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PERSPECTIVE

CORONIS symposium 2023: Scientific and clinical frontiers in ocular surface innervation

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Abstract

The 5th CORONIS Symposium, held during the 2023 Congress of the European Association for Vision and Eye Research (EVER), highlighted the growing importance of ocular surface innervation in eye surface disorders. This article summarises the insights and perspectives shared during the symposium, which focused on the clinical relevance of ocular surface innervation, as well as on the development of innovative diagnostic and therapeutic approaches for ocular surface pathologies linked to disturbed sensory innervation. Through robust interdisciplinary collaborations, these developments hold great potential to improve patient outcomes and quality of life.

KEYWORDS

aniridia-associated keratopathy, blood-derived eye drops, chronic ocular pain, corneal nerves, dendritic cells, dry eye disease, in vivo corneal confocal microscopy, neuropathic ocular pain, ocular surface disorders, ocular surface innervation, ocular surface neurobiology, sub-basal nerve plexus

1 | INTRODUCTION

For the past 7 years, the CORONIS FOUNDATION has been sponsoring ophthalmic symposia in association with the presentation of its 'Endre A. Balazs' Medal for Achievements in Ophthalmology, awarded biennially to a distinguished researcher for outstanding clinical or experimental studies of the eye (Bron et al., 2022). These meetings provide the members of the ophthalmological community with an opportunity to speculate and hypothesise about the future of eye research and to freely express new and provocative ideas.

The 5th CORONIS Symposium was held during the 2023 Congress of the European Association for Vision and Eye Research (EVER) and focused on ocular surface innervation.

Ocular surface sensory innervation, which was traditionally overlooked in ocular surface disorders, has gained more attention in recent years (Bron et al., 2022; Labetoulle et al., 2019). This shift is in part driven by the increasing concern about the ocular discomfort and pain that accompanies several common eye surface pathologies, as well as that associated with modern anterior segment surgical procedures. The clinical importance of ocular sensory innervation is further reinforced by the confirmation of a trophic dependence of peripheral sensory nerves on eye tissues and of dynamic crosstalk between ocular elements of the nervous and immune systems, an interaction that often becomes maladapted in pathological conditions (Al-Aqaba et al., 2019; Frutos-Rincon et al., 2022; Wu et al., 2022).

This article reflects the insights shared during the last CORONIS symposium. It focuses on the clinical significance of ocular surface innervation and provides insight into future directions. The first section focuses on the different aetiologies and the management strategies for chronic human ocular pain, with an emphasis on the neuropathic mechanisms. The second part provides an up-to-date overview of the molecular, cellular and integrative properties of ocular surface trigeminal ganglion neurons, emphasising their role in adaptive responses that protect the eye from environmental stress, both in health and disease. The third section discusses advances in microscopy that have arisen to study the morphological aspects of ocular surface innervation and their relevance in clinical settings. Finally, the article highlights novel therapeutic strategies for the treatment of sensory innervation disorders associated with ocular surface pathologies.

2 | AETIOLOGIES AND THE MANAGEMENT STRATEGIES FOR CHRONIC OCULAR PAIN. AUTHOR: A. GALOR, RECIPIENT OF THE 'ENDRE A. BALAZS' MEDAL, 2023

Chronic Ocular Pain (COP) is a common complaint in the general population (Stapleton et al., 2017). It is often described using terms such as 'dryness', 'grittiness', 'aching' and 'burning'. The severity of COP can fluctuate throughout the day, with extremes of light, wind and temperature commonly triggering an intensification of pain (Kalangara et al., 2017). In the past, ocular pain was described as a manifestation of 'dry eye', with aqueous tear deficiency a clear source of COP (Mehra et al., 2020). However, it is becoming increasingly clear that there are many aetiologies other than tear abnormalities that can contribute to COP, including nociceptive, neuropathic and nociplastic mechanisms (Mangwani-Mordani et al., 2023). Indeed, identifying the sources of pain in an individual is crucial to being able to adequately manage COP.

2.1 | Identifying the source of COP

Nociceptive sources of pain (i.e. pain resulting from tissue damage) (*IASP terminology,* n.d.) can include tear abnormalities, inflammation, anatomical irregularities (e.g. pterygium, Salzmann nodular degeneration) and toxic insults (e.g. benzalkonium chloride–BAC–in eye drops for glaucoma, air pollution). On the other hand, nerve abnormalities can also contribute to COP, either in the context of a lesion, through diseases affecting the peripheral or central nerves that connect the ocular surface to the brain (i.e. neuropathic pain), or in cases where no lesion has been identified (i.e. nociplastic pain) (*IASP terminology,* n.d.). Identifying the sources of pain in the individual patient is a fundamental issue in COP management, with a focus on both ocular surface and nerve abnormalities, as is devising a personalised management plan. Determining the nociceptive sources of pain commences with an analysis of the individual's medical history, capturing important co-morbidities like autoimmune disease, sleep apnoea and thyroid abnormalities, to name just a few. In addition, the ocular examination should capture information on periocular skin health, eyelid anatomy and function, as well as on ocular surface health (as evaluated with the use of fluorescein and/or Lissamine green dyes). Adjuvant tests may include qualitative assessment of the Matrix Metalloproteinase 9 (MMP9: InflammaDry, Quidel, San Diego) and the assessment of tear production through a Schirmer's test. However, it is just as important to evaluate nerve health and to identify potential neuropathic or nociplastic contributions to pain. Indeed, there may be several indications where Neuropathic Ocular Pain (NOP) contributes to COP.

Other clues regarding the source of COP may also be hidden in the individual's medical history, for example when pain began immediately post-surgery, post-viral infection or post-trauma, suggesting that nerve dysfunction contributes to pain. In addition, co-morbidities like migraine or fibromyalgia also provide clues to the presence of NOP, as chronic pain conditions often overlap and are driven by central sensitivity (Galor, Covington, et al., 2016). Questionnaires can also be used to tease out features of NOP, such as pain characterised as 'burning', or pain evoked by wind or light (Kalangara et al., 2017). These aspects of pain can be established using validated questionnaires, such as the Ocular Surface Disease Index (OSDI) used to detect Dry Eye Disease (DED) (Schiffman et al., 2000), which includes questions about light sensitivity and other features of NOP, or with pain-specific questionnaires like the Ocular Pain Assessment Survey (OPAS) (Qazi et al., 2016) or the Neuropathic Pain Symptom Inventory-Eye (NPSI-Eye) (Farhangi et al., 2019). Other clues regarding the presence of NOP include subjective symptoms that are disproportionate to evident ocular surface features (Ong et al., 2018) and the failure of pain to improve with treatments that target the ocular surface (Galor, Batawi, et al., 2016). Corneal sensitivity should be evaluated in all individuals. This can be done using tools that stimulate corneal nerves by direct mechanical contact, such as a cotton tip or dental floss (rated qualitatively as 0 'no', 1 'reduced', 2 'normal',

3 'increased' sensitivity), as well as the handheld Cochet-Bonnet esthesiometer. Non-contact devices, such as the corneal esthesiometer BRILL, can also be used for this evaluation (Merayo-Lloves et al., 2024). Abnormal sensitivity (either enhanced or dampened) provides further clues regarding the presence of NOP (Spierer et al., 2016).

Other tests have been developed to localise regions of nerve abnormalities, such as peripheral or central. Microneuromas detected by In Vivo Confocal Microscopy (IVCM) have been proposed as an indicator of peripheral NOP (Aggarwal et al., 2015; Hamrah et al., 2017). By contrast, a positive result in the 'anaesthesia challenge' and the presence of cutaneous allodynia have been proposed as signs of central NOP. For the 'anaesthesia challenge', individuals are asked to rate pain intensity prior to anaesthesia, and to then re-rate their pain between 30 s and 2 min after anaesthesia (e.g. proparacaine or tetracaine). Pain that resolves significantly with anaesthesia is suggestive of a nociceptive or peripheral neuropathic source, whereas persistent pain suggests a central or non-ocular contribution to pain. The test is inconclusive if no pain is reported prior to anaesthesia. Cutaneous allodynia, that is pain induced by normally non-painful stimulation of the skin, is a hallmark of central sensitisation. It reflects increased excitability of neurons within the central nervous system that receive input from trigeminal sensory neurons. This condition can be assessed by lightly touching the periorbital skin and observing any increase in sensitivity to light pressure in the area (Crane et al., 2017).

2.2 | Addressing NOP in ocular pain

Treatment of NOP involves adequately treating the sources of nociceptive pain. For example, chronic anti-inflammatory therapies (e.g. cyclosporine and lifitegrast) could be considered for those with systemic auto-immune conditions and/or a significant ocular surface inflammatory component, and Meibomian gland treatments can be recommended for individuals with Meibomian gland dysfunction, correcting anatomical abnormalities when possible. However, if pain persists or if minimal nociceptive sources are identified, neuromodulatory approaches with eye drops, oral medications and/or adjuvant strategies should be considered. Importantly, neuromodulation, whether applied peripherally or centrally, is a slow process that requires prolonged and repeated therapy for at least 3 months, with continued improvement often seen for up to one to 2 years. Topical neuromodulatory approaches are considered first-line therapies in individuals with suspected peripheral NOP. However, the optimal drug and formulation for several of the topical therapies that have been studied over the years are yet to be determined. Autologous blood products are those most commonly used due to their availability (Aggarwal et al., 2015, 2019). However, there is interest in other therapies for the treatment of COP, including topical insulin, recombinant human Nerve Growth Factor (rhNGF) (Pflugfelder et al., 2020), Transient Receptor Potential Vanilloid 1 (TRPV1) antagonists (Baiula & Spampinato, 2021) and Transient Receptor Potential Melastatin 8 (TRPM8) agonists (Weyer & Lehto, 2017; Yang et al., 2018; Yoon et al., 2021). Further studies in humans with suspected peripheral NOP will be needed in order to establish which approach is optimal for each patient population.

In individuals with a central component to their pain, peripheral neuromodulation may not be sufficient and the modulation of central nerves should be considered. Oral neuromodulators like ligands that target the $\alpha 2\delta$ subunit of voltage-gated calcium channels (e.g. gabapentin and pregabalin) and tricyclic antidepressants are commonly used to treat chronic pain conditions in which central neuropathic mechanisms participate. These conditions, such as chronic migraine and fibromyalgia, may share mechanisms with NOP (e.g. central sensitivity) (Mehra et al., 2020). Gabapentin and pregabalin are ligands of the α2δ subunit of voltage-gated calcium channels that dampen calcium influx, thereby inhibiting the release of excitatory neurotransmitters like glutamate and norepinephrine (Tzellos et al., 2010). Treatment with gabapentin typically starts at 300 mg daily, slowly titrated to a dose of 600–900 mg three times a day. Pregabalin dosing typically starts at 75 mg once nightly and is titrated to a dose of 150 mg twice daily. To date, $\alpha 2\delta$ ligands have been used in individuals with NOP with a good safety profile and efficacy (Small et al., 2020; Yoon et al., 2020). These medications are often combined with topical and other oral medications, including duloxetine (Small et al., 2020) or low dose naltrexone (Dieckmann et al., 2021). Tricyclic antidepressants are divided into secondary (e.g. nortriptyline) and tertiary amines (e.g. amitriptyline). Secondary amines inhibit noradrenaline reuptake, while tertiary amines inhibit noradrenaline and serotonin reuptake (Moraczewski et al., 2022). Tricyclic antidepressants are used to manage major depressive disorder and they have also been used to treat individuals with neuropathic pain, including NOP (Ozmen et al., 2020). The starting dose for tricyclic antidepressants is typically 10 mg nightly, with escalation to 50–75 mg. One potential side effect of tricyclic antidepressants is dryness due to off target effects, as such there may be some reservations when prescribing these to individuals with aqueous tear deficiency.

Adjuvant agents can be considered based on certain NOP features. In individuals with light sensitivity or a history of headaches, approaches used to manage chronic migraine are often beneficial, including Transcutaneous Electrical Nerve Stimulation (TENS) or botulinum toxin A (Botox) treatment (Patel et al., 2021). While the mechanisms underlying the benefits of TENS remain unclear, the Gate Control Theory postulates inhibition of peripheral nociceptors and ascending central pathways (Melzack & Wall, 1965). Other theories point towards the modulation of the descending pain pathways (Zayan et al., 2020). One TENS device studied for NOP is Cefaly® (Cefaly Technology), a FDA-cleared unit for the abortive and prophylactic treatment of migraine, with beneficial responses in some patients (Mehra et al., 2021; Riederer et al., 2015). However, studies are needed to further evaluate this approach and to study other TENS modalities (e.g. Scrambler therapy) that can be applied to NOP. Botulinum toxin A has also been approved for chronic

migraine and it has been speculated to modulate pain by inhibiting the release of Calcitonin Gene-Related Peptide (CGRP) (Becker, 2020). This approach has been assessed in individuals with NOP, both with (Diel et al., 2018) and without (Venkateswaran et al., 2020) chronic migraine. As with all neuromodulatory therapies, a beneficial response to therapy is seen in some individuals, yet more work needs to be done to identify which NOP subtypes benefit most from neurotoxin injection. Periocular nerve blockage can also be considered in NOP, especially in individuals with cutaneous allodynia (Dermer et al., 2020). This approach has been used to block terminal branches of the supraorbital, supratrochlear, infratrochlear and infraorbital nerves, employing a combination of a long-acting anaesthetic and corticosteroid (Small et al., 2020). Less information is available on the utility of autonomic ganglion blockade (e.g. sphenopalatine and stellate) as a treatment for NOP (Mehra et al., 2020), although these approaches have had variable success in individuals with head and neck pain (Gunduz & Kenis-Coskun, 2017). An important area of research is to identify when autonomic mechanisms contribute to NOP, as this will clarify when these approaches should be considered.

2.3 | Towards effective management of COP

Different mechanisms may drive the development of COP, including nociceptive, neuropathic or nociplastic phenomena. Individuals with COP must have their treatment tailored to the source(s) of their pain, which may involve topical, oral and adjuvant therapies. Given the increasing number of treatment options, better diagnoses are necessary to define when NOP contributes to pain and to identify the site of the nerve abnormalities (e.g. peripheral or central). Moreover, prospective studies will be necessary to robustly test these treatment modalities, in isolation or in combination, as most studies to date have been retrospective. Furthermore, novel therapies specifically targeting sources of NOP are needed, while it will also be essential to tailor therapies to each patient. These avenues of research are important because COP can be a debilitating condition, affecting the individual's quality of life and emotional health. Better diagnosis and therapeutic tools, tailored to the individual patients, will hopefully reduce the morbidity in these patients and improve their quality of life.

3 | UNDERSTANDING OCULAR SURFACE NEUROBIOLOGY THROUGH PRECLINICAL RESEARCH. AUTHORS: J. GALLAR, M.C. ACOSTA, V. MESEGUER

To develop effective treatments for ocular surface conditions and improve their management, it is essential to understand the neurobiology of the ocular surface. This includes understanding how the neurons that innervate the cornea, lid margin and conjunctiva contribute to generating ocular sensations and responses that protect the surface from harmful environmental factors. In addition, it is crucial to recognise the changes in neural morphology and activity that occur during inflammation and nerve regeneration. In this context, preclinical models are invaluable.

3.1 | The different types of sensory neurons innervating the ocular surface and the sensations they evoke

The cornea, conjunctiva and lid margin are innervated by the distal projections of a small number of primary sensory neurons whose cell bodies reside in the ophthalmic region of the trigeminal ganglion (V1). Most ocular sensory axons access the eyeball via the long and short ciliary nerves, extending to all ocular tissues except the lens and retina. The cornea is innervated extensively by sensory nerves that are organised into four strata parallel to the ocular surface, from the penetrating stromal nerve trunks to the intraepithelial nerve terminals. Some V1 neurons have heavily myelinated axons that end in the corneoscleral limbus or at the eyelid margin, where they associate with structures identifiable as low-threshold mechanoreceptors similar to those responsible for touch sensation in the skin. By contrast, all the nerves inside the cornea have a similar appearance, with unmyelinated axons that branch and terminate as free nerve terminals within the corneal epithelium. Nevertheless, these corneal nerves do vary in terms of the neuropeptides and other neurotransmitters they contain, many of which have important trophic roles in corneal tissue. Moreover, ion channel expression differs in corneal nerves, influencing the transduction of different stimuli or action potential (AP) generation. Consequently, there is considerable functional diversity among corneal sensory nerve fibres (Gallar & Acosta, 2024).

Ocular sensory nerves can be classified into five main functional types based on their response to different stimuli applied to their receptive fields at the ocular surface (Figure 1): (1) polymodal nociceptor nerve fibres that may be activated by mechanical energy, heat, protons and chemical irritants; (2) mechanonociceptor fibres activated by a mechanical stimulus; (3) cold thermoreceptor nerve fibres activated by tear film hyperosmolarity and mild (High Background-Low Threshold-HB) or intense (Low Background-High Threshold-LB) cold thermoreceptors, which sense temperature reductions and that are located in the cornea, sclera and conjunctiva (Acosta, Belmonte, & Gallar, 2001; Belmonte et al., 1991; Gallar et al., 1993, 2003; Parra et al., 2014); (4) low-threshold mechanoreceptor nerves activated by weak mechanical forces that are located in the corneoscleral limbus and at the lid border (Belmonte et al., 1997); and (5) pruriceptor nerve fibres that are activated by pruritogenic substances and that are located in the tarsal conjunctiva, close to the lid margin (Comes et al., 2021).

The ability of sensory nerve terminals to respond to different stimuli reflects the specific molecular machinery for sensory transduction at their nerve endings. Voltage-gated calcium, sodium and potassium channels, as well as cyclic nucleotide-gated channels, help determine the number of APs induced, along with

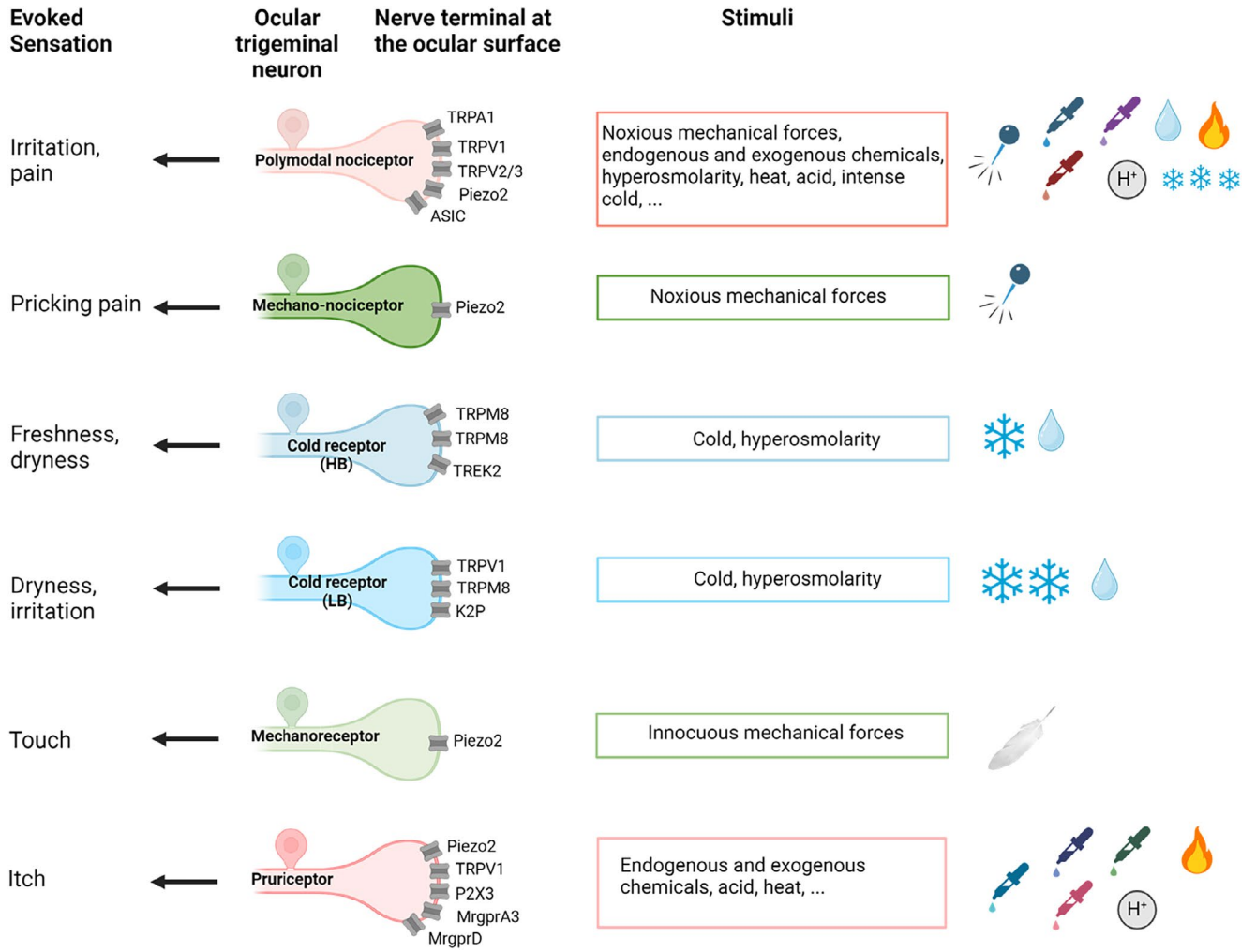

FIGURE 1 Functional characteristics of ocular surface innervation. Scheme representing the peripheral nerve endings of the different types of primary trigeminal sensory neurons innervating the ocular surface, with an indication of the ion channels involved in the detection, transduction and encoding of the different stimuli at their nerve terminals. The sensations evoked when the sensory information originated at the ocular surface reaches the brain cortex are also indicated. The nerve endings of the first four types of neurons located in the cornea, while the low-threshold mechanoreceptive nerve endings are located in the corneoscleral limbus, the conjunctiva and the lid margin. Finally, pruriceptor nerve terminals are located in the tarsal conjunctiva, near the eyelid border. Figure created with BioRender.com.

the firing frequency and patterns that characterise a stimulus (Acosta, Belmonte, & Gallar, 2001; Belmonte et al., 2004; Velasco et al., 2024). Among the ion channels expressed by polymodal nociceptor nerve terminals are TRPV1 and Piezo-Type Mechanosensitive Ion Channel Component 2 (Piezo2) (Acosta et al., 2013, 2014; Callejo et al., 2015; Comes et al., 2021; Viana & Belmonte, 2008). These channels open when the terminals are exposed to noxious heat, high-intensity mechanical forces, low pH or some chemical substances, consequently depolarising the terminal. If the stimulus is sufficiently intense, depolarisation surpasses the threshold and initiates an AP, which travels along the axon to the central nervous system, and after processing, evokes a sensation of burning, irritation and pain. Mechanonociceptor nerve terminals only express Piezo2 channels (Bron et al., 2014; Fernandez-Trillo et al., 2020), which open in response to mechanical forces, and their activation evokes pricking pain. Cold thermoreceptor nerve terminals express TRPM8 channels (Alcalde et al., 2018; Parra et al., 2010), which open in response to a reduction in the ocular surface temperature. Activation of the different classes of cold thermoreceptors, which differ in the types of potassium channels they express and in the density of their TRPM8 channels, elicits sensations of coolness, dryness and even pain. Conjunctival and limbal low-threshold mechanoreceptors also express Piezo2 channels, whose activation is responsible for the touch sensation from these tissues (Acosta, Tan, et al., 2001). Some conjunctival nerves express the MAS-related G protein-coupled receptor member D (Mrgprd) and the MAS-related G protein-coupled receptor member A3 (MrgprA3), characteristic of the pruriceptor nerve terminals responsible for evoking itching (Comes et al., 2021).

In summary, the specific ion channel types at the nerve terminal of each class of ocular sensory neurons enable them to detect and encode one or more stimulus modalities, giving rise to the different sensations evoked at the ocular surface.

3.2 | The many roles of ocular surface innervation

Sensory nerves innervating the ocular surface are responsible for the sensations evoked in response to stimulation,

and they regulate essential mechanisms to maintain ocular surface homeostasis, such as blinking and tearing. Reflex blinking and reflex tearing are driven by the activation of polymodal nociceptors (Acosta et al., 1999, 2004) as the afferent arm of reflex responses integrated by the brainstem. By contrast, basal blinking and the rates of tearing are under the control of cold thermoreceptors (Diaz-Tahoces et al., 2024; Frutos-Rincon et al., 2023; Parra et al., 2010; Quallo et al., 2015). Ocular surface sensory nerves are also involved in the trophic maintenance of ocular tissues. The response of corneal sensory nerve terminals to adequate stimuli conveys APs to the brainstem, where glutamate and neuropeptides are released. These APs also invade all the branches of the activated corneal axon, inducing the local release of neuropeptides like Substance P (SP) and CGRP. These neuropeptides, either alone or in combination with other growth factors present in the corneal tissue or corneal nerves (e.g. Epidermal Growth Factor–EGF– or NGF), are important agents in maintaining corneal epithelial integrity and promoting corneal wound healing (Bonini et al., 2003). Hence, the occasional activation of corneal nerves in response to environmental stimuli effectively releases molecules that act locally as essential trophic factors. Thus, any circumstance that provokes partial or total loss of corneal innervation will lead to weaker corneal nerve activity and, consequently, trophic problems within the corneal tissue.

3.3 | Changes in ocular surface innervation in the healthy eye or associated with eye inflammation or lesion

Corneal sensory nerves undergo continuous morphological remodelling of their axons throughout life (Harris & Purves, 1989). Deep stromal nerve trunks maintain a relatively constant position and configuration, whereas the nerves at the level of the basal epithelium, and especially intraepithelial nerve terminals, experience considerable rearrangements (Alcalde et al., 2018; Patel & McGhee, 2008). The molecular mechanisms driving these changes are not well understood. However, it is clear that ocular surface nerve density declines with age, resulting in decreased corneal sensitivity and altered blinking and tear regulation in older individuals (Acosta et al., 2006; Alcalde et al., 2018; Chirapapaisan et al., 2021). Cold sensory nerves innervating the mouse cornea that express TRPM8 undergo significant morphological and functional changes with age. A drastic reduction in intraepithelial nerve terminal branching, as well as reduced nerve impulse activity due to aberrant transduction, encoding and ion channel expression at corneal cold nerve terminals of aged mice, probably underlie the age-related sensations of discomfort and abnormal tearing in older adults (Alcalde et al., 2018).

After damage to the eye and inflammation, the morphology and activity of corneal sensory nerves is altered. Their recovery depends on the extent, depth and type of lesion. After nerve transection, axons distal to the lesion site degenerate, whereas the central stumps begin to regenerate, eventually producing a nerve pattern that differs somewhat from the original corneal nerve architecture (Muller et al., 2003; Rozsa et al., 1983; Yu & Rosenblatt, 2007). After a corneal incision, whether accidental or surgical, the denervated area is first invaded by sprouts from adjacent intact nerve fibres and later, the central stump of the injured axons begins to regenerate, forming microneuroma-like structures from which newly formed sprouts develop. These regenerating axons have enhanced sodium channel expression, such that they fire spontaneously, also explaining the continuous pain and dysesthesia experienced in situations like ocular surgery (Kovacs et al., 2016; Luna, Mizerska, et al., 2021).

Inflammatory mediators and other molecules, such as the ATP released during inflammation and injury, modify the activity of the ion channels responsible for transducing and encoding stimulation, making the nerve terminals more (sensitisation) or less excitable (inhibition) to stimuli. Under inflammatory conditions, the activity of corneal nociceptor nerves is enhanced in response to stimulation, while that of thermoreceptor nerves and the response to cold are dampened (Acosta et al., 2013, 2014; Zhang et al., 2012). Nerve damage sensitises nociceptor and cold thermoreceptor nerves around the lesion (Bech et al., 2018; Kovacs et al., 2016; Luna, Mizerska, et al., 2021). Interestingly, nerve activity is altered in both eyes, even if the injury or inflammation only affects one eye (Luna, Quirce, et al., 2021). This explains the bilateral pain reported by some patients with unilateral injury or inflammation, evidence of the influence of the immune system on corneal nerve activity (Frutos-Rincon et al., 2022).

In summary, abnormal activity of ocular surface sensory nerves forms the basis of the transient or permanent sensations of discomfort, dryness and pain that arise upon injury and inflammation and with ageing. In these conditions, the protective mechanisms controlled by ocular nerve activity are also altered, such as blinking and tearing.

4 | TECHNICAL INNOVATIONS AND FUTURE CLINICAL APPLICATIONS OF IN VIVO CORNEAL CONFOCAL MICROSCOPY. AUTHORS: J.M. BENÍTEZ-DEL-CASTILLO, O. STACHS

IVCM has attracted considerable interest in modern ophthalmology thanks to its ability to non-invasively capture high-resolution images of various cell structures in the living cornea, sparking interest in its diagnostic potential. A multitude of studies using IVCM have already evaluated and identified changes in corneal elements in health and disease. A good example is the studies of the Sub-basal Nerve Plexus (SNP), the network of corneal nerve fibres that runs parallel to the cornea at the level of the basal epithelium. This plexus is the densest and most recognisable component of mammalian corneal innervation. IVCM is still undergoing innovations with a view to enhancing both its clinical utility and ease of use. Moreover, while SNP imaging continues to be of

interest, there is an increasing emphasis on understanding the dynamic interactions between different elements in the cornea.

4.1 | Clinical applications of IVCM

Patients with DED have a compromised SNP, often with abnormal morphological features that include bead-like formations, tortuosity, irregular branching, microneuromas and abnormal loops. These changes are probably the result of nerve degeneration and regeneration. A loss of corneal sensitivity is correlated with lower SNP fibre density, whereas enhanced sensitivity is linked to nerve abnormalities (Benitez del Castillo et al., 2004, 2007; Labbe et al., 2013; Shetty et al., 2023). Currently, it is not possible to differentiate between DED subtypes by analysing SNP parameters, which can be achieved more reliably by assessing corneal dendritic cells (DCs). In DED, DC density is enhanced, with DCs present in the central corneal epithelium in contrast to their normal peripheral distribution (Figure 2), a finding that is more frequent in patients with severe symptoms. More and larger DCs are evident when aqueous-deficient DED is associated with an immune disorder (Hwang et al., 2021; Kheirkhah et al., 2015; Shetty et al., 2016).

IVCM has also been used to evaluate the SNP and other corneal elements in relation to diseases and conditions other than DED, serving as a useful tool to establish a differential diagnosis, and to better understand the aetiology and pathophysiology of these diseases. For example, in patients with corneal pain of a neuropathic source, there is a higher density of corneal DCs and a lower SNP fibre density. The presence of microneuromas is a key parameter to distinguish corneal neuropathic pain from other conditions, with different morphologies of microneuromas having been described: spindle, lateral and stump types (D'Souza et al., 2022; Ross et al., 2020). The SNP is depleted after Laser-Assisted in Situ Keratomileusis (LASIK) and Photorefractive Keratectomy (PRK), and recovery may require several years, although the loss of corneal sensitivity may be recovered within months. Abnormalities in the SNP after these procedures are more pronounced in patients with corneal neuropathic pain (Erie et al., 2005; Villani et al., 2014). Furthermore, a reduction in SNP fibre density in the central cornea has been observed in wearers of orthokeratology lenses (Lum et al., 2012), whereas the current consensus indicates that the use of contact lenses does not significantly alter corneal nerve morphology, although it is associated with changes in corneal sensitivity (Jalbert et al., 2009). Interestingly, keratoconus patients who wear contact lenses have a lower SNP fibre density relative to those keratoconus patients who do not use contact lenses (Patel et al., 2009).

Imaging of the SNP using IVCM offers a unique, non-invasive method to monitor peripheral nerve status. In patients with diabetes, SNP fibres are shorter, less dense and with fewer branches, in conjunction with greater tortuosity. These changes correlate with disease duration and notably, corneal nerve alterations often precede the onset of diabetic retinopathy. Indeed, there may then be an association between nerve changes and the progression of this disease, such that SNP parameters have emerged as valuable biomarkers for diabetic neuropathy. Nevertheless, some issues still remain to be resolved, such as how changes in SNP and HbA1c (glycated haemoglobin) levels are related (Roszkowska et al., 2021). The diagnosis and management of non-diabetic small fibre polyneuropathies has also benefitted from IVCM. These include a range of neurological disorders that are characterised by neuropathic pain and autonomic symptoms, a consequence of the selective involvement of thinly myelinated A-fibres and unmyelinated C fibres. The existing gold standard for diagnosing these conditions is the measurement of intraepidermal nerve fibre density, although IVCM offers comparable if not superior diagnostic returns. Furthermore, studies indicate that fibre loss correlates with disease progression in different types of small fibre polyneuropathies (Roszkowska et al., 2023).

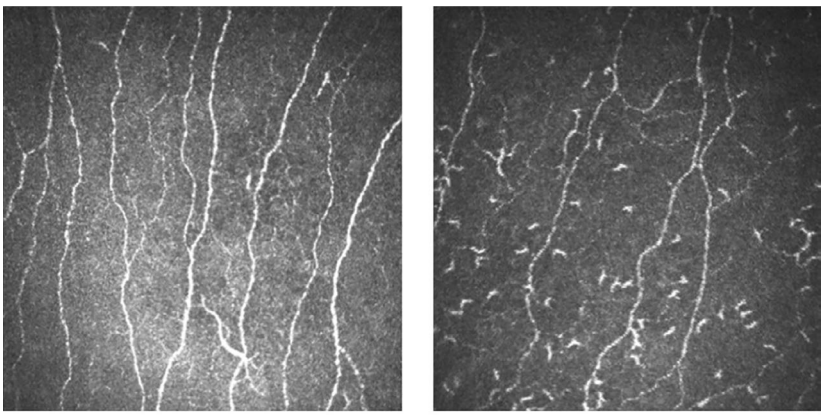

FIGURE 2 Sub-basal nerve plexus in a control subject and a patient with dry eye disease. Representative In Vivo Confocal Microscopy (IVCM) images of the Sub-basal Nerve Plexus (SNP) in the central cornea of a control subject and a patient with Dry Eye Disease (DED). In DED patients there is a lower SNP fibre density, but the Dendritic Cell (DC) density increases. The field of view of this type of image is in the order of 0.10–0.16 mm^2.

Importantly, IVCM is also clinically relevant to evaluate the response to treatment. For example, after a treatment of more than 6 months with cyclosporine eye drops (0.05%), patients with DED showed less tortuosity and reflectivity of SNP fibres (Shetty et al., 2023). IVCM has also been used to evaluate the efficacy of treatments for neurotrophic keratopathy, such as corneal neurotization, where partial recovery of the SNP may be seen (Rathi et al., 2022).

4.2 | Recent advances in IVCM

IVCM was boosted in 2002 by the introduction of Confocal Laser Scanning Microscopy (CLSM), and by combining the Heidelberg Retina Tomograph (HRT) and the Rostock Cornea Module (RCM). The HRT was introduced by Heidelberg Engineering GmbH in the early 1990s as a pioneering commercial confocal laser scanning ophthalmoscope, and it has been used widely to diagnose glaucoma. It was subsequently updated by versions HRTII (1998) and HRT3 (2005). In the early 2000s, an optical attachment for HRTII was introduced at the Rostock Eye Clinic, enabling microscopy imaging of the cornea that effectively converts the system into a CLSM (Stave et al., 2002). Building on this innovation, Heidelberg Engineering developed and commercialised (from 2004) the RCM, and currently, the HRT3/RCM provides corneal imaging capabilities with a field of view of up to 400×400 µm, providing detailed 2D transverse images of corneal cells and structures (Stachs et al., 2019).

This CLSM system is widely used for qualitative and partly quantitative analysis of corneal structures in experimental and clinical ophthalmology, providing images with sufficient depth discrimination such that 3D reconstructions of volumetric data can be generated. It can be used to analyse the cornea of laboratory animals, to assess stromal changes in keratoconus patients before and after cross-linking, for experimental full-thickness corneal imaging, and to quantify epithelial cell layers and the SNP. Although Optical Coherence Tomography (OCT) imaging may be used for cross-sectional imaging of the cornea, CLSM still offers superior lateral resolution and image quality. Several 3D reconstruction techniques for confocal image stacks have been published, including volume imaging using the HRT/RCM system (Stachs et al., 2019).

Recent advances in IVCM are promising, paving the way for broader applications in medical imaging and diagnostics, with a special emphasis on assessing corneal nerves to better understand their roles in ocular diseases and as a read-out of systemic diseases. This is particularly significant as a non-invasive technique to quantify changes to corneal nerves in diabetic individuals. Recent advancements in this direction include automated focal plane control to obtain a mosaic of the SNP (Figure 3) (Allgeier et al., 2018), enhancing the reliability of quantification and offering promise as a biomarker for diabetic peripheral neuropathy.

A novel method for volumetric reconstruction of the cornea at the cellular level has also been devised (Bohn et al., 2020), enabling image acquisition of volume up to $250 \times 300 \times 400\,\mu m^3$ in size. This method features automated focal plane control and utilises a unique contact cap to minimise eye movements, enhancing the quality and volume of the 3D reconstructions generated. By offering sectional views in any orientation, this approach enables slit lamp microscopy to be performed at the cellular level, which is proving invaluable in current research endeavours.

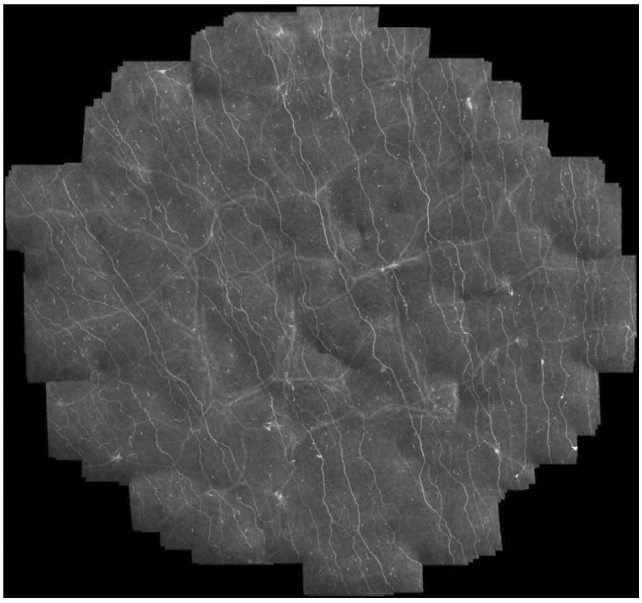

FIGURE 3 Sub-basal nerve plexus mosaicking in a healthy subject. Sub-basal Nerve Plexus (SNP) imaging in the central cornea using an automated technique that combines guided eye movements for rapid expansion of the acquired SNP area and axial focal plane oscillations. This solution provides a more complete image of the SNP than the currently available commercial solutions (HRT/RCM), with a larger field of view. The area visualised with this imaging technique is typically in the range of 2–4 mm^2.

To address the limitations associated with the field of view and image location specificity in IVCM, a multimodal imaging platform has been developed, combining OCT guidance with CLSM (Bohn et al., 2019). This set up permits simultaneous imaging at high frame rates with improved usability. Real-time assessment of image plane location and orientation within the cornea enhances location-based diagnosis, providing unprecedented insights into corneal structure and pathologies. Further optimisation of this system design and the OCT scan patterns will be necessary to maximise the potential of this innovative approach.

Cutting-edge CLSM technology allows the community to scrutinise the ocular surface down to the cellular level, facilitating both 2D mosaicking and 3D reconstruction. A major challenge currently facing the field is the upcoming end of development and sales of the HRT3/RCM in the European Union, although support for this technology will continue until the end of 2029. Realising the potential of this technology will require seamless collaboration between foundational research, clinical expertise and industrial stakeholders. Clearly, only through concerted efforts can the immense promise of this technology be truly unlocked.

5 | NOVELTIES IN THE MANAGEMENT OF OCULAR SURFACE DISEASES RELATED TO INNERVATION. AUTHORS: N. SZENTMÁRY, P. VERSURA

Traditionally, treatment of ocular surface diseases typically involves the use of tear substitutes as a front-line therapy, although recent studies have focused on the efficacy and safety of blood-derived eye drops (Bernabei et al., 2019). For example, the use of Plasma Rich in Growth Factors (PRGF) was recently investigated to alleviate symptoms of ocular dryness in patients with congenital aniridia (Lozano-Sanroma et al., 2024). As research in this area continues, it becomes increasingly important to understand the role of ocular surface innervation in disease, as well as its interplay with other systems like the immune system. This is important to understand the mechanisms by which products like blood-derived eye drops promote healing and regeneration.

5.1 | Corneal innervation in congenital aniridia

Congenital aniridia is a rare condition that affects both eyes, and it is usually caused by mutations in the *PAX6* gene, a key factor in eye development. PAX6 dysfunction disrupts the formation and maintenance of various ocular structures, including the corneal nerve network (Graw, 2010; Robinson et al., 2008; Shaham et al., 2012). Altered PAX6 activity might prevent proper nerve development, and damage to these nerves provokes a range of ocular complications (Versura et al., 2018), potentially contributing to Aniridia-Associated Keratopathy (AAK) (Leiper et al., 2009).

A recent study employed CLSM to image the cornea in congenital aniridia patients, analysing their corneal nerve patterns and tissue structure (Csorba et al., 2024). The study examined 31 eyes from 18 congenital aniridia patients and 46 eyes from 29 healthy individuals, with a similar age and gender distribution in each group. Among the aniridia patients, *PAX6* mutations were verified in 18 individuals from 12 families. All participants underwent slit-lamp biomicroscopy, and the severity of aniridia-associated keratopathy was graded according to (Lagali et al., 2018). CLSM images were analysed using ACCMetrics software (Manchester University), focusing on the morphology of the SNP. Moreover, DC and stromal cell densities were evaluated at different stromal depths (anterior, middle and posterior), assessing changes to the latter (e.g. keratocyte alterations and microdot deposition) that were graded (Utheim et al., 2020).

In patients with congenital aniridia, the SNP displays a significantly lower Corneal Nerve Fibre Density (CNFD), Corneal Nerve Fibre Length (CNFL) and Corneal Total Branch Density (CTBD), yet there is an enhanced Corneal Nerve Fibre Width (CNFW) than in controls. Moreover, there was a significantly higher DC density in aniridia eyes and with a more complex morphology. Keratocyte density was significantly lower in the anterior, middle and posterior stroma of aniridia patients relative to the controls (Csorba et al., 2024). Morphological changes to the stroma were observed in 45.2% of aniridia eyes, including confluent keratocytes in 6.5% and keratocytes with long extensions in 22.6%. Hyperreflective microdots were detected in 25.8% of aniridia eyes. However, no significant correlation was found between the severity of aniridia and corneal nerve parameters, DC density or keratocyte density (Csorba et al., 2024).

Corneal neuropathy is common in congenital aniridia, and it affects nerve fibres that are crucial for maintaining corneal health. Damage to these fibres disrupts corneal epithelial function, leading to instability and irregularities (Eden et al., 2012; Lagali et al., 2020). Despite conflicting reports on SNP morphology, the reduced CNFD and enhanced CNFW in aniridia patients are consistent with neuropathy. A higher DC density suggests ongoing inflammation, while changes in DC maturation may indicate disease progression. Corneal nerve damage disrupts the delicate balance between nerves and immune cells, potentially exacerbating aniridia-associated keratopathy (Belmonte et al., 2004; Brines et al., 2018; Du et al., 2017; Giannaccare et al., 2019; Lagali et al., 2018; Shtein et al., 2012; Szalai et al., 2016). Microdot deposition, observed predominantly in the anterior stroma, suggests increased cell death and degeneration in aniridia (Falke et al., 2009; Jafarinasab et al., 2010; Patel et al., 2001; Trittibach et al., 2004; Watson et al., 2003). Collectively, these findings highlight the complex interplay between nerve damage, inflammation and cellular changes in congenital aniridia, emphasising the need for further research into the underlying disease mechanisms and into treatment strategies.

5.2 | Blood-derived trophic factors to treat ocular surface diseases

The purpose of using blood-derived eye drops to treat ocular surface disease is to create a biological substitute that mimics the lubricating and nourishing properties of natural tears. This approach aims to support ocular surface renewal and enhance immunological defence (Figure 4) (Rauz & Saw, 2010). The presence of a wide range of growth factors in these preparations is of particular interest, especially those with an epitheliotropic role (e.g. EGF) in the healing of the corneal epithelium (Ljubimov & Saghizadeh, 2015). The levels of neurotrophins like NGF are also important. The rationale for supplying the ocular surface with these trophic factors stems from the need to replenish their levels, which are often diminished in patients with ocular surface problems. This is essential to facilitate corneal epithelium healing (Valente et al., 2022; Versura et al., 2016; Yoon et al., 2007).

Recent studies have shown that several blood-derived eye drop preparations significantly improve corneal nerve morphology in patients with ocular surface disease (Barros et al., 2023; Giannaccare et al., 2017). This might result from enhanced epithelial healing, which facilitates corneal nerve regeneration, the direct impact of growth factors on the corneal nerves, or a combination of these

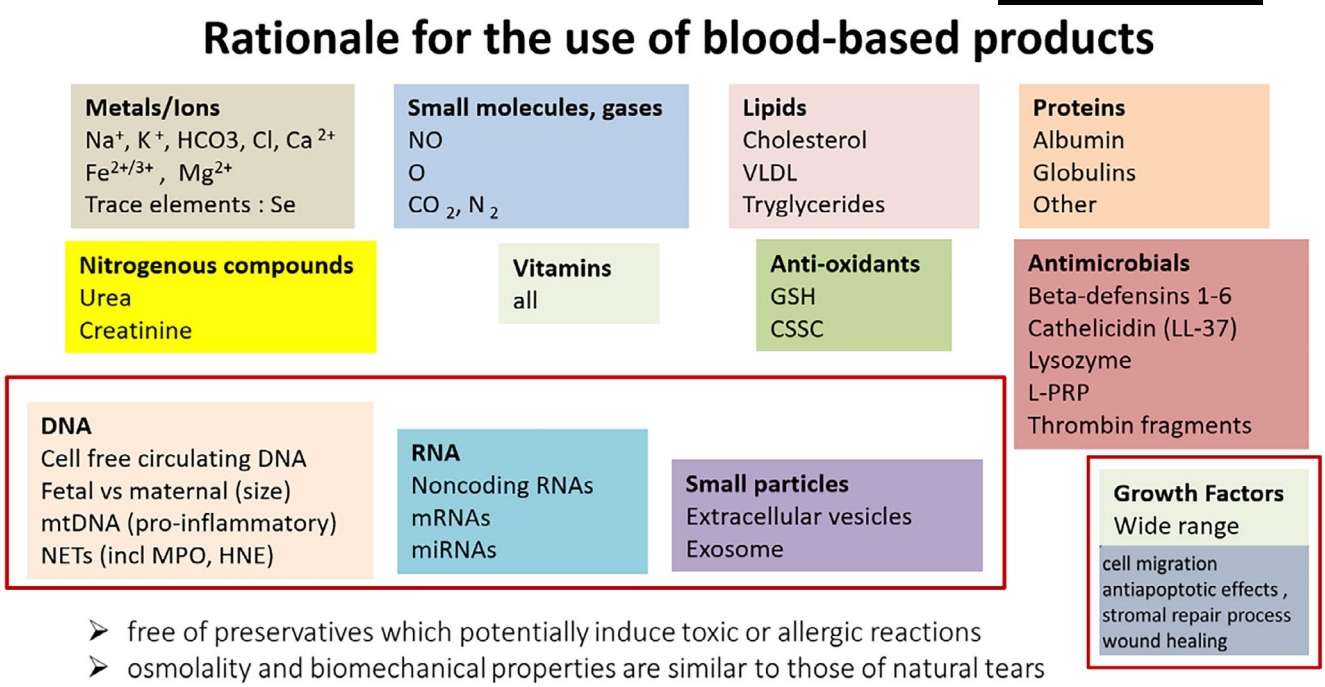

FIGURE 4 The substances in eye drops prepared from blood. Comprehensive list of the substances contained in blood-derived eye drops, emphasising the wide range of growth factors. Additionally, the emerging roles of small extracellular vesicles and exosomes, along with various types of RNA and DNA (such as cell-free circulating DNA), are currently under investigation (Yang et al., 2020).

mechanisms. Moreover, blood-derived eye drops have attracted attention as a promising therapeutic option for NOP (Aggarwal et al., 2015, 2019), with a growing number of studies investigating their potential applications. Beyond their action through neurotrophic factors, several other mechanisms may explain the therapeutic effects of blood-derived eye drops on NOP. Interestingly, certain components present in blood-derived eye drops have been postulated to modulate neuronal excitability, neuronal ion channels, inflammation and synaptic plasticity (Anam et al., 2024).

Autologous products (obtained from the patient's own blood) have been gaining traction for several decades, but now allogeneic products (obtained from donors, either adult peripheral blood or umbilical cord blood collected at birth) are also emerging (Anitua et al., 2017; Badami & McKellar, 2012), overcoming the disadvantages of autologous sources. Depending on how the blood is collected and prepared, each blood product may have a different final composition, making the decision to use serum or plasma important. If plasma is chosen, which type should be used? Serum products are cell-free fluids obtained after clotting and centrifugation of blood samples without anticoagulants, whereas plasma products are derived from blood samples with anticoagulants that are processed distinctly to obtain either platelet-rich plasma (PRP), platelet-poor plasma (PPP) or PRGF. There is an ongoing debate about which type of preparation is the best option for particular patients to provide truly personalised medicine. The lack of standardisation and the use of ad hoc products (often without any description of their contents) in published studies makes it impossible to compare the efficacies of the different products. One important issue could be that the presence of clotting proteins in plasma-based products may cause haze and/or interfere with the healing process, so their use should be avoided in the treatment of extensive keratopathy or corneal ulcers. It is also recognised that different products perform distinctly in vitro (Liu et al., 2006; Valente et al., 2022) and 'too much doesn't always mean better', as suggested by the downregulation of clonal growth in primary limbal corneal cells with increasing concentrations of EGF (Kruse & Tseng, 1993).

Blood-derived eye drops have been used to treat various ocular surface diseases, including DED, persistent corneal epithelial defect, corneal ulcer, neurotrophic keratitis, ocular surface burns, recurrent corneal erosion and limbal stem cell deficiency (Bernabei et al., 2019). They are recommended as a third-line option when conventional tear substitutes or medications like corticosteroids or cyclosporine fail to improve both the signs and, perhaps more importantly, subjective symptoms of discomfort and pain (Jones et al., 2017). Autologous and allogeneic eye drops appear to have comparable efficacy and tolerability in patients with severe DED (van der Meer et al., 2021). In the initial choice of blood source, the use of autologous products could be suggested for patients with 'only' ocular surface disease, while the allogeneic source might be better indicated for patients with concomitant systemic and/or infectious diseases.

Although there is a large body of scientific literature on prescription indications, and there is a general agreement on the success of blood-based eye drops in healing the corneal epithelium and reducing subjective pain, there are still very few randomised clinical trials. Moreover, we still await internationally recognised harmonised guidelines to improve the quality of the evidence, as well as the end products. Such efforts would lead to the more widespread use of these therapies in daily ophthalmological practice. In addition, more studies are needed to analyse short-, medium- and long-term

follow-ups to determine if any benefits are maintained after treatment and for how long.

Many unanswered questions remain about what might be considered optimal treatments, outlined as 'The 5 Ws and 2 Hs for blood-based eye drops' (Bernabei et al., 2019). These can be summarised as: (1) Who is the patient to be treated, in terms of disease type, severity and stage? (2) Why is a blood-based treatment needed, in terms of a target indication? (3) When is it appropriate to prescribe a blood-based eye drop, as too late is not always a good option? (4) Where will the products be dispensed? Is a national/regional programme a viable solution to optimise resources? (5) What is the product of choice, which source and preparation best targets a particular patient? Is self-reporting by the patient sufficient, or should the clinician who prescribed the product follow-up with the patient and report over the appropriate time course, as surgeons do for organ transplants? (6) How is the product standardised in terms of processing to ensure its optimal dilution, vehicle, dispenser and storage time? (7) How is the treatment delivered to the ocular surface, in terms of posology, dose-size modulation, duration of treatment and number of cycles?

Persistent pain throughout the day, which may even interfere with sleep at night, is the primary concern of patients. Therefore, pain reduction should be prioritised as the main outcome of treatment, rather than the traditional focus on reducing corneal signs. More pre-clinical studies are needed, and interdisciplinary work with specialists in transfusion medicine will be imperative in order to share information and skills, with a view to achieving standardised and targeted product that improves the ophthalmological patient's clinical management.

6 | CONCLUSIONS

The important role of sensory innervation in ocular surface health and disease is widely recognised, yet significant challenges remain. Overcoming these challenges requires robust interdisciplinary collaboration between preclinical and translational researchers, clinicians in their practices and the developers of new technologies, all with a view to achieving innovative diagnostic and therapeutic advances. By fostering partnerships and engaging clinicians, research associations, charitable foundations and industry stakeholders, progress can be accelerated to significantly improve patient outcomes and their quality of life.

AUTHOR CONTRIBUTIONS

AG, JG, MCA, VM, JMB-D-C, OS, NS and PV contributed to the writing of the original sections and the preparation of accompanying figures. WGKM-L, CB and JP-M were responsible for the original concept, drafting the introductory sections, editing, reviewing and supervising the preparation of the article. All authors approved the final version of their respective sections and collectively agreed on the submission of the final version of the manuscript for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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