INFLAMMATORY MOLECULES IN THE TEARS OF PATIENTS WITH KERATOCONUS

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Presented in part at the 2003 Association for Research in Vision and Ophthalmology meeting, Fort Lauderdale, FL, USA.

The authors have no proprietary interest in any of the products mentioned in this article.

Running head: Inflammatory molecules in keratoconus

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Précis: Tears of patients with keratoconus contain high levels of IL-6, TNF-α and MMP-9, indicating that subtle inflammatory events may be an ingredient in the complex pathogenesis of keratoconus.

SUMMARY

Purpose: To determine levels of a panel of inflammatory molecules and matrix-metalloproteinases in the tears of patients with keratoconus.

Design: A prospective, case-controlled study.

Participants: 28 patients (one eye from each) diagnosed with keratoconus at the Instituto Galego de Oftalmoloxía, Santiago de Compostela, Spain, during the period from September 2001 to June 2002, and 20 normal controls subjects (one eye each) were studied.

Methods: Keratoconus patients were routinely examined and keratometric readings were taken to monitor the degree of ectasia. 15 μl of tears were collected by capillarity from each eye.

Main outcome measures: The concentrations of cytokines (IL-4, IL-6, IL-10 and TNF-α), cell adhesion molecules (ICAM-1, VCAM-1) and matrix-metalloproteinase-9 were measured by enzyme-linked immunoadsorbent assay.

Results: Keratoconus patients presented significantly higher levels of IL-6 (6.7 [4.8-10.8] pg/ml *vs.* 2.2 [1.0-4.1] pg/ml in control subjects ($p < 0.0001$)), TNF-α. (3.8 [2.9-14.4] pg/ml *vs.*1.8 [1.5-2.3] pg/ml in control subjects ($p < 0.0001$)) and MMP-9 (66.5 [49.2-139.3] ng/ml *vs* 6.1 [3.9-8.3] ng/ml in control subjects. The extent of the increase was found to be associated with the severity of keratoconus.

Conclusions: IL-6, TNF-α and MMP-9 are overexpressed in the tears of keratoconus patients indicating that the pathogenesis of keratoconus may involve chronic inflammatory events.

Keratoconus is a disorder characterized by a conical ectasia of the cornea in the absence of clinical signs of corneal inflammation. Typically, the pathology is characterized by a central or inferior corneal thinning with increased curvature at the apex of the cone.[1] The etiology of this disease is unknown, but it has been classically described as being associated with certain systemic diseases, such as atopy and connective tissue disorders.[2,3] In most cases, keratoconus occurs bilaterally, but asymmetrically.[4] It generally affects young adults and has an incidence of about 1:2000 of the general population.[5] Many cases progress slowly and gradually in severity, but the rate of progression and the length of time that keratoconus remains actively progressive vary considerably. The factors governing the progression and stabilization of keratoconus are currently unknown.

In recent years, extensive studies of the biochemical and pathological changes which occur at the structural and cellular levels of the cornea have been carried out.[6-8] Nevertheless, the specific mechanisms underlying the development of keratoconus and its relationship to heredity or environment are still not fully understood. Although there may be a relationship between keratoconus and several conditions of allergic etiology,[9,10] the influence of these conditions on the pathogenesis and natural history of keratoconus remains unclear. For example, atopy is associated with elevated levels of serum immunoglobulin E (IgE). Likewise in keratoconus patients, an increased incidence of elevated serum IgE has been reported.[11] An association between keratoconus and eye rubbing, particularly in patients with an associated allergic condition, has also been reported. It has been estimated that 66 to 73 % of keratoconus patients rub their eyes frequently.[4,10,12-14] Moreover, Zadnik et al found that many patients (53.9 %) who enrolled in the Collaborative Longitudinal Evaluation of keratoconus (CLEK) study reported a

history of allergy.[11] However, the significance of these observations remains to be clarified.

The cornea is part of an integrated system - the ocular surface - which contains specific and non-specific immune molecules. Tissue degradation in thinning disorders, such as keratoconus, involves the expression of inflammatory mediators, such as proinflammatory cytokines, cell adhesion molecules and matrix metalloproteinases.[16-19] With a view to contributing to our understanding of the factors which govern the etiology and development of keratoconus, we evaluated the levels of proinflammatory cytokines {interleukin-4(IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10) and the tumor necrosis factor alpha (TNF-α)}, cell adhesion molecules {intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1)} and matrix metalloproteinase-9 (MMP-9) in tears from control subjects and keratoconus patients.

MATERIALS AND METHODS

Subjects and Examinations

We have designed a prospective, case-controlled study, in which 28 keratoconus patients and 20 normal subjects were enrolled. Keratoconus patients had never worn contact lenses or had not worn lenses for a period of more than one year (52.3% males, mean age 22.4 ± 6.5 years). Normal subjects had never worn contact lenses (47.8% males, mean age 22.6 ± 6.6 years). We studied one eye from each patient, generally the right eye. Exclusion criteria included the existence of active inflammatory or infective systemic or ocular disease and current treatment with systemic or local anti-inflammatory drugs.

Keratoconus patients and normal subjects were recruited from the Contact Lens Unit, Instituto Galego de Oftalmoloxía (INGO), Complejo Hospitalario Universitario de Santiago de Compostela (CHUS), Spain, from September 2001 through June 2002. The protocol was approved by the Ethics Committee and informed consent was given by all patients. All examinations were performed by the same researcher (IL). Data collected included sex, age, the patient's ocular, medical history (allergy, eye rubbing) and family history. Careful attention was paid to evaluate the presence of a clinical history of atopy.

Ophthalmic examinations consisted of best-corrected visual acuity measurements, slit-lamp examination and corneal topography. Slit lamp biomicroscopy involved examination of the external adnexa and cornea (Vogt's striae, Fleischer's ring, keratoconic scars). An Eyesys unit (Corneal Analysis System CAS, Eyesys laboratories, Houston TX, USA) was used for corneal topography. Topographic data was evaluated by means of Rabinowitz criteria for the diagnosis of keratoconus; central corneal power, inferior-superior dioptric asymmetry and central corneal power differences between the two eyes.[5] The assessment of keratoconus progression was performed by using steep keratometric reading categories described in the CLEK Study Baseline.[15] The stage of keratoconus was graded as mild when the steepest keratometric reading (K_2) was < 45 diopters (D), moderate if K_2 was between 45 and 52 D and severe with K_2 >52 D. We considered K_2 the quantitative clinical variable to assess the severity of keratoconus.

Tear analysis

Tear samples were obtained by capillarity attraction, without nasal stimulation (the stimulation of reflex tearing was maintained at a minimum), or previous instillation of drugs or vital dyes. Anesthetic drops were not instilled. The samples were collected

atraumatically from the inferior meniscus (near the exterior canthus). Care was taken to avoid touching the corneal and conjunctival surface. We collected 15 μl of tear sample in micropipettes (Disposable Micro Sampling Pipettes, Corning, NY, USA) and placed them in microtubes (Micro Titertube natural 845-TP, TTE Laboratories, Hopkinton MA, USA). A new capillary tube was used for each tear sample. Within 1 hour of obtaining the samples, they were frozen and stored at - 70ºC.

The concentrations of cytokines (IL-4, IL-6, IL-10 and TNF-α) and cell adhesion molecules (ICAM-1, VCAM-1) in tear samples were measured with commercially available quantitative sandwich enzyme-linked immunoadsorbent assay kits (Quantikine, R&D Systems, Minneapolis, MN, USA) and the assays were performed in accordance with the supplier instructions. Matrix metalloproteinase-9 levels were determined by commercially available enzyme linked immunoadsorbent assay (ELISA) kits (Biotrack, Amersham Pharmacia Biotech, Buckinghamshire, UK). ELISAs were performed according to the manufacturer's instructions. All determinations were carried out without knowledge of the corresponding clinical data (blind test).

Statistical analysis

Descriptive statistical analyses were performed with percentage for categorical variables. Discontinuous variables were expressed as median [quartiles]. Graphic expressions were elaborated by Box and Whisker plots.

Statistical significance for intergroup differences was assessed by the χ^2 test for categorical variables. Inflammatory molecular markers values were not normally distributed (Kruskal-Wallis analysis). The Mann-Whitney test was performed for

comparison between groups. The Spearman correlation coefficient was used to analyze the statistical significance between keratometric readings (K_2) and the concentrations of molecular markers. A multiple linear regression analysis was performed using both dependent (K_2) and independent variables (IL-6, TNF-α, MMP-9). A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Clinical Features

No age- or sex-related statistical differences were detected between keratoconus patients and control subjects. The duration of patient-reported keratoconus varied from 1 to 16 years (mean 7.0 ± 6.2 years). Only one patient (5%) from the control group was diagnosed as having allergic disease while 16 patients (57.1%) from the keratoconus group had experienced at least one allergic episode. Eighteen keratoconus patients (64.3%) admitted frequent and vigorous eye rubbing; no control subject presented this behavior. Eight keratoconus patients (28.5%) had a familiar history of keratoconus.

Slit-lamp biomicroscopy of the cornea revealed keratoconus characteristics (Vogt's striae, a Fleischer ring of at least 2 mm arc or corneal scarring characteristic of keratoconus) in 23 keratoconus eyes (82.1%). Six eyes (21.4%) presented mild keratoconus ($K_2 < 45$ D), 14 eyes (50%) had moderate keratoconus (K_2 between 45 D and 52 D), and severe keratoconus ($K_2 > 52$ D) was diagnosed in eight eyes (28.6%).

Inflammatory mediators

Levels of inflammatory molecules in control and keratoconus tears are shown in Table 1. Mean values of IL-4 and IL-10 were similar in control and keratoconus samples. However, keratoconus patients presented significantly higher levels of both IL-6 (6.7 [4.8-10.8] pg/ml *vs.* 2.2 [1.0-4.1] pg/ml in control subjects ($p < 0.0001$)) and TNF-α (3.8 [2.9-14.4] pg/ml *vs.* 1.8 [1.5-2.3] pg/ml in control subjects ($p < 0.0001$)).

As illustrated in Table 1, no significant differences were found in the concentrations of ICAM-1 and VCAM-1 in keratoconus patients and control subjects. In contrast, increased values of matrix metalloproteinase-9 (MMP-9) were found in keratoconus (66.5 [49.2-139.3] ng/ml) *vs.* control subjects (6.1 [3.9-8.3] ng/ml). This difference was very statistically significant ($p<0.0001$).

In this study, we did not find a relationship between patients with and without a clinical history of allergy as mean levels of upregulated inflammatory markers were found to be similar in both groups ($p = 0.732$ for IL-6; $p = 0.767$ for TNF-α and $p = 0.760$ for MMP-9). A correlation between eye rubbing and different concentrations of these markers was not found either (IL-6, $p = 0.395$; TNF-α $p=0.978$; MMP-9, $p=0.371$).

The association between increased levels of inflammatory markers and the severity of keratoconus (mild, moderate or severe) was also analyzed. As indicated in Fig. 1, higher concentrations of cytokines (IL-6, TNF-α) and MMP-9 were associated with severe keratoconus rather than with the mild or moderate stages ($p<0.001$). Differences between the mild and moderate stages were not statistically significant.

A significant correlation was found between the concentration of IL-6, TNF-α and MMP-9 in keratoconus tears and the steep keratometric reading (K_2) by simple regression analysis. The Spearman coefficient associated with the concentration of IL-6 and K_2 was 0.570 (p=0.002); that for TNF-α was 0.474 (p=0.011) and that for MMP-9 was 0.796 (p<0,0001). (see Fig. 2).

As shown in Table 2, when all the inflammatory molecules were included in a multiple regression analysis, MMP-9 was found to be the only single, independent variable associated with the degree of keratoconus.

DISCUSSION

Despite extensive basic and clinical studies of keratoconus in recent years, the precise mechanisms underlying this pathology still remain largely unknown. The majority of these studies employed corneas which had been obtained from transplants and erroneous conclusions have been drawn from a number of these studies.[20] Moreover, it has been shown that the reported increased levels of TNF-α and IL-1 are not specific to keratoconus, but are present in other pathologies of the cornea as well.[8] Nevertheless, despite contradictory reports,[21] it is becoming clear that mediators of inflammation are

present in the keratoconus cornea.[18,19,22,23] This finding contrasts with the generally accepted idea that keratoconus is a corneal ectasia which does not involve inflammation.[5]

Studies of tears are limited by methodological difficulties and by results which can be inconsistent and transitory. Nevertheless, events which take place on the ocular surface are likely to be reflected in the composition of tears. Thus, the presence of collagen degradation products has been reported in the tears of keratoconus patients.[24] In the present study, we demonstrate that tears, which are a part of the corneal surface, contain detectable levels of cytokines, adhesion molecules and matrix metalloproteinases. Moreover, the levels of some of studied cytokines (IL-6 and TNF-α) are significantly increased in the tears of patients with keratoconus. To our knowledge, this is the first study supporting an important increase of proinflammatory markers in tear film of keratoconus patients. Increased levels of MMP-9 in the tears of keratoconus patients have also been reported by others in studies of keratoconus and atopia.[22] Discrepancies between the present study and other published reports may be due to the fact that different data were analyzed and different methodologies (tear sample collection) were employed.

The lacrimal gland is the principal effector in the secretory immune system of the eye and plays a critical role in protecting the eye against allergic, inflammatory and infectious diseases.[16] The preocular tear film contains numerous specific and non-specific immune components. These include cytokines and cell adhesion molecules.[23,25] The increased levels of these molecules may be a consequence of increased secretion from the corneal epithelium or from another non-corneal cell type. Clarifying this issue would contribute significantly to determining the origin of the disease. Various cell types, including keratocytes, produce IL-6 in response to stimulation by IL-1 or TNF-α.[26] It is thus

conceivable that the tear may act as a reservoir of cytokines produced by the stroma and corneal or conjunctival epithelium; alternatively, it may act as a vehicle of cytokines produced by the lacrimal gland or other epithelia of the ocular surface.

In recent years, numerous clinical studies of keratoconus support the idea that its pathogenesis involves an inflammatory component. A variety of changes in the ocular surface of keratoconus patients have been found, including reduced corneal sensitivity, increased fluorescein and rose bengal staining scores and abnormal impression cytology such as squamous metaplasia and lower globet cell density.[27] In the same way, increased enzymatic activity has been demonstrated in the conjunctiva of keratoconus patients.[28,29] Despite the fact that the origin of these changes is not completely understood, Dogru et al have suggested recently that keratoconus may have an epithelial origin,[27] which would explain the reincident cases of keratoconus following keratoplasty. Overall, it cannot be ruled out that keratoconus originates in events which take place outside the cornea but which are ultimately responsible for the induction of its ectasia.

The association between keratoconus and allergy,[9-13] as well as the role played by eye rubbing in the development of ectasia are well established.[4,10,14] Eye rubbing may well contribute to the development of keratoconus by activating inflammatory mediators,[30] more so than by the physical pressure applied to the eyeball. Indeed, IL-1 has been implicated as a mediator of keratoconus in eye rubbing patients.[31] The present study included numerous patients who reported previous episodes of allergy and frequent eye rubbing. However, we did not find an association between either of these two parameters and the increased levels of inflammatory molecules in tears. It is conceivable that the concentration of interleukines increases only during episodes of itching and eye rubbing.

The tear may be a vehicle of some of the pathogenic protagonists of keratoconus, such as IL-6, TNF-α or MMP-9. Increased levels of these molecules may be sporadic, but sufficient to provoke slowly progressive ectasia.[20] Our results indicate that the concentration of inflammatory molecules in tears is associated with the intensity of keratoconus; however, a similar association with the progression of ectasia was not determined. Nevertheless, the presence of different classes of enzymes which play a role in the pathological process is not necessarily accompanied by an immediate presentation of corresponding clinical manifestations.[19] It is likely that keratoconus is a disease with a multivariable origin, in which corneal ectasia results from the degradation of stromal collagen. The results of the present study support this hypothesis and indicate that these processes may be accompanied by chronic inflammatory events. This would explain the findings of many studies which have reported the participation of all the layers of the cornea as well as other structures of the ocular surface in keratoconus.[8,27-29,32]

Thinning and ectasia of the cornea are suggestive of a degraded extracellular matrix. Many studies have described changes in corneas with keratoconus but only a few have studied the tear fluid of these patients. The small volume of the tear sample, the different methods employed and the possibility of modifying real parameters, may explain the different results reported in various studies. However, it has become clear by now that inflammatory events do take place in keratoconus. The present study reveals that high levels of some cytokines (IL-6 and TNF-α) and MMP-9 are associated with advanced keratoconus, but the precise role of each of these molecular players still needs to be defined. The pathogenesis of keratoconus seems to be multifactorial and governed by genetic, biological and biomechanical bases. It can also be concluded from our

current data that keratoconus cannot be defined any more as a non-inflammatory disorder.

Acknowledgements

The authors would like to express their thanks to ACTS (Academic Consulting and Translating Services http://www.euskalnet.net/acts) for having corrected the English of this paper.

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TABLES

TABLE 1: LEVELS OF INFLAMMATORY MOLECULES IN TEARS

MOLECULE	CONTROL	KERATOCONUS	P
Cytokines			
IL-4 (pg/mL)	6.1 [4.7 – 8.2]	6.5 [4.6 – 8.3]	0.975
IL-6 (pg/mL)	2.2 [1.0 – 4.1]	6.7 [4.8 – 10.8]	<0.0001
IL-10 (pg/mL)	1.7 [1.1 – 3.0]	2.0 [1.5 – 3.1]	0.315
TNF-α (pg/mL)	1.8 [1.5 – 2.3]	3.8 [2.9 – 14.4]	<0.0001
Adhesion molecules			
ICAM-1 (ng/mL)	8.9 [6.5 – 10.9]	8.8 [5.1 – 13.3]	0.950
VCAM-1 (ng/mL)	29.6 [25.3 – 36.0]	30.6 [23.2 – 38.4]	0.778
Matrix metalloproteinases			
MMP-9 (ng/mL)	6.1 [3.9 – 8.3]	66.5 [49.2 – 139.3]	<0.0001

Values are median [quartiles]

TABLE 2: MULTIPLE LINEAR REGRESSION ANALYSIS

Dependent variable: K_2

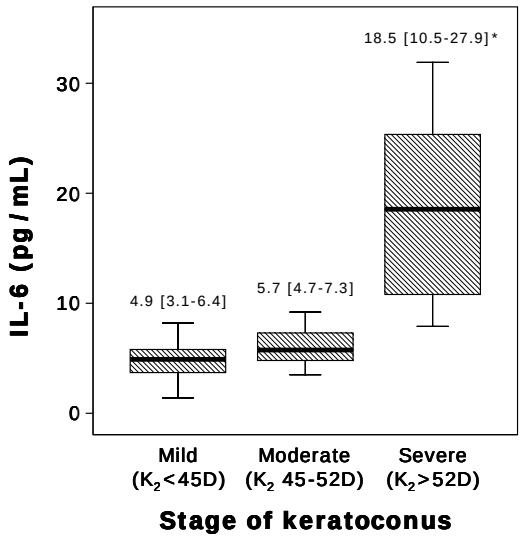
Independent variables	β	CI 95%	p
IL-6	0.25	-0.05 / 0.56	0.099
TNF-α	-0.02	-0.26 / 0.22	0.848
MMP-9	0.07	0.01 / 0.13	0.012

β = beta coefficient of linear regression

LEGENDS

Figure 1 Concentrations of inflammatory molecules in different degrees of ketatoconus. *p<0.001 in relation the other groups. 1a: IL-6 (interleukin-6); 1b: TNF-α (tumoral necrosis factor alpha); 1c: MMP-9 (metalloproteinase 9).

Figure 2 Correlation between the concentration of inflammatory molecules and the steep keratometric reading (K_2). 1a: IL-6 (interleukin-6); 1b: TNF-α (tumoral necrosis factor alpha); 1c: MMP-9 (metalloproteinase 9).



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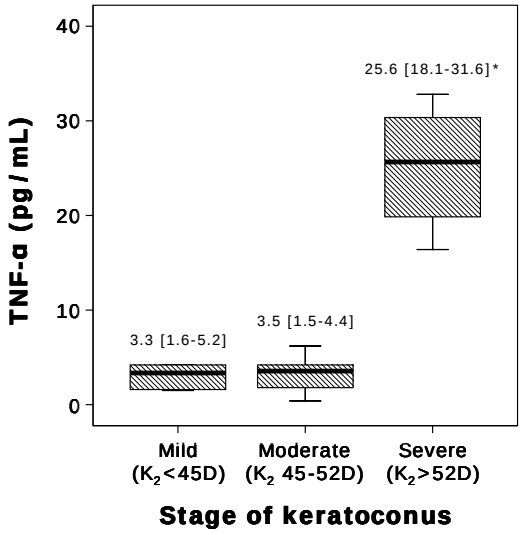
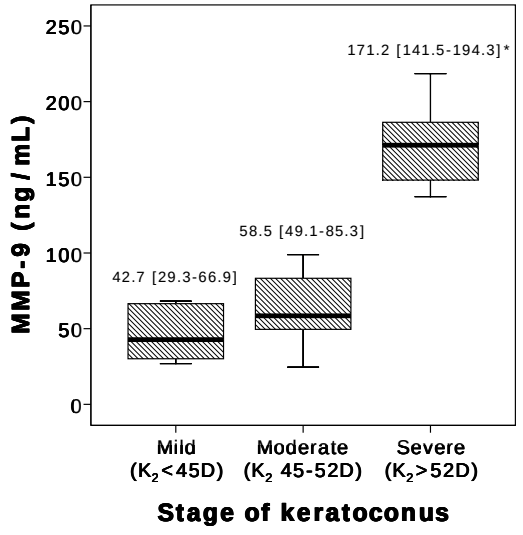
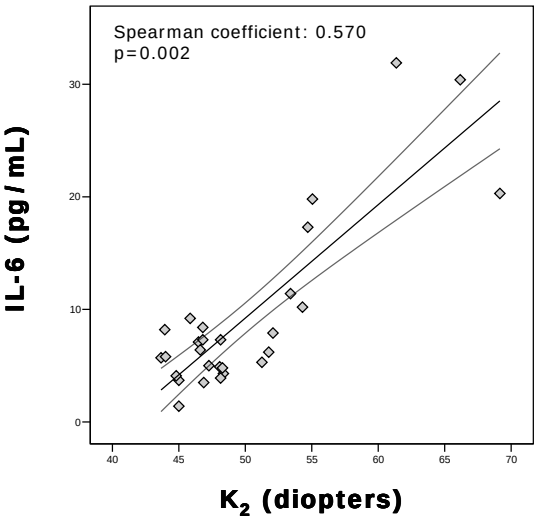


1b



1c

Figure 1



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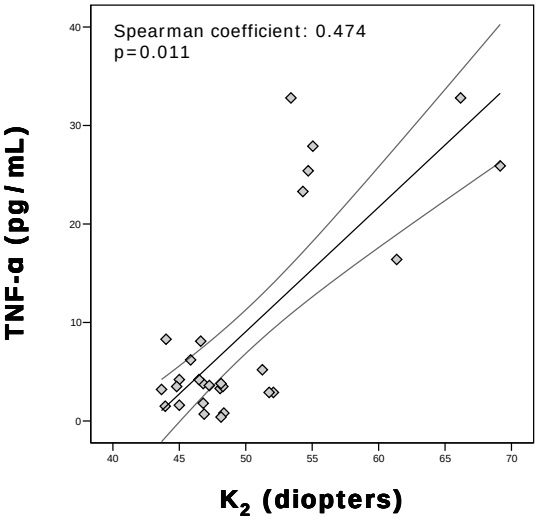

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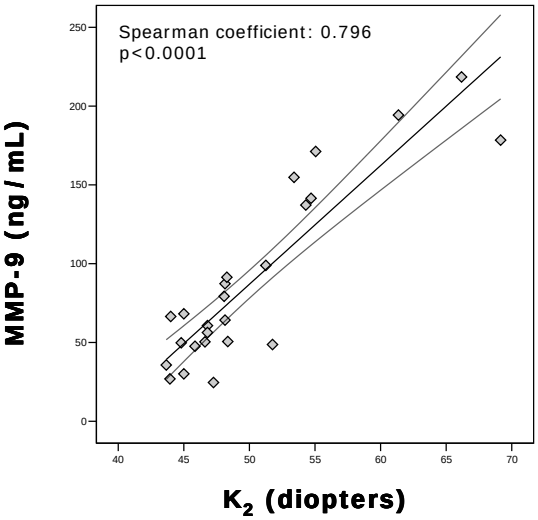

2c

Figure 2