

# The Pathological Relevance of Some Biochemical Changes in Keratoconus

## Focusing on serum lipid peroxidation products

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*Keratoconus (KC) is one of the most common degenerative corneal diseases. It is known that during KC degeneration the cornea gains conical shape and layers thinning, involving several molecular factors changes many of which consist in local or blood stream components. Furthermore, growing evidence show key implication of oxidative stress in degenerative processes observed in KC patients. In this way, malondialdehyde serum levels screening was made in order to find correlations between KC patients and controls. It has been shown that serum malondialdehyde (as a lipid peroxidation marker) level is increased in KC patients. Furthermore, we showed a decrease in lipid peroxidation levels during the monitoring time. These changes would indicate that an inflammatory process may be involved in keratoconus, but further analysis is needed.*

**Keywords:** malondialdehyde, thiobarbituric acid reactive substances, blood, keratoconus

Ophthalmological diseases are extremely diverse and can be due to both local and systemic causes. In both situations, there are the tears that transport or accumulate the products which can both help and damage the eyes. In dry eyes syndrome, for example, the small volumes of available tears retention lead to toxic products accumulation. In this way, the eyes are being intoxicated by their very need. There are many molecules reported in various eyes disorders patients as compared to normal controls: signalling factors, inflammation markers, blood cells, enzymes, free radicals [1]. Many disease markers could be also present in blood serum such as oxidative stress markers [2], collagen metabolism components [3], and inflammatory cytokines [4].

Keratoconus (KC) is one of the most common degenerative corneal diseases. It is known that during KC degeneration, the cornea exhibits conical shape and layers thinning. The clinical manifestations refer to irregular astigmatism and myopia [5]. However, the etiology and developmental factors of this disease are currently unknown. In this way, both systemic and local phenomena were reported including epithelial and stromal apical cone region thinning, Bowman's layer ruptures, and stromal keratocyte apoptosis [4]. Kenney et al. [6] suggest that altered extracellular matrix function lead to corneal degeneration. Furthermore, the basal cells degenerate next realising proteolytic enzymes which can destroy the underlying tissue [7].

Biochemical and immunohistological studies show that corneal stroma loss may also be due to increased proteases activity which comes to counteract proteolytic enzymes harm. Similarly, protease inhibitors such as  $\alpha$ 1-proteinase inhibitor and  $\alpha$ 2-macroglobulin are found in low concentrations [8]. Also, inflammation effects which lead to corneal destruction were considered by finding both IL-6 and TNF- $\alpha$  overexpression in KC patients as compared to controls [9].

A clear status regarding the serum lipid peroxidation levels of both normal and KC patients was barely reported. Many studies address the serum specific and non-specific protein levels in regarding the discrimination between inflammatory and structural impairments underlying KC development [10-12]. However, the malondialdehyde (MDA) levels are important in both inflammatory and structural impairments evaluation, MDA assessment being one of the common inflammatory markers [21].

Furthermore, growing evidence show that oxidative stress is an important player in KC development and processes. We previously showed that oxidative stress markers changes are common in KC patients [22]. This way, it is well possible that changes in protein concentration correlated with low or high enzymatic activity and high MDA levels lead to different conclusions in research. More than that, since antioxidant defence seems to be inhibited or overwhelmed, the histological degeneration may be due to the harmful effects of oxidative stress.

Therefore, we aimed to determine and compare serum MDA levels in KC and control subjects in order to find evidence regarding the correlation between corneal degeneration and oxidative stress. Also, due to the fact that few studies report the MDA levels dynamics in KC development, we aimed to track dynamic changes in MDA levels in KC patients.

### Experimental part

#### Materials and methods

The study cohorts consisted in ten 1 months-KC diagnosed non-smoking subjects, recruited from Oftaprop Clinic Iasi, Romania and 10 healthy control age, smoking habits and gender-matched subjects with normal ophthalmological examination. Each subject agreed by written informed consent approved in the local ethics committee and all the procedures complied Declaration of Helsinki tenets. From some of the KC patients blood

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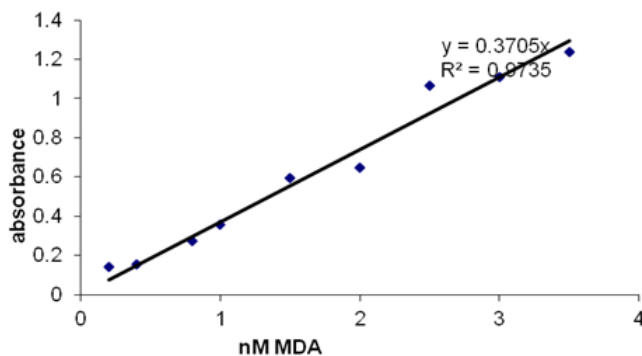


Fig. 1. Standard MDA curve

samples were also collected at 3 months, and 6 months of diagnosis.

All blood samples were collected *a jeun*, following standard procedures, allowed to clot, immediately centrifuged, and separated into Eppendorf vials. Lipid peroxidation was evaluated through the TBARS assay method: 200  $\mu$ L of serum was added in the reaction mix (1 mL of trichloroacetic acid at 50%, 0.9 mL of TRIS-HCl, pH = 7.4, and 1 mL of thiobarbituric acid 0.73%), vortexed. After a 20 min incubation (100°C), the samples were centrifuged at 3000 rpm for 10 min, and the supernatant was read at 532 nm. The signal was read against an MDA standard curve and the results were expressed as nmol/mL [13-17].

The MDA levels were statistically analysed by using one-way analysis of variance (ANOVA). All results are expressed as mean  $\pm$  SEM. F values for which  $p < 0.05$  were regarded as statistically significant.

## Results and discussions

In order to determine MDA levels, a standard malondialdehyde curve was performed (fig. 1). Also, MDA levels were read against a MDA standardized curve.

No significant gender and age differences between the control and KC groups were observed. Serum MDA levels found in KC patients were  $21.69 \pm 10.75$  nmol/mL, and in controls were  $15.41 \pm 3.06$  nmol/mL. Thus, it seems that MDA levels show a significant increase, as compared with controls ( $p < 0.05$ ).

Regarding the MDA levels dynamics in KC patients, we found that serum MDA concentrations exhibit a time-dependent increase. In this way, MDA levels were  $27.43 \pm 3.82$  nmol/mL at 1 month,  $26.97 \pm 0.83$  nmol/mL at 3 months, and  $18.00 \pm 4.62$  nmol/mL at 6 months from diagnosis. Thus, the time-dependent pattern means that MDA levels tend to decrease in time from diagnosis to chronicization. Interestingly enough, the pattern does not reach comparable level with the controls during our observation timeline.

In our study, we found no significant differences between female and male patients, and also no significant age-dependent variance was detected. It seems that in normal subjects, serum MDA may be age-dependent. In this way, Spittler [18] show that cell membrane structure changes occur in elders due to oxidative stress damaging effects. All the reactive oxygen species (ROS) found in high concentration in all old tissues equally contribute to tissue damage and destruction modulation. Furthermore, as a result of the high ROS activity lipid peroxidation is expected and observed in cell membrane structures impairments. However, it seems that no such differences can be made in our study cohorts since matched age and sex controls were selected.

Moreover, several studies report some gender and age differences in keratoconus patients. In this regard, Fink et al. [19] reported that women are more likely to exhibit ocular symptoms such as dryness, but they also reported that women declared more hours of near work per day. Conclusively, they reported that gender differences exist in patient history, vision, and ocular symptoms in keratoconus patients.

However, although the familial history is more frequent in women, no gender predilection for keratoconus was established. A possible hormonal influence on keratoconus was described by Gatziofufas et al. [20] showing that pregnancy could alter both keratoconus progression and thyroid gland dysfunction. This study was amongst the few that correlate keratoconus severity with endocrinological status [21]. Similarly, a more detailed study was carried by the Fink research group [23] showing that men are more likely to develop heavier corneal scarring, but no gender-dependent progression of scarring was shown. In the same study, they showed no significant differences regarding

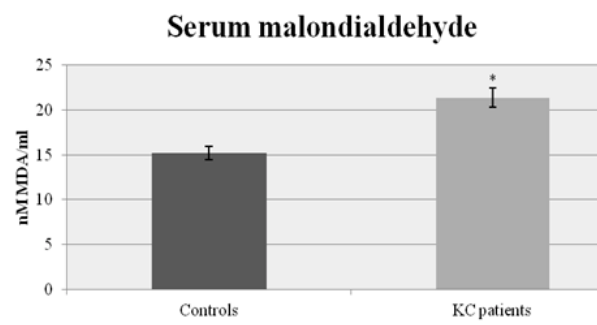


Fig. 2. Serum MDA levels in KC patients as compared to controls. The values are expressed as means  $\pm$  SEM. (n = 10 in control, and n = 10 in KC group) (\* $p < 0.005$ )

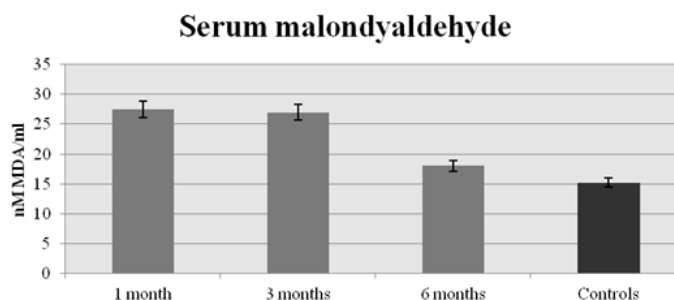


Fig. 3. Serum MDA levels dynamics in KC patients as compared to different moments in disease timeline and compared to controls

hormonal influence on keratoconus severity and progression. At the same time, it seems that normal aging could bring changes in tear film components and also in serum components levels [21].

Despite that, we found significant differences when comparing KC patients with healthy subjects. In this way, we found comparable changes in regarding oxidative stress and total antioxidant status [22] insinuating that several blood and humoral components are crucial in KC development. As a matter of fact, these changes may be also correlated with the MDA levels which we found increased in KC patients as compared with the healthy controls. Thus, it seems that lipid peroxidation may be associated with the harmful effects of the oxidative stress. Whereas total antioxidant status may be lower, the damaging effects of the uncontrolled ROS production may lead to lipid peroxidation which further causes cellular damage and corneal degeneration. Many studies show similar changes in inflammation markers [24, 25], collagen content and enzymatic activity [26]. Furthermore, it is now believed that some lipid peroxidation products are actively involved in inflammation modulation [27, 28]. Moreover, these results come to endorse the changes in total serum proteins among other parameters variation which we also found in our study (unpublished data).

Interestingly enough, almost all of the studies regarding these aspects analyse the changes occurred in tear film molecular component, but only few refer to blood components variation. In this way, we highlighted that even if important changes were shown in tear film components, serum molecular components levels, such as MDA levels, also exhibit modification patterns. Moreover, it seems that these changes occur in KC patients as a result of disease progression and could indicate further analysis on this track.

The fact that MDA levels increase in time may indicate alongside total oxidant status changes an inflammatory process which can lead to further need of investigation. However, it can be observed that the 6-months-KC patients MDA levels are still higher than the control subjects indicating that the molecular response tends to partially counter the lipid peroxidation processes during KC pathogenesis. In this way, serum inflammatory status markers, such as C reactive protein, immunoglobulin, or inflammatory complement system, may be screened. Since atopy and keratoconus were longly correlated [21], serum specific immunoglobulins levels may also be screened for changes or progression. Also, the correlation between the known lipid peroxidation products, inflammation pathways, and degenerative processes may further be analysed.

## Conclusions

Significant changes in serum malondialdehyde levels in KC patients as compared to healthy controls are shown by our results. Furthermore, we showed that MDA levels decrease in time, by comparing KC patients during the proposed observation timeline with age and sex-matched controls. Despite that, it seems that the serum MDA levels do not return to their normal levels as observed in control

patients. These changes could indicate that an inflammatory process may be involved in keratoconus, but further analysis is needed in this direction.

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