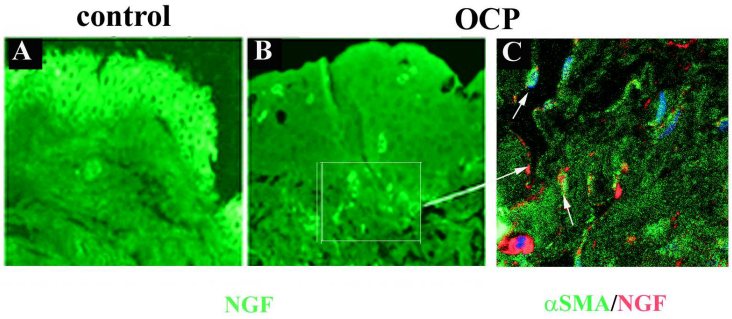

Fig 3. NGF immunoreactivity in OCP conjunctiva and FBs. Confocal analysis for NGF (green) in control (A) and OCP (B,C) conjunctiva. C. Immunofluorescence for NGF (red) and αSMA (green) expression in the conjunctival stroma. Note the presence of intracytoplasm and perinuclear staining. Nuclei were counterstained with toto3. Magnifications: AB, x400; C, x600 (oil immersion).

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±0.56 *vs*. 15.52±5.97 MFI; $p < .05$) as well as unchanged $trkA^{NGFR}$ (20.33±8.52 *vs*. 20.93±4.87 MFI; $p > .05$) protein expressions were observed upon NGF exposure. In *advanced* OCP-FBs, no significant changes of αSMA and $p75^{NTR}$ (respectively 17.60±1.15 *vs*. 17.00±6.12 and 31 ±4.77 *vs*. 26.83±3.84 MFI; $p > .05$) as well as $trkA^{NGFR}$ (2.93±0.83 *vs*. 2.41±1.27 MFI; $p > .05$) protein expressions were detected upon NGF exposure. With respect to $trkA^{NGFR}/p75^{NTR}$ expression, FCM analysis showed that 94.91% *early* OCP-FBs were $trkA^{NGFR}$ positive, with 57.24% co-expressing $p75^{NTR}$. Upon NGF exposure, 96.88% *early* OCP-FBs were still $trkA^{NGFR}$ positive, with 24.12% co-expressing $p75^{NTR}$ and 72.76% expressing only $trkA^{NGFR}$. The statistical analysis showed that a decrease of 57% in $trkA^{NGFR}/p75^{NTR}$ co-expressing cells occurred in association with a shift to $trkA^{NGFR}$ expressing cells. The $trkA^{NGFR}/p75^{NTR}$ immunoreactivity in NGF exposed early OCP-FBs is shown (Fig 6).

NGF modulation of OCP-activated FBs derived TGFβ1 and IL4 cytokines

Last, changes in TGFβ1 and IL4 profibrogenic factor release were also detected in the conditioned media from baseline and NGF treated OCP-FBs. TGFβ1 and IL4 levels in the conditioned media from *early* OCP-FBs were respectively 8-times (101.00±30.00 *vs*. 12.00±2.10 pg/mL TGFβ1, $p < .05$) and 6-times (308.00±7.00 *vs*. 55.00±40.00 pg/mL IL4; $p < .001$) higher as compared to control counterparts. Conditioned media from *advanced* OCP-FBs did not show difference in both TGFβ1 and IL4 levels, as compared to controls. Upon 10ng/mL NGF exposure, IL4 protein decreased in the conditioned media from *early* (26.00±10.00 *vs*. 101.00±30.00 pg/mL IL4; $p < .05$) and *advanced* (1.80±0.30 vs. 23.00±4.80 pg/mL IL4; $p < .05$) OCP-FBs. By contrary, TGFβ1 levels decreased only in the conditioned media from *early* OCP-FBs (63.00 ±40.00 vs. 308.00±7.00 pg/mL TGFβ1; $p < .05$).

Discussion

Increasing data indicate that the chronic inflammatory process occurring in OCP conjunctiva leads to FB activation and survival, with overt collagen deposition and excessive matrix deposition [3]. As a product of different structural/immune cells and FBs/myoFBs, both cytokines and growth factors actively contribute to subepithelial fibrosis and conjunctival scarring [25]. To date, different proinflammatory and profibrogenic factors have been investigated by different groups, which have focused their attention especially on receptor signalling [26]. While in a previous study we have described the $trkA^{NGFR}$ and NGF expression respectively in OCP

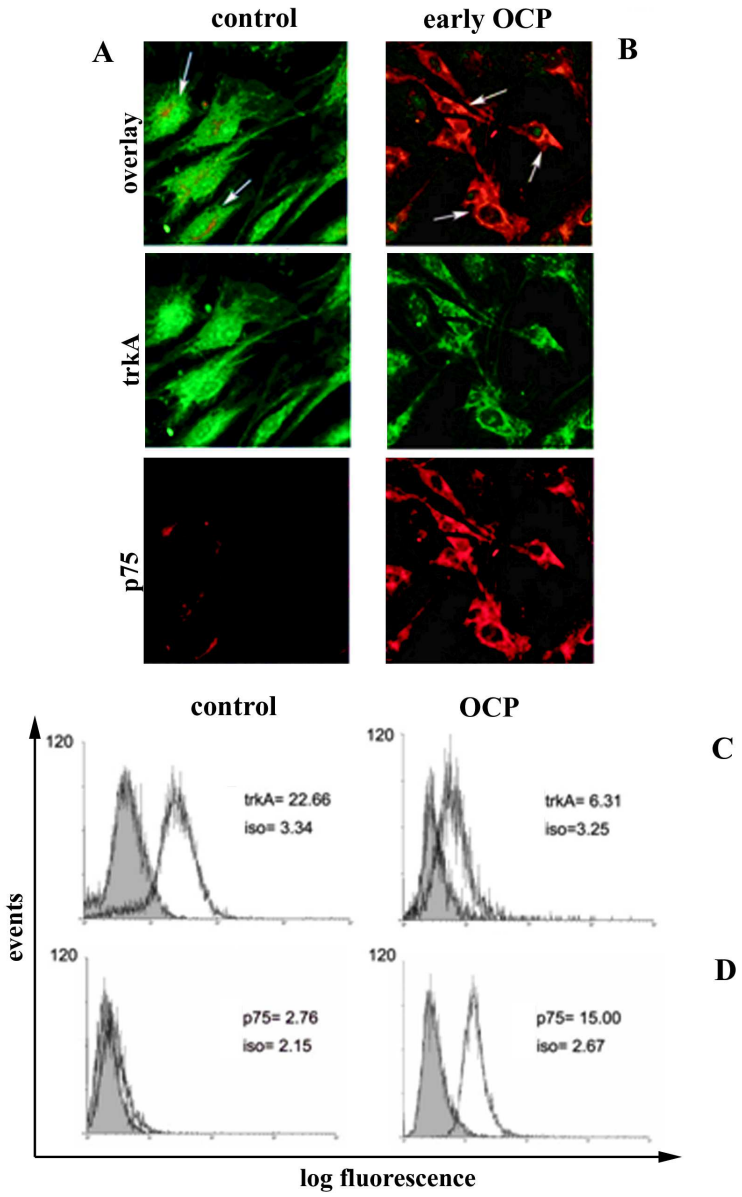

Fig 4. trkA^{NGFR} and p75^{NTR} in OCP conjunctiva and FBs. AB. Confocal analysis of control (A) and OCP (B) FBs double (overlays; x400) and single stained for trkA^{NGFR} and p75^{NTR} (see below). Relevant single immunoreactions are shown below and cross-reactivity of trkA^{NGFR} and p75^{NTR} are marked with white arrows (overlays). CD: Flow cytometry analysis of control (left) and OCP (right) FBs showing expression of trkA^{NGFR} (C) and p75^{NTR} (D). Related isotype fluorescence intensity data are shown (iso).

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conjunctivas and tears, herein we hypothesize a possible NGF role in the modulation of cultured OCP-FBs [8,9].

First of all, αSMA expression was detected in OCP conjunctiva and confirmed in primary cultures of FBs obtained from OCP explants. αSMA (α-Smooth Muscle Actin) represents the most reliable phenotypic marker for the majority of fibrotic states. Our findings indicate the presence of activated FBs inside inflamed/fibrotic OCP conjunctiva [7,27,28]. If these activated

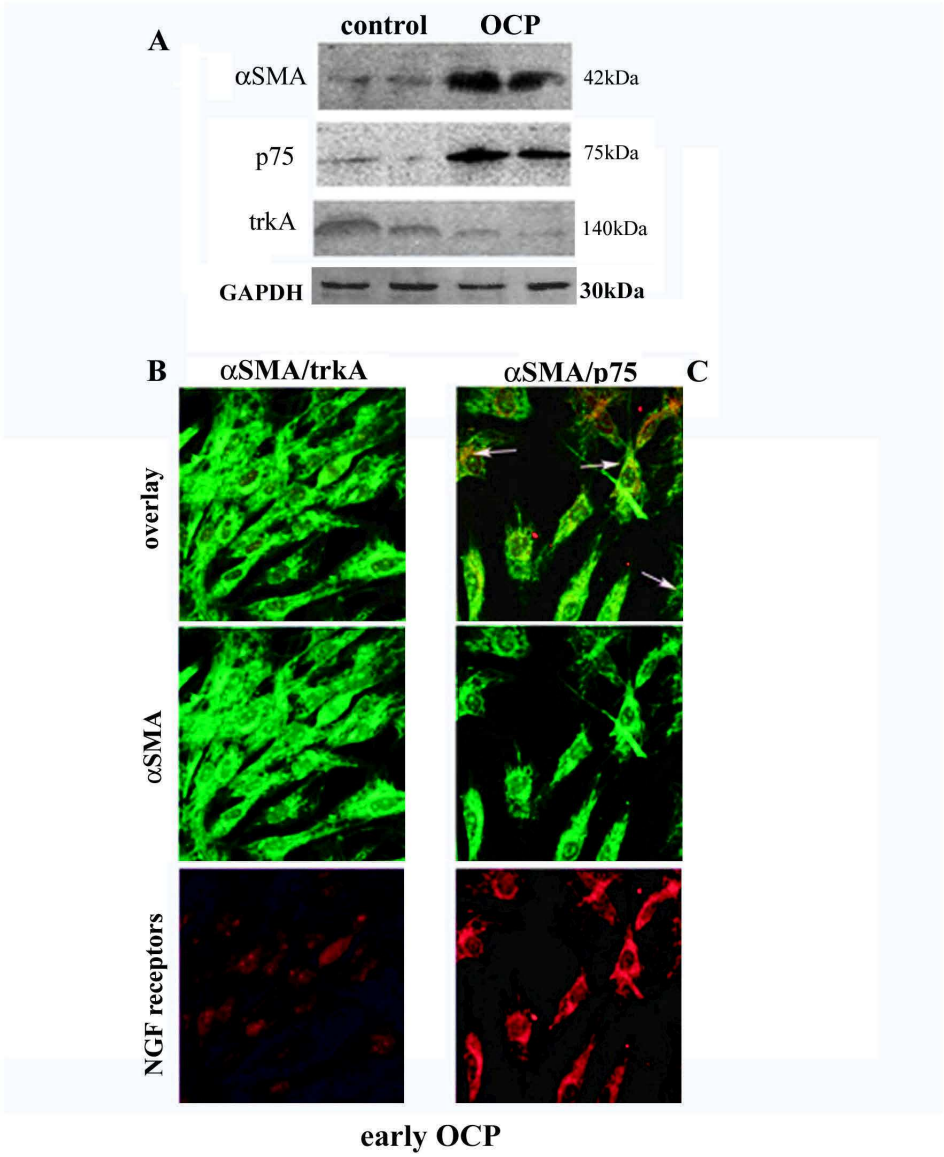

Fig 5. αSMA and trkA^{NGFR}/p75^{NTR} expression in OCP-FBs. A. Representative Western blot analysis specific for αSMA, p75^{NTR} and trkA^{NGFR} proteins in control (left) and OCP (right) FBs (n = 2/each group). Normalization was checked by GAPDH reprobing on the same gels. BC. Confocal analysis for α-SMA/trkA^{NGFR} (B) and α-SMA/p75^{NTR}(C) in OCP-FBs (overlays, x600). Respective single immunoreactions are shown below. p75^{NTR} and α-SMA cross-reactivity is highlighted with white arrows (C).

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FBs are αSMA-expressing myofibroblast (myoFBs) remains to be clarified since in previous studies the possible differentiation of OCP-FBs into myoFBs was not reported [29].

An increase in the stroma and a significant decrease in the epithelium were detected for NGF in OCP conjunctival biopsies (n = 7), as compared to control ones. To the best of our knowledge, this data has not been previously described and it is supported by our recent findings showing an increased NGF content in OCP tear fluids [8,9]. The observation of NGF-expressing OCP-FBs might suggest that the NGF increase in OCP stroma as well as the increased NGF levels in OCP tears might be partially due to local activated-FBs. On the other

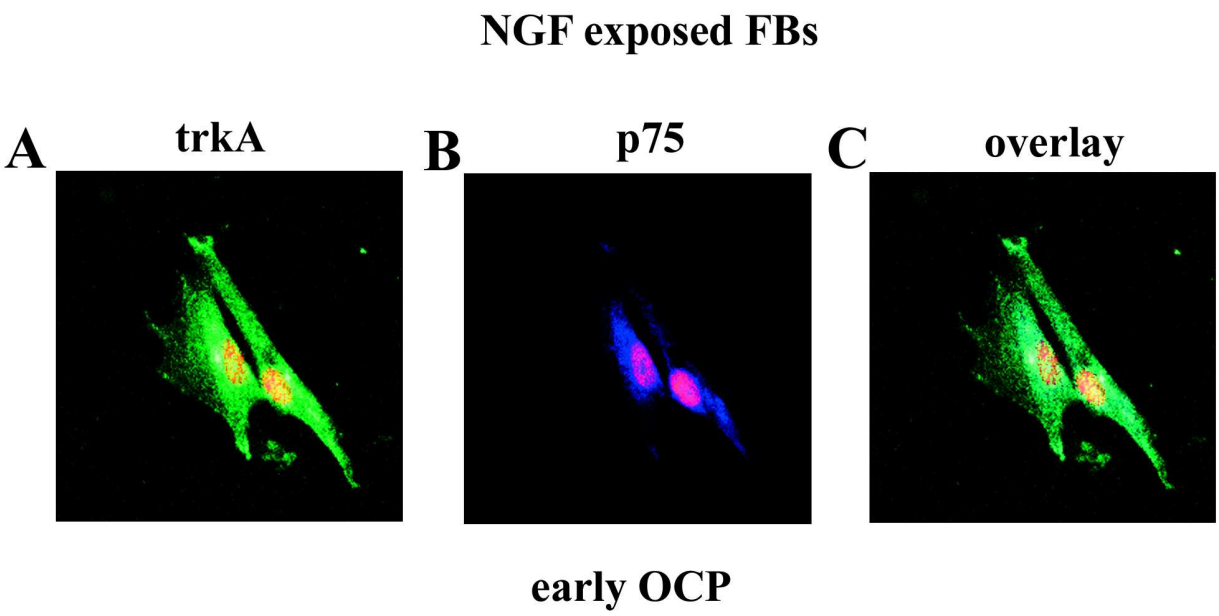

Fig 6. trkA^{NGFR}/p75^{NTR} expression in NGF exposed early OCP-FBs. Confocal images showing the trkA^{NGFR} (FC/green, A) and p75^{NTR} (Cy5/blue, B) immunoreactivity in early OCP-FBs exposed to NGF over 24hrs (overlays, C). Nuclei counterstained with propidium iodide are shown in all panels. The cytoplasmic and perinuclear distribution of both receptors is clearly visible. Magnifications: A-C, x400.

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side, the decreased NGF immunoreactivity in OCP epithelium is actually missing of explanation and not investigated/discussed in this study.

As product of stromal inflammation, it is reasonable to hypothesize that NGF might contribute to tissue remodeling by influencing the FB phenotype, as observed in previous studies conducted on other cell types [14,17,18,20]. With respect to the activated FB phenotype, NGF effects might cover either cell survival and/or soluble mediator release [14,17]. According to literature, NGF activity is driven by trkA^{NGFR} and p75^{NTR} receptors, which mediate NGF signal alone or in cooperation [30–35]. As a new finding, NGF, trkA^{NGFR}/p75^{NTR} and αSMA (co) expressions were detected in primary cell cultures alongside with sub-cultured OCP-FBs. Of interest, sub-cultured OCP-FBs showed the ability to retain FB phenotype upon few passages and were therefore suitable for stimulation studies. Interestingly, trkA^{NGFR}/p75^{NGF} expression was found strictly dependent to the *early/advanced* grouping of disease as well as αSMA phenotype correlated to p75^{NTR} and paralleled the severity of fibrosis. Particularly, FBs from *advanced* OCP showed higher αSMA and p75^{NTR} together with lower trkA^{NGFR}, as compared to *early* and control counterparts. This expression would imply a close association of p75^{NTR} with OCP-FB phenotype, and highlight a possible modulation of myoFB apoptosis, as observed in other systems [20,35]. To date, the role of trkA^{NGFR} and p75^{NTR} in tissue remodeling remains controversial. As documented, both trkA^{NGFR} and p75^{NTR} can mediate either survival or apoptosis, depending on their surface receptor (co)expression and microenvironment [33,36–38]. In early healing process, high levels of trkA^{NGFR} might drive both migration and differentiation (as initial matrix remodelling) while in late healing process the trkA^{NGFR} down-regulation might allow p75^{NTR} to mediate other biological activities, alone or eventually in cooperation with trkA^{NGFR} [33,39–41]. As described, fibrotic tissues appear characterized by low trkA^{NGFR} and high p75^{NTR} expression [17,18,20]. In this study, a higher trkA^{NGFR}/p75^{NTR} ratio (the outcome of a trkA^{NGFR} over-expression) was observed in *early* OCP-FBs while lower trkA^{NGFR}/p75^{NTR} ratio (the outcome of an increased p75^{NTR} expression) was detected in

advanced OCP-FBs, according to the clinical and histological features (infiltrates and remodelling features) [2,22,42]. To support our findings, the lower trkA^{NGFR}/p75^{NTR} ratio expression in *advanced* OCP-FBs (the outcome of an increased p75^{NTR} expression) has been also reported in other fibrotic conditions, either in vitro/ex vivo [7,17,18,28,43]. A down-regulation of both αSMA and p75^{NTR} expression was observed in NGF-exposed *early* OCP-FBs, while no effect was detected in NGF-exposed *advanced* counterpart. The observation that NGF modulated trkA^{NGFR}/p75^{NTR} ratio expression preferentially in early OCP-FBs would suggest that a potential control of activated FBs might be possible in early OCP showing a mild-moderate clinical facet, opening to potential NGF therapeutic applications. As shown, activated FBs disappear alongside "proper repair process" while αSMA-expressing activated FBs survive in pathological remodelling [7,28]. This process might be highly regulated by growth factors and cytokines, including NGF, all known to be increased in OCP tissues and tears [5,6,8,9]. Therefore, a possible cross-talk between NGF and other profibrogenic factors cannot be excluded. In line, TGFβ1 and IL4 were extensively investigated in fibrosis and are widely reported to contribute selectively to tissue remodelling and overt fibrosis in different disorders via an extensive sustaining of myoFBs [2,6,28,44]. Therefore, we wonder whether NGF might influence TGFβ1 and IL4 release from sub-cultures of OCP-FBs. The biochemical analysis highlighted a significant decrease of TGFβ1 and IL4 in the conditioned media from NGF-exposed *early* OCP-FBs, while only a decrease of IL4 was monitored in *advanced* counterparts. This selective effect holds up the potential NGF involvement in OCP remodelling, through a modulation of inflammatory/fibrogenic soluble factors, at least in *early* stage of disease.

Overall, OCP is a chronic inflammatory disease that slowly evolves in severe conjunctival scarring and visual impairments [1,2,45]. Most of the current OCP therapies target the suppression of inflammation, as counteracting the recurrent inflammation represents the main way to reduce progressive remodelling [46–48]. The findings of this *in vitro* study suggest a possible NGF effect on early OCP-FBs having a low trkA^{NGFR}/p75^{NTR} ratio, highlighting the possible NGF effect in the modulation of FB activity during the *early* stages of disease. Since the topical NGF application has been suggested as a therapeutic tool in some ocular surface disorders [11,49], these findings encourage further studies to understand the underlying NGF mechanism in OCP conjunctiva in order to develop alternative strategies to counteract fibrosis.

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Author Contributions

Conceived and designed the experiments: AM BS AL SB. Performed the experiments: AM BS ADZ RS EMN AL SB. Analyzed the data: AM BS ADZ RS MC AL SB. Contributed reagents/materials/analysis tools: AM SB. Wrote the paper: AM BS AL SB.

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