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Distribution of αB-Crystallin in the Central Retina and Optic Nerve Head of Different Mammals and its Changes during Outer and Inner Retinal Degeneration

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Abstract

Purpose: To investigate species differences in the distribution and localization of alpha B-crystallin (ABC) in the normal retina and optic nerve head region, and to describe changes during outer and inner retina degeneration.

Material and methods: Animals studied included mice, rats, cats, pigs, cows, and monkeys. Sections of the optic nerve and central retina were labeled with antibodies against ABC and glial fibrillary acidic protein (GFAP).

Results: ABC was located in astrocytes and Muller cells with different intensities. During outer retina degeneration (dystrophic rat and Abyssinian cat), only late stages showed an increase in ABC in the retina and optic nerve head. Inner retina degeneration in the glaucoma mouse model showed no increase of ABC. In the monkey glaucoma model, only the innermost layer of the optic nerve head showed increased labeling for ABC.

Conclusions: The distribution of ABC is species dependent and is (excluding the mouse) present in the nerve fiber layer of the retina and in the optic nerve head (localization of astrocytes). Chronic retinal degeneration does not necessarily lead to an over-expression of ABC. While in outer retinal degeneration induction was predominantly present in late stages, pressure-induced glaucoma led to a specific increase in ABC already in early stages indicating a local stress-response in this region.

Keywords: Alpha B-crystallin; Astrocytes; Optic nerve; Central retina

Introduction

The small heat shock proteins alpha B-crystallin (ABC) and Hsp27 are chaperone molecules that maintain the integrity of intracellular processes during various types of stress. While Hsp27 is rather specific for the human and has only modified related proteins in other mammals (e.g. Hsp25 in rodents and birds) [1,2], ABC is a highly conserved protein that exists in nearly any species examined [3-6]. In previous work we could show that ABC is present in the retrolaminar optic nerve of numerous species including human, monkey, cow, pig, rat, and mouse [7,8]. The amount of ABC showed species differences but was homogenous within different age groups of a single species.

Under pathological conditions the expression level of ABC frequently increases. For this reason it is considered to be a useful marker in a variety of neurodegenerative diseases [9]. Concerning ocular pathologies, an increase in ABC was described in the retrolaminar optic nerve of glaucomatous monkey eyes [8]. In vitro studies confirmed the increase of ABC in human optic nerve head astrocytes exposed to elevated hydrostatic pressure [10]. In the retina, intense light exposure increased the amount of ABC in rod outer segments and retinal pigment epithelium [11]. During retinal degeneration in the rd mouse the increased ABC expression was associated with glial cell reaction [12]. Only recently, ABC secretion was described from the retinal pigmented epithelium and a neuroprotective role suggested [13].

The present study was designed to answer three major questions. A. Are there species differences in the distribution and localization of ABC in the normal retina and optic nerve head region? B. Is an increase of ABC a general phenomenon during retinal degeneration? C. Is there a difference in ABC labeling between outer (photoreceptor type) and inner (ganglion cell type) retina degeneration?

Material and Methods

Tissue samples of the central retina and optic nerve head region were collected from mouse, rat, cat, pig, cow, and monkey eyes. Most of the tissues were left-over from various other protocols: the cat eye samples were from a collaboration with Kristina Narfström (Uppsala, Sweden) [14-16], the monkey eye samples from a collaboration with Paul Kaufman (Madison, USA) [17,18], the DBA2/NNia mouse eyes from a collaboration with Thomas Mittag (New York, USA) [19-21]. Rat and other mouse eyes were from own breeding (the animals sacrificed by neck dissection) [22-24], pig and cow eyes from the slaughterhouse in Erlangen (Germany). All animals were kept and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the local animal care statutes.

Normal eyes of six pink-eyed rdy +/- Royal College of Surgeons (RCS) rats (3 weeks to 22 months of age; RCS +/-), five European mixed breed cats (1-6 years of age), one Abyssinian cat (4 years of age), five pigs (6-12 months of age), four cows (1-2 years of age), and seven cynomolgus monkeys (6-20 years of age) were collected. To study eyes with outer retinal degeneration, seven RCS rdy -/- animals (3 weeks to

12 months of age; RCS -/-) and eight Abyssinian cats with various stages of retinal degeneration (1-8 years of age) were available. To study eyes with inner retinal degeneration, nine DBA2/NNia mice (2 to 23 months of age) and seven cynomolgus and rhesus monkeys (12-32 years of age) with unilateral laser-induced ocular hypertensive eyes were available.

All tissue samples were fixed 2-10 minutes after sacrifice in 4% paraformaldehyde for 4-6 hours, then either washed in phosphate buffered saline (PBS; pH 7.2-7.4) or shipped in 1% paraformaldehyde to Erlangen.

Sagittal 14μm thick cryo-sections through the central retina and the optic nerve head region were performed and mounted on glass slides. Incubation with a polyclonal rabbit anti alphaB-crystallin antibody (dilution 1:400; antibody kindly provided by Hans Bloemendal, Nijmegen, The Netherlands) or a polyclonal rabbit anti glial fibrillary acidic protein (GFAP; dilution 1:500; BioGenex Laboratories, San Ramon, CA, USA) was performed over night in a moist chamber at room temperature. After rinsing in PBS, a fluorescein-conjugated goat anti rabbit Cy3 antibody (dilution 1:1000; Dianova, Hamburg, Germany) was added for 1 hour. The slides were rinsed again in PBS, mounted with Kaiser's glycerin gelatine and viewed with a fluorescence microscope (Aristoplan; Leica) at 40X to 400X magnifications.

Results

Species differences in normal eyes with no ocular pathology

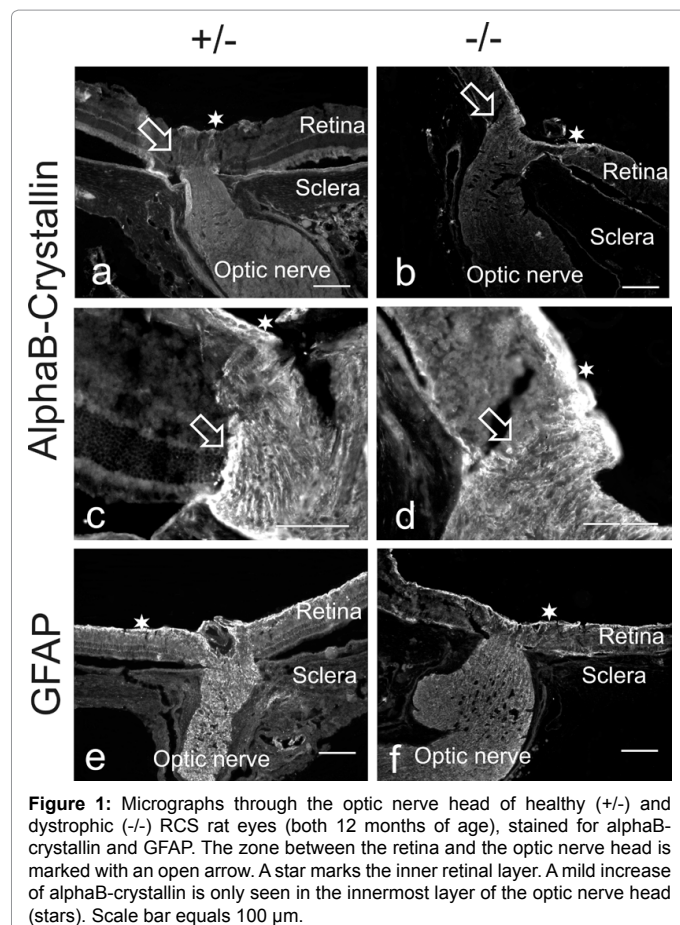Immuno fluorescence labeling of ABC revealed clear species differences in the retina and optic nerve head:

Figure 1: Micrographs through the optic nerve head of healthy (+/-) and dystrophic (-/-) RCS rat eyes (both 12 months of age), stained for alphaB-crystallin and GFAP. The zone between the retina and the optic nerve head is marked with an open arrow. A star marks the inner retinal layer. A mild increase of alphaB-crystallin is only seen in the innermost layer of the optic nerve head (stars). Scale bar equals 100 μm.
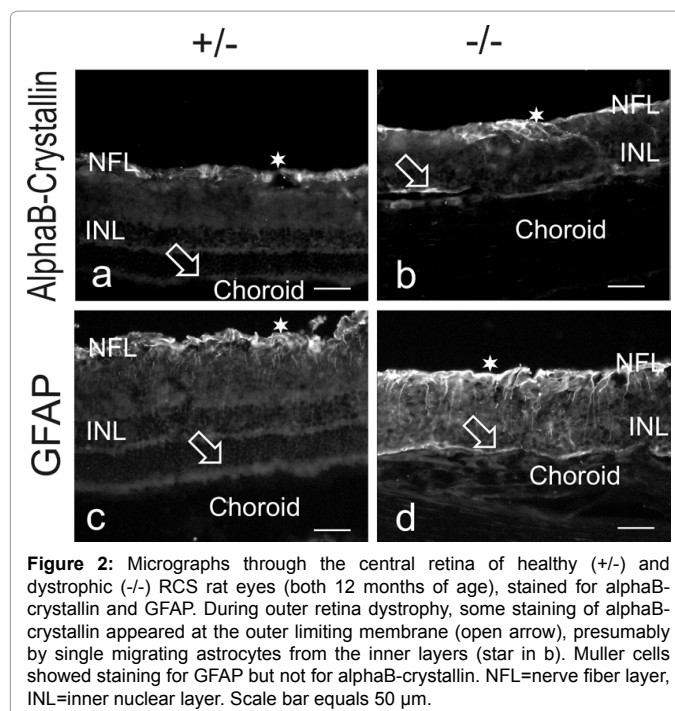

Figure 2: Micrographs through the central retina of healthy (+/-) and dystrophic (-/-) RCS rat eyes (both 12 months of age), stained for alphaB-crystallin and GFAP. During outer retina dystrophy, some staining of alphaB-crystallin appeared at the outer limiting membrane (open arrow), presumably by single migrating astrocytes from the inner layers (star in b). Muller cells showed staining for GFAP but not for alphaB-crystallin. NFL=nerve fiber layer, INL=inner nuclear layer. Scale bar equals 50 μm.

In the rat optic nerve, cell processes in the lamina cribrosa and prelaminar region labeled for ABC (Figure 1a and 1c). The estimated density of these processes was less than that of processes labeled for GFAP (Figure 1e and 1f). In the innermost layer of the papilla region towards the inner limiting membrane, consistent labeling for ABC and GFAP was seen. Only single processes, presumably of astrocytes, in the nerve fiber layer (NFL) of the retina labeled for ABC; the Muller cells were not stained (Figure 2a). The distribution of GFAP was more intense in the NFL compared to ABC and indicated astrocytes and inner Muller cell processes (Figure 2c).

Consistent labeling for ABC was found in optic nerve head sections of the cat, pig and cow. Numerous positive staining was seen in the lamina cribrosa and prelaminar region of the optic nerve (Figure 3a and 3e), revealing a similar labeling pattern as GFAP (Figure 3c). Labeling of the innermost layer of the papilla region was more intense than the other optic nerve head regions. In the central retina, cell processes in the NFL (all animals) as well as single Muller cell processes (pig and cow) labeled positive for ABC (Figure 3b and 3f). GFAP showed intense labeling of the NFL of the central retina with no increase in the papilla region. Muller cells showed a much lower intensity in GFAP labeling (Figure 3d and 3g). Data of the cat not shown.

In normal monkey optic nerve specimens, the labeling distribution of ABC was comparable to that found in the cat, pig and cow (Figure 4a and 4c).

Eyes with inherited outer retinal degeneration

In the dystrophic RCS -/- rat, labeling for ABC in the retro laminar optic nerve was similar to that observed in the normal animals. No changes occurred during retinal degeneration. At the innermost layer of the papilla region, an increase in labeling intensity was seen in the 12 months old dystrophic animals (Figure 1b and 1d). Within the central retina, clear ABC labeling was seen only in the NFL (Figure 2b). The Muller cells, characterized by their long processes through the whole retina, became positive for GFAP only (Figure 2d).
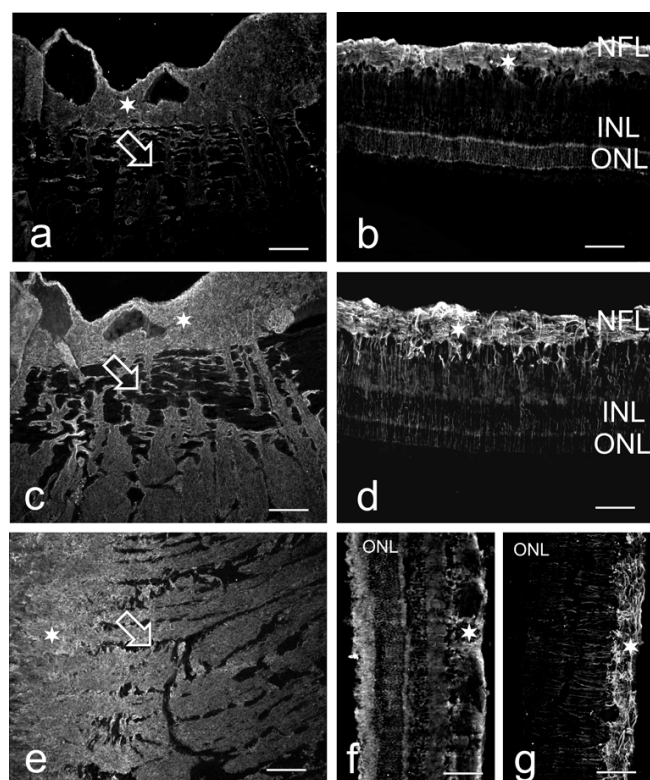
Figure 3: Micrographs through the optic nerve head (a,c,e; scale bar 200 µm) and central retina (b,d,f,g; scale bar 100 µm) of the porcine (a-d) and bovine (e-g) eye, stained for alphaB-crystallin (a,b,e,f) and GFAP (c,d,g). Astrocytes in the lamina cribrosa (open arrow) and in the prelaminar region (stars in a,c,e) as well as in the nerve fiber layer of the retina (stars in b,d,f,g) staine positive for alphaB-crystallin. Single Muller cells show also alphaB-crystallin immunoreactivity, but less regular than GFAP (parallel stained lines through the retina in d,g). NFL=nerve fiber layer, INL=inner nuclear layer, ONL=outer nuclear layer.

In the Abyssinian cat, early stages (stage 0 and1) of retinal degeneration showed no changes in labeling for ABC and GFAP (data not shown). At later stages (stages 2-4), however, increase in ABC labeling intensity at the papilla region and of the Muller cells occurred (Figure 5). GFAP positive Muller cells, in contrast, were seen only in the very late stages of retinal degeneration (stage 4; data not shown).

Eyes with inner retinal degeneration (glaucomatous eyes)

In rhesus monkeys, increase in intraocular pressure lead to an increase in ABC and GFAP staining mainly in the innermost layer of the papilla region towards the inner limiting membrane (Figure 4b and 4d). This increase was consistent in all glaucomatous papillae studied, while the labeling intensity of the neuronal prelaminar and lamina cribrosa region showed inter-individual differences that were not correlated to the duration or severity of glaucomatous damage. In the central retina, labeling for ABC was restricted to the astrocytes in the NFL. No increase was noted in different stages of glaucomatous damage.

In the DBA2NNia strain, the overall distribution of ABC was not changed even in old animals with late stages of glaucomatous damage. The labeling was restricted to the retroocular myelinated part of the optic nerve. No labeling appeared in the papilla region or in the retina even in 20 months old animals with severe loss of retinal ganglion cells and optic nerve fibers (data not shown). There was also no visuable increase of ABC in the retina.

A semi-quantitative analysis summarizing the above described distribution of ABC is outlined in Table 1.

Discussion

1. The results of the present study show clear differences in the distribution of ABC in the retina and optic nerve head region between the different mammalian species studied. 2. During photoreceptor degeneration, an increase in ABC was only seen in late stages when the inner layers of the retina were also affected. 3. Retinal ganglion cell degeneration led to a specific early staining of the inner prepapillary region in the monkey glaucoma model.

A general problem when comparing different species and diseases is the affinity of the antibodies used. Proteins can become modified
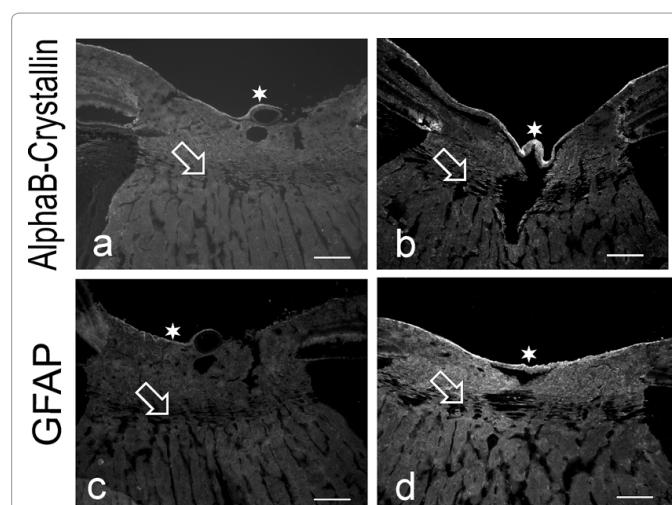

Figure 4: Micrographs through the optic nerve head of a healthy (a,c) and a glaucomatous (b,d) monkey eye (one animal, 12 years of age), stained for alphaB-crystallin and GFAP. The lamina cribrosa region (open arrow) and prelaminar region showed no staining difference between glaucoma and control for alphaB-crystallin, but a clear increase in GFAP. Only the innermost line of the optic nerve head (star) showed an increase in alphaB-crystallin staining. Scale bar equals 200 µm.
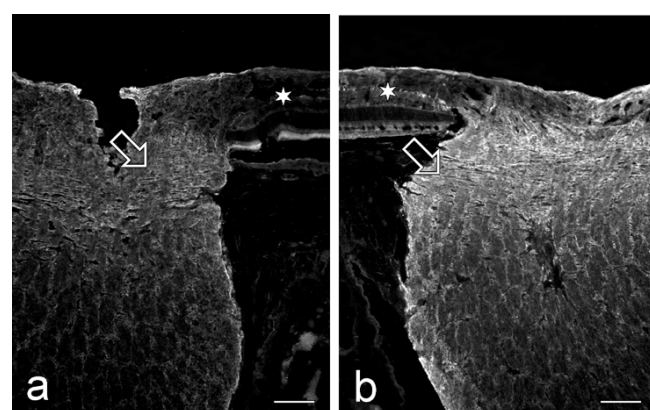
Figure 5: Micrographs through the optic nerve head of a healthy (a) and a dystrophic (stage 2, b) Abyssinian cat eye (both animals 4 years of age), stained for alphaB-crystallin. The homogenous staining in the normal eye (lamina cribrosa as indicated by the open arrow, prelaminar region, rim of the optic nerve bundles) is somewhat increased in the dystrophic eye. A star marks the retina which shows alphaB-crystallin staining in the nerve fiber layer. Scale bar equals 200 µm.

Animals	Retina			Optic nerve head			
	Nerve fiber layer	Muller cell processes	Outer limiting membrane region	Inner layer towards inner limiting membrane	Pre-lamina region	Lamina cribrosa region	Post-lamina region
mouse (data from reference 39)	0	0	0	0	0	not existent	+
Glaucoma mouse (n=18)	0	0	0	0	0	not existent	+
RCS +/- rat (n=12)	+	0	0	+	+	+	+
RCS -/- rat (n=14)	+	0	e 0 l (+)	++	+	+	+
European mix breed and Abyssinian cat (n=6)	+	0	0	++	+	+	+
RP Abyssinian cat (n=8)	+	e 0 l (+)	0	++	e + l ++	e + l ++	+
pig (n=5)	++	(+)	0	++	+	+	+
cow (n=4)	++	(+)	0	++	+	+	+
cynomolgus monkey (n=12)	++	0	0	++	+	+	+
Glaucoma cynomolgus and rhesus monkey (n=7)	++	0	0	+++	+	+	++

Table 1: Semiquantitative evaluation of alphaB-crystallin distribution in the retina and optic nerve head of various animals (n= number of eyes evaluated). 0 = no staining; (+) = single or inconsistent staining; + = consistent mild staining; ++ = consistent moderate staining; +++ = consistent strong staining. e = early stages of disease, l = late stages of disease.

with age, truncations can occur in degenerative conditions, and thus compromise the affinity. Unfortunately, there is no way to exclude these processes which could lead to misinterpretation. The following discussion is based on the described results and therefore reflects the actual staining performed with the specific antibody. Since the results do not contradict other statements and descriptions of ABC, they might reflect the real situation.

Ad 1. Species differences were prominent between rodents and the other animals investigated in this study. Since chaperone molecules are highly related to various types of stress, a pure size-dependence of the eye (small versus large eyes) does not sufficiently interpret the findings. Two stress factors might more likely be causative: one is the absolute life span of the animals, the other one is the normal activity rhythm (nocturnal versus diurnal). A counter-example for the latter hypothesis is the fact that intense light exposure was shown to stimulate ABC expression in rod outer segments but not in the inner retina [11].

For all animals it was interesting to note, that there was no ABC staining located within the neurons, but only within glial cells (constantly astrocytes, occasionally Muller cells). This is in contrast to other crystallins and small heat shock proteins which were also described in photo receptor cells and retinal ganglion cells [25-30]. On the other hand, the close relation of ABC to glial cells is not unique in the retina but also known in other neuronal tissues [9,31].

Ad 2. Even though retinal degeneration leads to an imbalance of the local homeostasis and therefore to stress-like conditions, increase of ABC is not an early phenomenon during outer retinal degeneration. In this type of retinal degeneration, the inner layers are well preserved for a long time. Only at late stages morphological alterations are paralleled by a different expression of ABC. Heat shock protein protection seemed to appear earlier than fibrillar activation in glial cells, at least in the Abyssinian cat model. Increase of ABC was also reported in Muller cells and retinal astrocytes of the rd mouse with an accelerated type of degeneration [12].

Concerning other small heat shock proteins, a reduction of alphaA-crystallin seems to be present in the RCS rat already in early stages of the degeneration [32]. This lack of alphaA-crystallin might lead to a higher vulnerability of the photo receptors [33].

Ad 3. Increase of ABC in glaucomatous optic nerves was reported [8,10], but a precise localization has not been described so far. Interestingly, the mouse glaucoma model is not suitable for this type of investigations since ABC seems to play no major role in the mouse eye pathology. It is tempting to speculate that heat shock protein 27 might sufficiently serve the protective function in this species [34,35] and possibly in the rat, too [36].

The increase of ABC in the monkey glaucoma model showed a specific location at the inner papilla already in early stages of the disease. This unique location was not reported for other heat shock proteins [37] and might reflect a specific vulnerable region. It supports the hypothesized pathogenesis by Osborne [38] claiming the prelaminar region as the most sensitive part of the optic nerve fibers. An early and intense reaction of the local glial cells seems to take place, expressing not only ABC, but also GFAP and other factors like TGFbeta [39].

Numerous studies report an increase of ABC within the first few days following an acute insult (diabetes, retinopathy of prematurity, ischemia; reviewed in Fort and Lampi [40]) or optic nerve damage [41,42] but long term regulation is often neglected. In age-related macular degeneration ABC accumulation was described in drusen [43-45] and recent interest is focused on this protein in the retinal pigmented epithelium [46,47]. Increase of ABC was reported in the glaucomatous retrolaminar optic nerve [8] and trabecular meshwork [48] and in a mouse model of outer retinal degeneration [12]. In this study, several models with chronic retinal destruction were studied concerning their distribution of ABC. They show that during the process of outer retinal degeneration ABC expression is not greatly altered, suspecting no major protective mechanism of this molecule under these in vivo conditions. In contrast, the glaucoma monkey model showed an increase of ABC in the inner layer of the optic nerve head already in early stages of increased intraocular pressure which remained present in later stages. The early induction at this location points to an active role of ABC in vivo. Although effects of ABC are reported in and discussed for the retina [49], both types of retinal degeneration showed almost no changes of ABC labeling in the retina itself.

Since this study shows only mild and focused changes of ABC in a number of animal models with inner and outer retinal degeneration, future studies are required to more precisely describe the role of ABC in chronic, long-lasting situations. Chronic retinal diseases with vascular

involvement (e.g. diabetes) might help to further understand the role of ABC, since new data points to a vascular regulating role of ABC [50] which might be more important than pure neuronal aspect. Induction of diabetes in rats showed an early increase of alpha-crystallins with a reduction at later time points [51], supporting the low levels described only recently in the human [52]. The vascular aspect, however, was not taken into account in these studies.

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