
<tr>
<td>Pulmonary/Circumferential</td>
<td>3.59 (3.05-4.21)</td>
<td>3.34 (2.78-4.02)</td>
<td>4.18* (3.54-4.93)</td>
</tr>
<tr>
<td>Pulmonary/Radial</td>
<td>0.94 (0.81-1.10)</td>
<td>0.88 (0.72-1.07)</td>
<td>1.07 (0.91-1.25)</td>
</tr>
</tbody>
</table>

Values are mean or median (95% CI).
p-values comparison between species: *, $p < 0.05$ **, $p < 0.01$.

Figure 1: Maximum load (N) with 95% CI error bars for each valve in the circumferential direction. Significant differences between species were noted for the aortic valve ($p = 0.001$), but not for mitral and pulmonary valves ($p = 0.1$; $p = 0.3$, respectively). See Table I for further details.

Figure 2: Maximum slope (N) of the load-strain curve as a measure of maximum stiffness in the circumferential direction (95% CI error bars). A significant difference was noted between species in the aortic valve ($p = 0.001$), but not for mitral valves ($p = 0.6$) and pulmonary valves ($p = 0.3$). See Table I for further details.
direction no significant difference between species was noted in the aortic valve [0.01 (CI -0.02 to 0.04); p = 0.67], but the pulmonary valve showed a significant higher energy loss in giraffes than in calves [0.07 (CI 0.04 to 0.11); p <0.001].

**Collagen content per mm specimen (mg/mm)**

The collagen content per mm specimen (unit collagen) did not differ significantly between giraffes and calves with regards to the aortic valve [0.12 mg/mm (CI -0.07 to 0.32 mg/mm); p = 0.22] and pulmonary valve [-0.001 mg/mm (CI -0.20 to 0.20 mg/mm); p = 0.99]. However, for the mitral valve the unit collagen was higher in the giraffe than in the calf [0.37 mg/mm (CI 0.14 to 0.59 mg/mm; p = 0.002].

**Maximum load adjusted for collagen**

In order to determine whether collagen variations in the cross-section of the specimens (unit collagen) may explain the difference in tensile strength, a normalized tensile load value (maximum load/unit collagen) was calculated. After normalization for collagen, no significant differences were found in strength between giraffe and calf with regards to valve type or direction [aortic valve: 4% (CI -18 to 33%; p = 0.36; mitral valve: 34% (CI -28 to 151%; p = 0.036; pulmonary valve: 10% (CI -15 to 43%; p = 0.48).

**Collagen density**

Collagen content per cubic millimeter of specimen (density, mg/mm$^3$) was measured only for the aortic and pulmonary valves. Density was found to be significantly greater in giraffe than in calf with regards to the aortic valve [40% (CI 25 to 57%); p <0.0001]. A higher collagen content was also noted in giraffe versus bovine pulmonary valves [18% (CI 3-34%); p = 0.014].

**Collagen content per dry weight (%)**

The collagen content per dry weight in giraffe valves was significantly higher than in calf valves [aortic valve 10.94% (CI 7.77 to 14.12%); p <0.0001; pulmonary valve 4.31% (CI 1.03 to 7.59%); p = 0.01; mitral valve 9.55% (CI 3.45 to 15.64%); p = 0.002] (see Table II).

**Elastin content per dry weight (%)**

Both, aortic valves [1.46% (CI 0.08 to 2.85%); p = 0.04] and pulmonary valves [2.46% (CI 1.03 to 3.88%); p = 0.001] contained significantly more elastin per dry weight in giraffes than in calves. No significant differences between species were found for mitral valves [1.38% (CI -1.44 to 4.19%); p = 0.34] (see Table II).

### Table II: Collagen and elastin in percent of dry defatted weight.

<table>
<thead>
<tr>
<th>Valve</th>
<th>Collagen (%)</th>
<th>Elastin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Giraffe</td>
<td>Calf</td>
</tr>
<tr>
<td>Aortic</td>
<td>55.29*</td>
<td>44.25</td>
</tr>
<tr>
<td></td>
<td>(53.34-57.23)</td>
<td>(41.78-46.72)</td>
</tr>
<tr>
<td>Mitral</td>
<td>51.05*</td>
<td>41.52</td>
</tr>
<tr>
<td></td>
<td>(47.34-54.76)</td>
<td>(36.28-46.76)</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>44.92*</td>
<td>40.59</td>
</tr>
<tr>
<td></td>
<td>(42.98-46.85)</td>
<td>(37.98-43.19)</td>
</tr>
</tbody>
</table>

Values are mean (95% CI). p-values comparison between species: *, p <0.05.

![Figure 3: Average cross-sectional area of valve tissue (95% CI error bars) with a fixed width as a measure of specimen thickness (mm$^2$). Specimens from aortic and pulmonary valves in the radial direction. A significant difference was noted between species for the aortic valve (p = 0.006) and pulmonary valve (p = 0.01).](image)
Morphology

The three valve types investigated were principally of the same construction, with no major differences between giraffes and calves (Fig. 4). The surface of the valve is lined by endothelial/endocardial cells, with a dense collagenous layer (holding face) at the side of the valve facing the heart chamber with the higher pressure. A layer of mainly elastic fibers (the deformed face) is seen at the side of the valve opposing the heart chamber. Between these two layers - at the center of the valve cusp - a more loose arrangement of fibrous tissue and elastic fibers is prevalent, with relatively large amounts of proteoglycans and glycosaminoglycans (spongiosa) (Fig. 5). Pathological changes such as inflammation, degenerative changes or tumors were not seen in any specimen.

Lymph and blood vessels were each visible in the heart valves. In both giraffes and calves, the vessels can be followed half-way to the tip of the valve, or even further (Fig. 6). No nerves were visible in the valve tissue per se.

Smooth muscle cells were a constituent of the vessel wall, but not of the valve structure per se.
Discussion

The present study is the first to provide details of the mechanical and morphological features of giraffe heart valves – structures which are subjected constantly to high-pressure conditions. The demonstration that the valves of the left side of the giraffe heart were stronger than in the calf most likely reflects an evolutionary adaption to the very high blood pressures encountered in the giraffe circulation.

The aortic valve tissue was shown to be significantly stronger (70%) in giraffes than in calves, in both radial and circumferential directions, and the same was true for the mitral valve (75%), but not statistically significantly so. However, as it was not possible to harvest more than one sample per mitral valve due to the irregularity of the valve, a type II statistical error cannot be ruled out.

The aortic valve in giraffes is significantly thicker than in calves, which intuitively is the cause of the increased strength of giraffe heart valves. The maximum load when adjusted for cross-sectional area displayed a species difference, with higher values for giraffe for both aortic and pulmonary leaflets. The difference in cross-sectional area does not explain the higher maximum load found for the giraffe aortic leaflets, however. In contrast to cross-sectional area, the high collagen content of the leaflets seems to better explain the species difference in maximum load. The increased collagen density found in aortic leaflets is also in line with the increased strength of the giraffe leaflets being caused by a greater collagen content rather than thickness (cross-sectional area).

The elastin content was also significantly more prominent in the aortic and pulmonary valves of giraffes than of calves, and this was also the case for mitral valves, though not significantly so. The function of elastin is to pull the heart valve back to its closing position after ejection, and the force required is intuitively greater in a high-pressure system.

The pulmonary leaflets in the giraffe were found to be significantly thinner than in the calf. This point proved surprising due to the fact that both the collagen and elastin contents were greater in the giraffe. Thus, the higher pulmonary leaflet thickness in calves could be caused by a greater amount of extracellular matrix, such as glycosaminoglycans/proteoglycans. The reason for this variation is unknown, as the conditions (i.e., pulmonary blood pressure) on the right side of the circulation are considered to be the same in both species. Rather, a more global influence on heart valve development caused by a higher blood pressure on the left side in giraffes might be the cause of this inconsistency. This influence could be genetically induced, or it may be due to systemically circulating ‘growth factors’.

The aortic valve was also found to be significantly stiffer in the giraffe than in the calf. This was in concordance with previous results regarding the strength of the aortic valve, and was most likely a result of the high-pressure system. No difference was found between species regarding the pulmonary and mitral valves.

Several vessels were identified at the base of the valves, despite very few blood vessels being seen primarily at the base of valve structures in humans (17). In giraffes and calves, these vessels could be followed more peripherally on the valves compared to humans, the suggested explanation for this being that the animal valves are thicker than human valves. A
nutritional diffusion of oxygen from the surface might, therefore, not be sufficient. No difference was found between calves and giraffes regarding the numbers of blood vessels.

No nerves were identified in the valve leaflets of either giraffes or calves. These findings were in contrast with observations in human valve tissues, where Misfeldt and Sievers described nerve cells in both aortic and mitral valves (17). This difference may be related to species, although since both calves and giraffes lack nerves this could not be related to the blood pressure.

It can be argued that neither calf nor giraffe hearts can be compared directly to humans, despite their anatomical resemblances. Yet, the calf has been chosen in order to probably had the greatest impact on the blood pressure of giraffes, as calves and giraffes are both cloven-hoofed animals and have similar body weights. Furthermore, the relative heart mass of the giraffe is similar to that of calves and other mammals (typically 0.5% of body mass) (10,18). Thus, the dimensions of the heart should be comparable, the only difference being the hemodynamics. Any measured difference between giraffes and calves would most likely be a result of differences in blood pressure.

**Study limitations**

A major limitation of the study was the small number of animals included, and as giraffes are not common experimental animals the valve sampling had a natural limitation. A second limit was the small number of tissue slices available from the mitral valve, and this probably had the greatest impact on the results obtained. In addition, as only young animals were investigated it is impossible to elaborate on any changes that might occur to the giraffe heart valves over their life span.

A future pathway for advances in biological valve prosthetic design would be tissue engineering which, in contrast to conventional biological valve prosthetic designs, is not limited to using available tissues of either bovine or porcine origin. New biological valve leaflets may be designed in a variety of ways, and the present findings may serve as an inspiration for the design of future prostheses with longer durabilities. The use of tissue engineering may lead to the design of valve leaflets composed of high-density collagen layers targeted at increasing resistance not only to shear stresses but also to compressive and tensile forces. In addition, increasing the elastin layer will preserve elastic efficiency.

In conclusion, the present investigations with giraffe heart valves have led to the evolution of several interesting characteristics. Notably, the aortic valve is stronger, most likely due to a compact collagen construction. Likewise, the contents of both collagen and elastin were found to be higher in giraffes than in calves, a construction that would cause giraffe valves to be more resistant to the high-pressure forces that naturally characterize these animals. Unfortunately, such a construction also leads to stiffening and thickening of the valves. The elastic efficiency was similar in the leaflets of giraffe and calf hearts, while mitral leaflets showed similar - but mostly insignificant - trends in strength, stiffness, and collagen/elastin content.

**Acknowledgements**

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