Ultrastructural study of peripheral and central stroma of keratoconus cornea

Aljoharah Alkanaan,1 Robert Barsotti,2 Omar Kirat,3 Turki Almubrad,1 Adnan Khan,1 Saeed Akhtar1

ABSTRACT
Purpose Assess the lamellar organisation of the peripheral and central stroma of the keratoconus (KC) and normal cornea.
Methods Five normal and three KC corneas were fixed in 2.5% glutaraldehyde and processed for electron microscopy. The ultrathin sections were observed under JEOL 1400 TEM, and digital images were taken with a bottom-mounted 11-megapixel Quamisa camera, using the iTEM software. Measurements of the lamellae were carried out using the iTEM software. Statistical analysis was performed using the SPSS software.
Results The lamellar organisation at the centre and periphery of the KC cornea was disrupted by the presence of multiple undulations, which were more aggressive at the posterior stroma. Among the KC cornea, the mean lamellar thickness of the peripheral middle (1030.32±86.25 nm) and posterior (615.68±30.94 nm) stroma was also significantly (p<0.05) thinner than their corresponding areas of the central KC cornea (1151.1±48 nm; 783.57±31.10 nm). At the periphery of KC cornea, just above the Descemet’s membrane (DM), small undulations appeared to emerge out from the DM. Furthermore, the anterior stroma of the peripheral cornea contained several lamellar sutures. The mean lamellar thickness of the peripheral and central KC cornea was significantly (p<0.0001) thinner than the corresponding areas of the normal cornea.
Conclusion The present study reveals the involvement of lamellae in the peripheral stroma in the pathogenicity of the KC cornea. The emergence of small undulations in the DM suggests that the formation of undulation might be starting from the DM.

INTRODUCTION
Keratoconus (KC) is the most common ectatic corneal disorder, with an incidence ranging from 2 to 22.3 per 100 000 population.1–2 KC is a progressive bilateral asymmetrical corneal disorder characterised by localised corneal thinning and conical protrusion, leading to high myopia, irregular astigmatism, corneal scarring and visual impairment.3 Ultrastructural studies on the KC cornea have revealed a loose interconnectivity in the anterior stroma, the presence of multiple undulations in the lamellar outline and disruption in the organisation of collagen fibrils (CFs) and proteoglycans (PGs).4–8
Previous studies have focused on the central area of the KC cornea, which is the area of the cone, leaving the peripheral area understudied. Mathew et al9 conducted a study to investigate the histopathological changes in the anterior cornea at both the apex of the cone and the adjacent mid-peripheral corneal region. Interestingly, they have reported pathological changes in the mid-peripheral area of the KC cornea that are similar, but to a lesser extent, than that in the conical area. These changes include variation in epithelial thickness and size, thinning and ruptures in Bowman’s layer (BW) and occasional kinks in the lamellar outline. Peripheral involvement was also investigated clinically by Brautaset et al10 by measuring corneal thickness in the peripheral and central corneal regions of patients with KC. The results of their study show that corneal thickness in patients with KC was significantly less than that of normal controls in all corneal regions. These findings provide clinical evidence that the peripheral cornea is also affected by the disease process.

Moreover, frequent reports of KC reoccurring in an originally normal graft11–12 have raised the question of peripheral involvement in the disease process. Bergmanson et al12 suggested that this might be due to the presence of subclinical KC in the graft, which progressed and manifested after the surgery rather than an actual recurrence of KC. Nevertheless, this does not fully eliminate the possibility of peripheral involvement and migration from the host tissue to the donor’s graft.

The earlier clinical and laboratory studies together with reports of KC recurrence drove us to consider the possibility of peripheral involvement of the corneal stroma in the disease process. We hypothesise that changes in the CFs and PGs in the peripheral stroma initiate the pathogenicity of KC cornea, leading to alteration and slippage of the lamellar organisation. Due to slippage of the lamellae, the pathogenicity spread from the peripheral stroma towards the central part of the stroma of the KC cornea, causing the cone formation. We conducted the current study to assess the lamellar organisation of the peripheral and central parts of the KC cornea and compare it with that of the normal cornea.

METHODS
Tissue procurement and use adhered to the tenets of the Declaration of Helsinki and local ethical regulations. The study was approved by the ethical committees in both King Saud University and King Khaled Eye Specialist Hospital, Riyadh, Saudi Arabia.

Five normal and three KC corneas were used in this study (table 1). The normal and KC corneas were fixed in 2.5% glutaraldehyde in 0.1 M
phosphate buffer at pH 7.0 for 2 hours, then washed three times in 0.1 M phosphate-buffered (15 min × 3). Tissues were post-fixed in 1% osmium tetroxide for 1 hour, then washed thrice (15 min × 3) with distilled water and serially dehydrated in 30%, 50%, 70%, 90% and 100% ethanol for 30 min in each case. Tissues were infiltrated in three 1-hour changes in a mixture of 100% ethanol and Spurr resin, and in 1-hour Spurr resin, then 8-hour Spurr resin. The samples were polymerised in Spurr resin at 70°C for 8 hours.

The blocks were then sectioned with an RMC ultracut microtome (Reichert-Jung ultracut microtome) to acquire ultrathin (75 nm) cross-sections. Ultrathin sections were then collected on 200 mesh copper grids and stained with 2% uranyl acetate (10 min) and lead citrate (10 min), then observed using the transmission electron microscopy JEOL 1400 transmission electron microscope (TEM) (JEOL, Akishima, Japan). Digital images of the lamellar organisation were captured using a bottom-mounted Quamisa camera and iTEM Soft Imaging System (Soft Imaging System, Münster, Germany). To analyse the lamellar thickness, three digital images were taken from the lamellae of the anterior, middle and posterior stroma. The iTEM analysis program was used to measure the lamellar thickness.

Data were exported on to Excel spreadsheets from the imaging software and then analysed using the SPSS statistical software Version 18. The mean lamellar thickness of the anterior, middle and posterior stroma at the centre and periphery of the normal and KC cornea was calculated and then compared using the Mann-Whitney and Wilcoxon tests.

**RESULTS**

**Lamellar organisation in the normal cornea**

In the normal human cornea, the lamellae ran parallel to the corneal surface and were laid in an orthogonal arrangement. In the central part of the cornea, the lamellae of the anterior stroma were thin (1065.03 nm, n=143), tightly packed and were interlacing each other (figure 1A). The lamellae in the middle and posterior stroma were thicker (2289.28 nm, n=174 and 2050.86 nm, n=107, respectively) and laid in layers parallel to each other (figure 1B). In the periphery, the lamellar organisation is similar to that in the centre, except that the lamellar interlacing extended deeper into the mid-stroma and the overall lamellar thickness increased. The CFs within each lamella also ran parallel to each other and were highly organised with a regular diameter and inter fibrillar spacing.

**Lamellar organisation in the KC cornea**

The lamellar organisation in the KC cornea was degenerated and disoriented in the central as well as in the peripheral part of the cornea. At the subepithelial region of the central part, the BW and basement membrane were degenerated and the lamellae were adjacent to the basal epithelial cells (figure 1C). We observed variable changes in the epithelial basement membrane that ranged from marked thickening to complete degeneration (figure 1C). There were several localised breaks of BW in which the basal epithelial cell layer appears to be resting directly over the anterior stromal lamellae (figure 1D). Just below the BW, the lamellae at the central part of the KC cornea were loosely interlaced and the CFs within these lamellae were running randomly (figure 1E). The undulations were rarely seen in the anterior stroma but more frequently in the deeper layers of the posterior stroma (figure 1F,G). The keratocytes between the lamellae were crushed due to the severity of the undulations of these lamellae in the middle and posterior stroma (figure 1G). The undulations in the posterior were also observed just above the Descemet’s membrane (DM) in the central part of the KC cornea (figure 1H).

The peripheral cornea also had undulating lamellae similar to the central cornea. The lamellae in the anterior stroma were thin and contained disoriented CF, which were surrounded by electron-dense material (figure 2A). The presence of the undulations was also limited to the middle and posterior lamellae (figure 2B). In the anterior stroma, some lamellae were running almost at a 90° angle to the corneal surface forming sutures (figure 2C). These sutures appeared to be branching out from the horizontal-running undulated lamellae (figure 2D). The basal part of these sutures was embedded in the disorganised undulating lamellae and surrounded by patches of electron-dense granular material and disoriented CF (figure 2D). The lamellae of the posterior stroma were thick but undulated with disorganised CF (figure 2E). These posterior lamellae also contained sutures (figure 2F). At the interface of the posterior stroma and DM, the origin of the undulations (white arrowheads) of the CF was observed (figure 2G). Later on, these CFs are fully developed in the form of undulating lamellae (figure 2H).

**Lamellar thickness analysis**

The mean lamellar thickness between corresponding stromal zones within the same cornea and between KC and normal corneas is displayed in tables 2 and 3. A comparison between the normal and KC cornea revealed that the central KC lamellae of the anterior (600.84 nm, n=233), middle (1151.1 nm, n=204) and posterior (783.57 nm, n=240) stroma were significantly (p<0.0001) smaller than the mean lamellar thickness of the anterior (1065.03 nm, n=143), middle (2289.28 nm, n=174) and posterior (2050.86 nm, n=107) stroma of the normal cornea (table 2). The mean lamellar thickness of the anterior (686.84 nm, n=174), middle (1030.3 nm, n=153) and posterior (615.68 nm, n=225) stroma of the peripheral KC cornea was also significantly (p<0.0001) smaller than the mean lamellar thickness of the anterior (1168 nm, n=130), middle (2244.95 nm, n=113) and posterior (2264.88 nm, n=93) stroma of the peripheral part of the normal cornea (table 2).

Within the KC cornea, the mean lamellar thickness of the peripheral middle (1030.3 nm, n=133) and posterior (615.68 nm, n=225) stroma was also significantly thinner (p<0.05) than that of the central middle (1151.1 nm, n=204) and posterior (783.57 nm, n=240) stroma. However, the mean lamellar thickness of the anterior stroma did not differ significantly between the peripheral and central parts of the KC cornea (p=0.538) (table 3). Nonetheless, the number of lamellae in the peripheral part (375) was higher compared with the number of lamellae in the central part of the KC cornea (254).

**Table 1** Details of the normal and keratoconus (KC) specimen used in the present study

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Age</th>
<th>Gender</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₁</td>
<td>26</td>
<td>Male</td>
<td>Eye bank</td>
</tr>
<tr>
<td>N₂</td>
<td>25</td>
<td>Male</td>
<td>Eye bank</td>
</tr>
<tr>
<td>N₃</td>
<td>65</td>
<td>Male</td>
<td>Eye bank</td>
</tr>
<tr>
<td>KC₁</td>
<td>24</td>
<td>Female</td>
<td>Right eye, advanced KC, penetrating keratoplasty (PK), fixed in glutaraldehyde within 30 min</td>
</tr>
<tr>
<td>KC₂</td>
<td>26</td>
<td>Male</td>
<td>Left eye, advanced KC, PK, fixed in glutaraldehyde within 30 min</td>
</tr>
<tr>
<td>KC₃</td>
<td>23</td>
<td>Male</td>
<td>Left eye, advanced KC, PK, fixed in glutaraldehyde within 30 min</td>
</tr>
</tbody>
</table>

Figure 1  Electron micrographs of the central part of a normal cornea and keratoconus (KC) cornea. (A) Part of the anterior stroma in the normal cornea showing tight interlacing (white arrowheads) of the lamellae (L). (B) Part of the posterior stroma in the normal cornea showing parallel running lamellae (L). A keratocyte (KR) is lying flat between the parallel lamellar layers in the posterior stroma. (C) Part of the subepithelial region of KC cornea showing localised absence of basement membrane and Bowman’s layer, with the epithelial cell (EP) resting on the lamellae (L). A keratocyte (KR) with prominent nucleus (N) was present between lamellae. (D) Part of the subepithelial region (EP) of KC cornea showing a break in the Bowman’s layer (BW) resulting in a direct contact of basement membrane (BM) with anterior stroma (AS). (E) Part of the anterior stroma of KC cornea showing loose interlacing (white arrowhead) and severely disoriented collagen fibrils (black arrowheads) with patches of electron-dense granular material (*), below the Bowman’s layer (BW). Part of a keratocyte (KR) appears between the interlacing lamellae. (F) Undulating lamellae (U) in the anterior stroma of KC cornea crushing a keratocyte (KR). Please note the crease (C) in the Bowman’s layer (BW). (G) Part of the posterior stroma of KC cornea showing a keratocyte (KR) crushed between the severely undulated lamellae (U). (H) Part of the interface of the posterior stroma and Descemet’s membrane (DM) in KC cornea showing the presence of the undulations (U) just above the DM.
Figure 2  Electron micrographs showing the lamellar organisation in the periphery of keratoconus cornea. (A) Thin lamellae (L) in the anterior stroma with slightly disorientated fibrils (black arrowheads) surrounded by electron-dense granular material (*). (B) Part of the middle stromal showing undulations (U) in the lamellae and disorientation of the collagen fibrils (CFs; denoted by arrowheads). (C) Part of the anterior stroma showing suture (SU) running across the lamellar (L) at the anterior stroma. Part of a large keratocyte (KR) with prominent nucleus (N) was present between lamellae. (D) High magnification of the basal part of the suture shown in (C), showing the presence of the electron-dense granular material (*) around the disorganised CFs (black arrowhead). Notice the CFs (white arrowhead) in the middle part of the suture similar to the CFs of lamellae. (E) Part of the posterior stroma showing the undulation (U) lamellae and disorganisation of the CFs (black arrowheads). (F) Part of posterior stroma showing the presence of suture (white arrowhead) in between lamellae. Notice how the suture appears branching from a lamella with disorganised fibrils (black arrowheads) and patches of electron-dense granular material (*). (G,H) Origin of the undulations (white arrowheads) at the electron lucent (LU) interface of the posterior stroma and Descemet's membrane (DM). The lucent space was above the electron-dense banded Descemet's membrane (BDM). Please note that the CFs are fully developed in the form of undulating lamellae (black arrowhead). The homogeneous Descemet's membrane (HDM) was present below the BDM.
DISCUSSION

Our observations of the KC cornea revealed that the lamellae at the central part of the KC cornea were loosely interlaced in the anterior part of the stroma, whereas in the middle and posterior stroma run parallel to each other. The CFs within these lamellae were running randomly. Similar but less prominent changes were also observed at the peripheral area of KC cornea. The lamellar outline was disrupted by the presence of multiple undulations throughout the stroma both in the central and peripheral parts of the cornea. In the central part of the cornea, the undulations were occasionally seen in the anterior stroma but more frequently in the deeper layers of the posterior stroma, whereas in the peripheral stroma the presence of the undulations was limited to the middle and posterior layers. The observed generalised thinning of lamellae in the centre and peripheral region correlates well with the previously reported corneal thinning.\(^3\)\(^,\)\(^,\)\(^,\)\(^,\)\(^,\)\(^,\)\(^,\)\(^5\)\(^,\)\(^13\)\(^,\)\(^16\) Furthermore, the thickness of the lamellae in the middle and posterior stroma of the peripheral KC was markedly thinner than those in the central part of KC cornea. The thinning of the lamellae in the posterior stroma might be related to the disorder in the synthesis of the CF and PGs, which results in biomechanical weakness of the lamellae. It is presumed that these biomechanically weak and thin lamellae were prone (susceptible) to undulation.

An interesting finding in our sample was the presence of lamellae running at a vertical angle (suture) to the corneal surface at the periphery of the KC cornea. Under high magnifications, these lamellae had the same collagenous structure as neighbouring lamellae, and they appeared to be branching from the original horizontal-running lamellae. In orientation and structure, these lamellae resemble the sutures typically found in the corneal stroma of Elasmobranchii and salamanderfish.\(^5\)\(^,\)\(^7\)\(^–\)\(^9\) It is believed that sutures stabilise the corneal shape and prevent corneal swelling in these species. The observation of such sutures in our sample raises the question of their involvement in corneal shape support. Their observation in the periphery and not the centre, where the cornea is much more vulnerable to shape alteration, is another question that requires further investigation.

It is documented that the interconnectivity of the lamellae in the anterior stroma provides biomechanical strength to the cornea and regulates the curvature of the cornea.\(^5\)\(^,\)\(^12\)\(^,\)\(^13\)\(^,\)\(^14\) The interlamellar bonds physically link the entire adjacent lamellae of the entire corneal stroma, and this provides the mechanical strength. These physical interactions could occur between separate CFs both within an individual lamella and in adjacent lamellae.\(^12\)\(^,\)\(^13\) We believe that due to loss of interconnectivity of the lamellae in the anterior stroma, the biomechanical strength and stability of the cornea degenerated. This induces high pressure on the BW and eventually causes the breaks. The breaks in the BW are a common KC feature that has been observed in electron microscope and biochemical studies.\(^6\)\(^,\)\(^8\)\(^,\)\(^9\)\(^,\)\(^13\)\(^,\)\(^14\) Akhtar et al have reported the presence of BW breaks and basement membrane thickening only in severe KC corneas but not in mild cases. Accordingly, they suggested that these changes are reactive to the disease process. In our observation these alterations were exhibited only within the central area of the KC cornea where the cone protrudes, which may indicate that changes in the anterior cornea are secondary to the development of the cone.

The lamellar disorganisation in the anterior stroma, randomly running CFs and the presence of undulations have been described by previous investigators.\(^6\)\(^,\)\(^8\) The involvement of peripheral alteration described above in our study was not documented in previous studies. The changes in the architecture of the peripheral KC cornea in our study suggested its involvement in the pathogenicity of the disease. The presence of the undulating lamellae in the middle and posterior stroma but not in the anterior stroma of the mild KC cornea has been reported by Akhtar et al, who suggest the initiation of lamellar undulation from the posterior stroma rather than from the anterior stroma. Our observation showed the formation of undulation just above the DM in the periphery. An elevation emerged from the electron-dense material present just above the banded DM, and this electron-dense elevation was beginning to be transformed into CFs. Further development of the CFs around it results in the formation of undulation of the lamellae. We believe that alterations in the lamellar outline initiate from the DM and progress

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Table 2 Comparison of lamellar thickness of anterior, middle and posterior stroma between KC and normal corneas

<table>
<thead>
<tr>
<th>Stromal zone</th>
<th>Corneal area</th>
<th>KC (n=sample size)</th>
<th>Normal (n=sample size)</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lamellae thickness (nm) (mean±SE)</td>
<td>Lamellae thickness (nm) (mean±SE)</td>
<td></td>
</tr>
<tr>
<td>Anterior stroma</td>
<td>Centre</td>
<td>600.8±25.10 (n=233)</td>
<td>1065.0±50.40 nm (n=143)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Periphery</td>
<td>686.8±46.47 (n=374)</td>
<td>1168±62.03 nm (n=130)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Middle stroma</td>
<td>Centre</td>
<td>1151.1±65.48 (n=204)</td>
<td>2289.2±116.83 nm (n=114)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Periphery</td>
<td>1030.3±86.25 (n=153)</td>
<td>2244.9±106.79 nm (n=113)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Posterior stroma</td>
<td>Centre</td>
<td>782.5±21.10 (n=240)</td>
<td>2050.8±124.30 nm (n=107)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Periphery</td>
<td>615.6±30.94 (n=225)</td>
<td>2264.8±101.04 nm (n=93)</td>
<td>&lt;0.0001</td>
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<tr>
<td>Mean lamellar</td>
<td>Centre</td>
<td>828.7±25.75 (n=677)</td>
<td>1738.2±62.28 nm (n=364)</td>
<td>0.0001</td>
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<tr>
<td></td>
<td>Periphery</td>
<td>752.7±31.978 (n=552)</td>
<td>1834.0±58.97 nm (n=336)</td>
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</tr>
</tbody>
</table>

* Mann-Whitney test.

KC, keratoconus; n, number of lamellae; SE: standard error.

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Table 3 Comparison of lamellar thickness of anterior, middle and posterior stroma between the central and peripheral areas of the same cornea

<table>
<thead>
<tr>
<th>Stromal zone</th>
<th>Corneal area</th>
<th>Centre (n=sample size)</th>
<th>Periphery (n=sample size)</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lamellae thickness (nm) (mean±SE)</td>
<td>Lamellae thickness (nm) (mean±SE)</td>
<td></td>
</tr>
<tr>
<td>Anterior stroma</td>
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<td>600.8±25.10 (n=233)</td>
<td>686.8±46.47 (n=174)</td>
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<td>1065.0±50.40 nm (n=143)</td>
<td>1168±62.03 nm (n=130)</td>
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<td>Middle stroma</td>
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<tr>
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<td>2289.2±116.83 nm (n=114)</td>
<td>2244.9±106.79 nm (n=113)</td>
<td>0.822</td>
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<tr>
<td>Posterior stroma</td>
<td>KC</td>
<td>783.5±31.10 (n=240)</td>
<td>615.6±30.94 (n=225)</td>
<td>&lt;0.0001</td>
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<tr>
<td></td>
<td>Normal</td>
<td>2050.8±124.30 nm (n=107)</td>
<td>2264.8±101.04 nm (n=93)</td>
<td>0.02</td>
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<tr>
<td>Mean lamellar</td>
<td>KC</td>
<td>828.7±25.75 (n=677)</td>
<td>752.7±31.978 (n=552)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>1738.2±62.28 nm (n=364)</td>
<td>1834.0±58.97 nm (n=336)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Wilcoxon Test.

KC, keratoconus; n, number of lamellae; SE: standard error.
from the posterior stroma towards the anterior stroma. A gradual increase in the aggressiveness and number of undulations in the posterior stroma further support our hypothesis that the disease starts in the posterior stroma and progresses anteriorly.

Our present study has shown the changes in the peripheral part of the stroma and indicated that the thickness and number of lamellae was higher in the peripheral region than in the central part of the KC cornea. The presence of thicker and higher numbers of lamellae connected by sutures suggests that the peripheral anterior stroma had more biomechanical strength than the central anterior stroma of the KC cornea. The large number of undulations developing from the DM of the peripheral region suggests that pathogenicity might have started from the peripheral region and spread towards the central part of the cornea. The effect of pathogenicity and formation of undulations was more severe in the central part than in the peripheral part due to the weak lamellae organisation in the anterior stroma of the central part.

Roberts1,9 suggested that the progression of KC is due to a focal reduction in corneal biomechanical strength and consecutive localised increases in the stress induced by intraocular pressure (IOP). Thus, it is likely that this weak central part may be affected by IOP more than the peripheral part. As a result, severity of the undulation increases in the central part and causes the formation of cones in the KC cornea rather than in the peripheral part of the cornea.

Contributors AA and SA hypothesised and designed the experiments. AA, AK, SA and TA performed the experiments. AA, OK, TA, AK and SA analysed the data. AA, AK and SA contributed reagents/materials/analysis tools. AA, RB, OK, TA and SA prepared the manuscript. All the authors reviewed the manuscript and provided intellectual input for finalising discussion and interpretation of data.

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Competing interests None declared.

Patient consent Tissue used were anonymous.

Ethics approval Ethical Committee of King Saud University, Ethics Number: CAMS 19-36/37.

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