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Exosomal cargo including microRNA regulates sensory neuron to macrophage communication after nerve trauma

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Following peripheral axon injury, dysregulation of non-coding microRNAs (miRs) occurs in dorsal root ganglia (DRG) sensory neurons. Here we show that DRG neuron cell bodies release extracellular vesicles, including exosomes containing miRs, upon activity. We demonstrate that miR-21-5p is released in the exosomal fraction of cultured DRG following capsaicin activation of TRPV1 receptors. Pure sensory neuron-derived exosomes released by capsaicin are readily phagocytosed by macrophages in which an increase in miR-21-5p expression promotes a pro-inflammatory phenotype. After nerve injury in mice, miR-21-5p is upregulated in DRG neurons and both intrathecal delivery of a miR-21-5p antagonim and conditional deletion of miR-21 in sensory neurons reduce neuropathic hypersensitivity as well as the extent of inflammatory macrophage recruitment in the DRG. We suggest that upregulation and release of miR-21 contribute to sensory neuron–macrophage communication after damage to the peripheral nerve.
Neuropathic pain is a debilitating condition and the efficacy of current treatment strategies, which include opioids and anticonvulsants, is limited by the extensive side effect profiles observed in patients. Thus, there is a necessity for novel mechanisms and therapeutic targets to be identified. Compelling evidence supports a critical role of immune cells in the mechanisms underlying neuropathic pain at the site of nerve damage in the periphery, in the dorsal root ganglia (DRG), and in the dorsal horn of the spinal cord. At the site of injury and in the DRG, monocytes/macrophages infiltrate in response to chemokines produced by Schwann cells and satellite cells. Pro-inflammatory macrophages release mediators such as cytokines and chemokines, which activate the vascular endothelium and alter the sensory transduction properties of nociceptive axons and cell bodies, causing continual activity (peripheral sensitization). In the spinal cord, microglia proliferate, change their morphology, undergo changes in gene expression, and release pro-nociceptive mediators, which can sensitize neurons and contribute to central sensitization.

Fig. 1 Expression of miR-21 is increased in DRG neurons following spared nerve injury. a–d Upregulation of miR-21 expression detected by fluorescence in situ hybridization (FISH) in ipsilateral L5 DRG neurons 7 days after SNI compared to sham injury and contralateral DRG neurons. Scale bar = 100 μm. e Quantification of miR-21+ neurons in L4/5 DRG. f–g Immunostaining for large-diameter DRG neurons (NF-200, red) and FISH for miR-21 (green) in sham and SNI ipsilateral L5 DRG. Scale bar = 100 μm. h Quantification of large cell bodies NF-200+ neurons that also express miR-21 in L4/5 DRG. i–j Immunostaining of small-diameter DRG neurons (CGRP, red) and FISH for miR-21 (green) in sham and SNI ipsilateral L5 DRG. Scale bar = 100 μm. k Quantification of CGRP+ neurons that also express miR-21 in L4/5 DRG. l–o Immunostaining of macrophages (F4/80+ cells, red), FISH for miR-21 (green), and nuclei (4′,6-diamidino-2-phenylindole (DAPI), blue) in sham and SNI DRG. Scale bar = 100 μm. Representative example of high-magnification merge (×63), a puncta (yellow) can be seen in macrophages (F4/80+ red cells), scale bar = 10 μm. Data are means ± S.E.M., n = 3 mice/group. ***P < 0.001, one-way ANOVA, post hoc Bonferroni.
Fig. 2 Capsaicin induces release of extracellular vesicles that include exosomes containing miRs from sensory neurons in culture. 

**a** Intracellular expression of miRs 21-5p, Let7b-5p, 124-3p, and 134-5p normalized to SNORD 202 (housekeeping non-coding RNA; n = 5 cultures). 

**b**-**d** Representative western blot and quantification of exosomal markers TSG101, Flotillin-1, and MFG-E8 in the culture media of DRG neurons incubated with buffer control (0.001% dimethyl sulfoxide (DMSO) in HEPES buffer + glucose 1 mg/ml; CON) or Capsaicin (1 µM; CAPS) for 3 h. Data are means ± S.E.M., n = 4 cultures; *P < 0.05 and **P < 0.01 compared to control, Student’s t-test.

**e**-**g** ImageStream™ analyses. 

**e** Pseudocolour dot plots of carboxyfluorescein succinimidyl ester (CFSE) fluorescence against side scatter (SSC) for EVs isolated from culture media of neurons incubated with buffer control or CAPS for 3 h. 

**f**, **g** Representative images and quantification of EVs. Data are means ± S.E.M., n = 4 cultures; **P < 0.01 compared to control, Student’s t-test. 

**h** NanoSight detection of exosomes isolated from culture media of neurons incubated with buffer control or CAPS for 3 h. Representative outcome is shown. Each colored line represents a single frame recording with a total of 3 frames for each sample. The inset data indicate mean diameter, mode diameter and D90 values represent the diameter of 90% of the particles. 

**i** Expression of miRs 21-5p, Let7b-5p, 124-3p and 134-5p in the exosomal fraction of DRG neurons media treated with buffer control or CAPS for 3 h. Data are means ± S.E.M., n = 4 cultures; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control, Student’s t test.
sensitization\textsuperscript{5–7}. Both central and peripheral sensitizations are fundamental for the generation of allodynia, hyperalgesia, and spontaneous pain\textsuperscript{8,9}.

The manipulation of neuron–macrophage/microglia communication is proving to be a viable instrument with which to halt the development of neuropathic pain, and both macrophage and microglia targets are being considered for novel therapeutic approaches\textsuperscript{1,2,9}.

Here we investigate the mechanisms by which neurons and macrophages communicate in the DRG and modify the inflammatory infiltrate after peripheral axon injury. Specifically, we focus our attention on the release of extracellular vesicles (EVs), including exosomes, from sensory neuron cell bodies in the DRG. Exosomes are EVs that are secreted by all types of cells, including immune cells and neurons\textsuperscript{10}. While initially thought to be a cellular mechanism of waste disposal, EVs are now also considered to be highly specified enablers of intracellular and intercellular communication\textsuperscript{11}. Exosomes derive from multivesicular bodies (MVBs) and secretory exosomes contain a specific cargo composition\textsuperscript{10}. Current evidence indicates that MVBs are present in the cell bodies of sensory neurons in the DRG rather than in peripheral or central axonal terminals\textsuperscript{12}, suggesting that cell bodies may release EVs, including exosomes under appropriate conditions. However, evidence for the ability of primary sensory neurons to secrete exosomes is yet to be established. Although electrical excitability of the cell bodies in the DRG is not necessary for signal conduction to the central nervous system, their cell membranes are electrically excitable and peripherally generated spikes, which propagate centrally, invade, and provoke activity of the soma, which also has the capacity to fire spontaneously\textsuperscript{13}. Recent in vivo imaging studies demonstrate that neuronal coupling in DRG contributes to pain hypersensitivity after peripheral injury\textsuperscript{14}.

Exosome cargo includes a variety of microRNAs (miRs), and recent evidence indicates significant deregulation of miRs in the DRG and spinal cord after nerve injury\textsuperscript{15–17}. These miRs can modulate nociception and, for instance, intrathecal delivery of miR-124, miR-103, and miR-23b attenuates inflammatory and neuropathic pain by altering intracellular neuronal, astrocytic, and microglial functions\textsuperscript{18–20}. Conversely, miR-let7b exerts a pro-nociceptive effect via mediation of neuron–neuron cross-excitation. Following its activity-induced release by DRG neurons, miR-let7b activates TRPA1 channels, thus providing positive feedback for sensory neurons\textsuperscript{21}. In addition, miR-134, which is also expressed in the DRG, is pro-nociceptive in chronic pain models\textsuperscript{22} and miR-183 cluster controls neuropathic pain-regulated genes in DRG\textsuperscript{23}.

To date, however, much of our understanding regarding miR-mediated effects on pain mechanisms is based on the use of “unpackaged” miRs. In order to advance our knowledge, it is now critical to assess the effect of miRs in a biologically relevant setting in which they are present as exosomal cargo. In this study we assess whether sensory neuron cell bodies in the DRG secrete exosome-containing miRs as a means to communicate with infiltrated macrophages after peripheral nerve injury.

Results

Exosomes containing miRs are released from DRG neurons.

Several miRs are dysregulated in sensory neurons after spared nerve injury (SNI) and in particular the expression of miR-21 increases after sciatic nerve axotomy\textsuperscript{24}. We confirmed that peripheral nerve injury induces upregulation of miR-21 expression in the lumbar DRG. Specifically, we observed relatively low expression of miR-21 in the cell bodies of sensory neurons under sham conditions (Figs. 1a, c, e) as well as contralateral to nerve injury (Figs. 1d, e) and significant upregulation of miR-21 in ipsilateral sensory neurons 7 days after SNI (Figs. 1b, e). No miR-21 expression in DRG was detected with a control scrambled probe (Supplementary Figs. 1a–d). Expression of miR-21 was elevated 7 days after SNI in both large-diameter neurons (NF200\textsuperscript{+}; Figs. 1f–h) and small-diameter peptidergic neurons (CGRP\textsuperscript{+}; Figs. 1i–k) compared to either contralateral or ipsilateral sham neurons. Predictably, while very few F4/80\textsuperscript{+} cells were found in sham injury DRG (Figs. 1l, m), in the DRG ipsilateral to injury, infiltrating macrophages (F4/80\textsuperscript{+} cells) were observed in the vicinity of sensory neuron cell bodies containing miR-21 (Fig. 1n). In addition, some macrophages expressed miR-21 fluorescence in SNI DRG (Fig. 1o) and miR-21-positive macrophages were more abundant in DRG ipsilateral compared to contralateral SNI and ipsilateral sham injury (Supplementary Fig. 1e).

In order to confirm that the endogenous expression of miR-21 and other selected miRs could be detected in DRG, we measured extracellular expression in dissociated DRG cultures. Expression of miR-21–5p was comparable to miR-let7b, miR-124, and miR-134, which were selected as positive-control miRs (Fig. 2a). As we were interested in determining whether sensory neurons release EVs and miRs from their cell bodies following noxious-like activation, we treated cultured DRG with capsaicin. Incubation of capsaicin for 25 min and 3 h resulted in a significant accumulation, in the extracellular fraction of the culture media, of the endosomal sorting complex required for transport (ESCRT-I) component Tumor Susceptibility Gene 101 (TSG101; Fig. 2b and Supplementary Fig. 2a), which is an intracellular protein central for exosomal sorting from MVBs\textsuperscript{25–27}. Capsaicin treatment for 3 h, but not 25 min, also induced a significant extracellular increase of the exosomal marker Flotillin-1 (Fig. 2c and Supplementary Fig. 2b), which belongs to the family of lipid raft-associated proteins and is involved in endosome and exosome recycling\textsuperscript{28}. Similarly, capsaicin incubation for 3 h, but not 25 min, significantly increased the extracellular expression of the exosomal protein Milk Fat Globule-E 8 protein (MFG-E8; Fig. 2d and Supplementary Fig. 2a), which is an adhesion molecule and acts as an opsonin for cells to be engulfed by phagocytes following binding to αvβ5 integrin in macrophages\textsuperscript{29,30}. Capsaicin incubation was not associated with neurotoxicity as determined by LDH assay (data not shown). Consistent with an activity-dependent release of exosomal markers, incubation of cultured DRG with depolarizing agent potassium chloride at 25 and 50 mM for 3 h promoted release of TSG101 and MFG-E8 (Supplementary Fig. 3). However, the highest concentration was required for Flotillin-1 band to become visible (Supplementary Fig. 3). Furthermore, treatment of pure sensory neurons in culture with capsaicin induced significant release of EVs compared to buffer incubation alone (Figs. 2e–g). We could readily observe neuronal EVs by ImageStream\textsuperscript{TM} flow cytometry (Figs. 2e, f) which were significantly elevated in capsaicin compared to control media (Fig. 2g). NanoSight tracking analysis confirmed that capsaicin incubation doubled the number of EVs in the media and revealed that both control and capsaicin media were rich in particles, 90% of which have a diameter below 125 nm with a mode of 74.7 nm and hence can be genuinely considered as exosomes (Fig. 2h and Supplementary Fig. 4a).

Expression analysis of miRs in the exosome fraction of cultured DRG media indicated that capsaicin significantly increased levels of miR-21–5p, let7b, miR-124, and miR-134 compared to control conditions (Fig. 2i). In keeping with this increase of miRs in EV fractions, in DRG whole-cell lysates, capsaicin induced a significant increase in Dicer mRNA and Dicer protein fragments isolated by immunoprecipitation (∼90 and 66 kDa; Supplementary Figs. 4b, c). As the RNase type III enzyme Dicer is the rate-
limiting enzyme in the miR formation from pre-miR, these data suggest that capsaicin may trigger Dicer activity in sensory neurons. The effect of capsaicin was TRPV1 receptor-mediated. In fact, capsaicin-induced release of TSG101 and MFG-E8 positive EVs was significantly reduced when dissociated DRG obtained from mice deficient in TRPV1 were used (Fig. 3a). Similarly, the release of miR-21-5p, let7b, miR-124 and miR-134 measured in the exosomal fraction of wild-type (WT) DRG was absent in DRG obtained from TRPV1 knockout (KO) mice (Figs. 3b–e). As TRPV1 is exclusively expressed by neurons and not satellite cells, we can confidently attribute these differences in release between WT and TRPV1 KO mice as neuronal.

These in vitro data indicate that exosomes containing miRs, such as miR-21-5p, are released from the cell bodies of TRPV1-expressing sensory neurons. In the in vivo scenario of the DRG after peripheral nerve injury, nociceptive neuron-derived exosomes may interact with macrophages, which infiltrate in response to nerve injury. Such macrophages may phagocytose miR-21-containing exosomes, especially those expressing the adhesion molecule MFG-E8, which we have identified in our EV preparations.

**Sensory neuron-derived EVs are phagocytosed by macrophages.** To assess whether macrophages phagocytosed neuron-derived exosomes, resulting in functional transfer of miR-21-5p, we incubated peritoneal macrophages with exosome-enriched media derived from pure sensory neurons in culture and made the following three observations. Firstly, EVs were effectively transferred into macrophages as confirmed by ImageStream™ analysis using fluorescently labeled vesicles. Neuron-derived EVs (CFSE-labeled; green) were rapidly engulfed by primary macrophages (F4/80+ in Fig. 4a) as demonstrated by both augmented positive events in the dot plots and representative images.
Quantification at single-cell level revealed that macrophages accumulated significantly more EVs following incubation with capsaicin-derived media compared to control media (Fig. 4c). Secondly, EV phagocytosis had an impact on macrophage phenotype, as evident by an increase in mRNA for inducible nitric oxide synthase (iNOS, Nos2, M1 marker) and decrease in mRNA for CD206 (mannose receptor, Mrc1, M2 marker) in macrophages incubated with neuron-derived EVs.

Fig. 4 Exosomes released from pure sensory neurons after capsaicin are phagocytosed by macrophages. a, b Representative scatterplots (gating strategy) and ImageStream™ images showing EVs (CFSE-labeled, green) uptake by macrophages (F4/80+, red). EVs were isolated from culture media after incubation of pure DRG neurons with buffer control or CAPS for 3 h. c Percentage-positive and median fluorescence intensity of CFSE+ macrophages incubated with neuron-derived EVs. Data are means ± S.E.M., n = 4. *P < 0.05, compared to control, Student’s t-test. d Nos2, Mrc1, and Spry2 mRNA expression levels in macrophages incubated with and without exosomes derived from CAPS-treated pure DRG neurons. e Nos2 and Spry2 mRNA expression levels in macrophages transfected with miR-21-5p antagomir or scrambled oligomer and incubated with EVs derived from CAPS-treated pure DRG neurons. Data are means ± S.E.M., n = 3; *P < 0.05 and **P < 0.01 Student’s t-test.
compared to macrophages not exposed to exosomes (Fig. 4d). Consistent with possible miR-21 transfer in macrophages, the expression of Sprouty2 mRNA (Spry2) a known miR-21 target was downregulated (Fig. 4d). Finally, inhibition of miR-21-5p prevented the effect of neuron-derived exosomes on cell phenotype as treatment of macrophages with a specific miR-21-5p antagonist resulted in lower expression of Nos2 concomitant to higher expression of Spry2, compared to treatment with scrambled oligomer (Fig. 4e). We have observed that macrophages expressed basal constitutive levels of miR-21-5p, although to a much lesser extent than other miRs, such as miR-155-5p (Fig. 5a). Thus, to further investigate possible transfer of miR-21, we silenced miR-21-5p (Figs. 5b, c) and incubated macrophages with exosomes isolated from either non-viral transfected or miR-21-overexpressing sensory neurons.

Under both conditions, we observed that in antagonist-transfected macrophages incubation of neuron-derived EVs produced a significant increase in miR-21-5p expression (Figs. 5b, c). Macrophage incubation with miR-21-overexpressing neuron EVs was associated with upregulation of Nos2 (Fig. 5d), whereas in the presence of the antagonist, incubation of miR-21-overexpressing neuron EVs resulted in higher expression of Mrc1 and Spry2 (Fig. 5e).

Altogether, these in vitro observations indicate that sensory neurons can transfer miR-containing exosomes to macrophages and this transfer results in changes of cell phenotype as well as intracellular levels of miR-21-5p and known miR-21 gene targets.

**Overexpression of miR-21-5p promotes M1 macrophage phenotype.** To define the effect of increased intracellular miR-21-5p on macrophage polarization, we transfected primary peritoneal macrophages with either fluorescence-labeled miR-21-5p (miR-21-5p mimic) or the scrambled sequence termed N4. Macrophage transfection with miR-21-5p mimic produced more than a 90% yield efficiency in F4/80+ cells and resulted in a significant increase in miR-21-5p expression relative to controls (Figs. 6a, b). Transfection with N4 displayed the same efficacy as miR-21-5p mimic, but, as expected, did not result in increased expression of miR-21-5p (Fig. 6b). The expression of Spry2 was downregulated in miR-21-5p mimic-transfected macrophages compared to control transfection and N4-transfected cells (Fig. 6c).

Subsequent analysis of polarization markers in miR-21-5p-transfected macrophages revealed upregulation of several pro-inflammatory markers compared to N4 transfection and control transfection. Specifically, we detected an increase of both protein and mRNA for iNOS and transcription nuclear factor κB p65 (NF-κB p65, Rela; Figs. 6d, e). Furthermore, miR-21-5p transfection, more significantly than N4 and control transfection, reduced the transcriptional levels of Mrc1 and Arginase-1 (Arg1; Figs. 6e). Consistent with these intracellular changes, extracellular levels of pro-inflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-6, were higher in the media obtained from miR-21-5p-transfected macrophages compared to either control transfection or N4 media (Fig. 6f).

These in vitro transfection data support evidence for a pro-inflammatory role of miR-21 by showing that an increase in intracellular miR-21-5p induces macrophage polarization toward a pro-inflammatory M1 phenotype.

In order to substantiate our finding, we performed flow cytometry analyses of miR-21-5p-transfected macrophages and
observed a significant shift in favor of the M1 population. Specifically, macrophages that were transfected with miR-21-5p were significantly polarized toward M1 (CD206−CD11c+) compared to both N4-transfected cells and control transfection (Figs. 7a, e) while the proportion of M2 cells (CD206+CD11c+) was not significantly altered (Figs. 7a, f). Notably, the numbers of CD45+, F4/80+CD11b+, and CD206+CD11c+ cell populations were comparable after control, N4 and miR-21-5p transfection (Figs. 7a–d).

Inflammatory macrophages infiltrate ipsilateral DRG. One week after nerve injury, inflammatory macrophages infiltrate in the DRG in higher numbers than in sham-injured DRG9. By performing flow cytometry analysis of leukocytes isolated from Day-7 sham-operated and neuropathic mice, we demonstrated a significant infiltration of leukocytes (CD45+ cells) in DRG ipsilateral to peripheral nerve injury compared to contralateral DRG (Supplementary Figs. 5b, c). This infiltration was also apparent under sham conditions, but to a lesser extent (Supplementary
miR-21 antagonist prevents nociceptive hypersensitivity. Having observed that (i) macrophages that had infiltrated into the DRG after injury displayed a pro-inflammatory M1 phenotype; (ii) DRG sensory neurons were able to release exosomes containing miR-21-5p in an activity-dependent manner; (iii) miR-21-5p overexpression in macrophages was associated with M1 phenotype, we hypothesized that in vivo the ongoing nociceptive neuron activity contributes to M1 polarization of macrophages through exosome release, which serves as a neuron–macrophage communication mediator.

Specifically, we postulated that neuronal miR-21 could contribute to the nociceptive hypersensitivity and influence the nature of the inflammatory infiltrate in the DRG after peripheral nerve injury. In order to test this hypothesis, we took a dual approach by (i) performing prolonged intrathecal delivery of the miR-21-5p antagonist and (ii) generating sensory neuron conditional miR-21 null mice. We observed that intrathecal delivery of the miR-21-5p antagonist, but neither the scrambled oligomer nor transfecting agent (vehicle), significantly prevented the development of nerve injury-associated nociceptive hypersensitivity from day 2 to 7 by ~50% (Fig. 8a). Critically, delivery of the miR-21-5p antagonist did not have an effect on mechanical thresholds contralateral to injury (Fig. 8b). The administration of the miR-21-5p antagonist to naive mice significantly enhanced expression of Spry2 compared to the scrambled oligomer (Supplementary Fig. 6a) and reduced miR-21-5p expression in the lumbar DRG (Supplementary Fig. 6b), confirming efficient delivery of the construct. As both the miR-21-5p antagonist and scrambled oligomer were fluorescently tagged, we examined their distribution. No fluorescence was observed in the spinal cord (Supplementary Fig. 7). However, both compounds had reached the ipsilateral and contralateral DRG where they accumulated preferentially in the cell bodies of sensory neurons (Figs. 8c, d, i and Supplementary Fig. 8). The constructs were found in up to 20% of the macrophages (F4/80+ cells, Figs. 8e, f, i) and 5% of satellite cells (GFAP+; Figs. 8g-i). Furthermore, immunohistochemical analysis revealed that miR-21-5p antagonist treatment significantly reduced the number of macrophages (F4/80+ expressing cells) in DRG ipsilateral to injury compared to treatment with the scrambled oligomer (Figs. 8j, k). Systemic administration of the same dose of miR-21-5p antagonist for 7 days via subcutaneous pumps altered neither SNI mechanical hypersensitivity nor the number of macrophages compared to scrambled oligomer (Supplementary Fig. 9), suggesting that the intrathecal oligomer was effective locally.

miR-21 antagonist modulates macrophage phenotype in DRG. We then went on to assess the phenotype of macrophages present in the DRG following peripheral nerve injury under the intrathecal treatment conditions. Flow cytometry analysis of the leukocyte population (CD45+ cells) in the DRG revealed, following injury, a significant higher number of M1 macrophages in ipsilateral compared to contralateral DRG when the scrambled oligomer was delivered continually for 7 days (Figs. 9a, c–f), similar to that observed in untreated neuropathic DRG (Supplementary Fig. 5). However, delivery of the miR-21-5p antagonist altered the macrophage profile in DRG ipsilateral to injury. We detected a reduction in CD45+ cells (leukocytes; Fig. 9b) and no longer observed a significant difference in this population between ipsilateral and contralateral DRG (Fig. 9c). We also observed a significant reduction in F4/80+CD11b+ cells (macrophages) as well as CD206+CD11c+ cells (M1 phenotype) compared to ipsilateral DRG treated with the scrambled oligomer (Figs. 9a, b, d, e). M2 macrophages (CD206+CD11c−) were significantly reduced in ipsilateral compared to contralateral DRG following injury when mice were treated with the scrambled oligomer, but not the miR-21-5p antagonist (Fig. 9f). Thus, 7-day-intrathecal administration of a miR-21-5p antagonist, which accumulated predominantly in the cell bodies of sensory neurons in the DRG, resulted in a reduction in pro-inflammatory macrophage infiltration in the DRG and a significant behavioral antinociceptive effect.

miR-21 deletion in sensory neuron prevents hypersensitivity. In order to establish the cellular source of miR-21 and further substantiate the proposed mechanism of neuroimmune interaction, we silenced miR-21 expression selectively in sensory neurons, and achieved a significant 50% downregulation of miR-21 expression in the miR-21 conditional KO (cKO) compared to WT littermates DRG (Fig. 10a). After peripheral nerve injury, in cKO we observed an attenuation of ipsilateral nociceptive hypersensitivity compared to WT, which reached significance on days 5–7 (Fig. 10b). Contralateral thresholds were comparable between cKO and WT (Fig. 10b). In the lumbar DRG of cKO the number of CD45+ cells, which had infiltrated by day 7 from nerve injury was significantly lower than in WT (Fig. 10c). Macrophage numbers (F4/80+CD11b+ cells; Figs. 10d, e) and M1 cells (CD206+CD11c+) were significantly reduced in cKO compared to WT ipsilateral DRG. Notably, numbers of M2 macrophages (CD206−CD11c−) were elevated in cKO ipsilateral DRG.
compared to WT DRG (Fig. 10g). To identify possible gene targets that are regulated by neuron-derived miR-21 to drive the macrophage response, we performed a genome-wide microarray analysis of macrophages (F4/80+CD11b+ cells) isolated from L4 and L5 DRG of WT and cKO at 7 days after SNI (Supplementary Fig. 10a). We found that 2207 genes were statistically and
significantly regulated in the sorted macrophage population (Supplementary Fig. 10b) and a number of pathways were significantly perturbed by neuronal miR-21 absence (Supplementary Fig. 10c). To gain further insight from these pathways, we examined the cohort of regulated genes and found that three known target genes of miR-21 were significantly regulated in the macrophages (Supplementary Fig. 10d). Specifically, we have identified genes coding for two binding proteins, Acta2, Tpm1, and one transcription factor, znf288, that are responsible for cytoskeleton remodeling and cell survival (Supplementary Fig. 10d).

**Discussion**
This study provides novel evidence for the dysregulation of non-coding miRs in sensory neurons after peripheral axon injury,
which regulates the nature of the inflammatory infiltrate in the DRG microenvironment and exerts a significant impact on the development of neuropathic hypersensitivity. Specifically, peripheral nerve injury induced upregulation of miR-21 in ipsilateral DRG neurons, which was associated with ipsilateral mechanical hypersensitivity. The intrathecal delivery of a miR-21-5p antagonir resulted in (i) downregulation of miR-21-5p expression and upregulation of Spry2 in DRG; (ii) prevention of the development of ipsilateral mechanical hypersensitivity; and (iii) reduction of inflammatory macrophage number in DRG. As predicted, systemic delivery of the same dose of the antagonir did not alter neuropathic hypersensitivity, suggesting that intrathecal...
delivery of the antagonir acted locally and systemic distribution of the same dose of antagonir possibly diluted the active concentration of the oligomer. It would be worthwhile to test whether higher systemic doses of the antagonir display antihyperalgesic efficacy and target miR-21 at sites additional to the DRG.

Consistent with the intrathecal antagonir data, deletion of miR-21 expression in sensory neurons resulted in development of a less severe mechanical hypersensitivity and a marked reduction of inflammatory macrophage infiltration in the DRG. Moreover, selective deletion of neuronal miR-21 was associated with a significant presence of anti-inflammatory macrophages that displayed significant alteration in known miR-21-5p target genes.

Furthermore, we show in vitro that capsaicin stimulation of sensory neurons causes the release of exosomes containing miR-21-5p, that when phagocytosed by macrophages, promotes an increase in the expression of pro-inflammatory genes and proteins. Therefore, we conclude that the release of exosomes containing miRs is a plausible mechanism of neuron-macrophage communication in the DRG after nerve trauma. Whether EVs transfer pre-miR-21 and/or induce transcription of miR-21 in macrophages remain interesting possibilities. However, these data are indicative of neuronal miR-21 being a critical regulator, either directly or indirectly, of large number of cellular processes that underpin the macrophage phenotypes.

The cell bodies of sensory neurons in the DRG are invaded by action potentials traveling from the periphery to the spinal cord. Following peripheral nerve injury, ectopic impulses generated in the DRG become substantial and neuron-to-neuron coupling contributes to neuropathic pain13, 14, 33. Sensory neurons can modulate immune cells’ function, for instance, by releasing inflammatory neuropeptides that mediate neurogenic inflammation34. We provide in vitro evidence that nociceptive neuron activity results in the secretion of EVs, including exosomes, which in vivo could serve the novel function of providing a mode of communication with nearby macrophages. The formation of MVBs and secretion of exosomes remain exclusive to sensory neuron cell bodies, as peripheral nervous system axons are unlikely to anteroretrogradely transport MVBs, which are rarely detected in PNS axons in vivo12. In dissociated DRG culture, neurons are mixed with satellite cells; however, we provide imaging evidence that pure sensory neurons release EVs, including exosomes, after capsaicin, which activates neuronal TRPV1 receptors leading to upregulation of intracellular Dicer. Thus, we demonstrate that sensory neuron cell bodies can perform regulated exocytosis of EVs in response to noxious-like activation and specific intracellular signaling pathways. Furthermore, detection of exosomal markers TSG101, Flotilllin-1, and the adhesion molecule MFG-E8 in dissociated DRG culture media indicates the presence of exosomes, which can interact with phagocytic cells such as macrophages. Exosomal cargo can vary depending on cell type and stimulus, and we observed that sensory neuron-derived exosomes contain several miRs including miR-21, which is upregulated in the DRG following peripheral nerve injury. miR-21 has been shown to play a significant intracellular role in promoting neurite outgrowth through downregulation of the Spry2 protein7 and it is conceivable that miR-21 regulates targets in sensory neurons that can influence nociceptors’ function directly or indirectly at peripheral and central terminals.

Our data provide evidence for an extracellular role of neuron-derived miR-21, which is released as part of exosomal cargo. Released upon DRG neuron soma activity in exosomes, when phagocytosed and overexpressed by macrophages, miR-21 would polarize macrophages toward a pro-inflammatory over an anti-inflammatory phenotype as evident by several validated markers. We acknowledge evidence that miR-21 may upregulate IL-10 in macrophages after 24h-TLR4 stimulation, which mimics an infective status35. It is conceivable that the modulatory roles of miR-21 on macrophage polarization are plastic as well as dependent on time, stimulus, and specific microenvironments. Following traumatic nerve injury we could demonstrate a functional link between overexpression/deletion of miR-21 with pro-/anti-inflammatory macrophages both in vitro and in vivo.

Inflammatory macrophages infiltrate the DRG after peripheral axon injury and release cyto/chemokines that contribute to neuronal sensitization3, 9. We suggest that in vivo neuron-macrophage transfer of exosomes containing miR-21 may serve as a regulator of macrophage phenotype and consequentially promotes a pro-nociceptive environment. Herein, we make two observations that demonstrate that DRG neurons are a critical cellular source of exosomes containing miR-21. Specifically, stimulation of sensory neurons in vitro with capsaicin induced release of miR-21-5p in a TRPV1-mediated manner, suggesting that nociceptive neurons are a likely source of miR-21. Intrathecally delivered miR-21-5p antagonir in vivo, which significantly prevented the development of neuropathic hypersensitivity and showed preferential tropism toward neuronal cells over satellite cells and macrophages. Notably, the antagonir showed limited distribution to the spinal cord and probably higher centers.

Consistent with a neuronal origin of miR-21, the deletion of miR-21 expression in sensory neurons was associated with antinociceptive behavior, polarization of macrophages toward an anti-inflammatory phenotype, and alteration in macrophages of known miR-21-5p target genes that are responsible for cytoskeleton remodeling and cell survival. As the macrophage phenotype is associated with change in their shape that depends on contractility within the actin cytoskeleton36, future studies will determine potential direct association between cytoskeletal changes and downstream effects of miR-21-5p on macrophage polarization and function.

In summary, this study demonstrates not only a novel mechanism by which neuron–macrophage communication occurs, but also a potential function of DRG neuron excitability in the context of nerve trauma-associated pain. Cell bodies of sensory neurons in the DRG release exosomes and selected miRs, including miR-21-5p, following specific activation of nociceptive neurons by capsaicin. Once released, exosomes can be phagocytosed by infiltrating macrophages where elevated miR-21-5p expression is accompanied by an increase in pro-inflammatory and decrease in anti-inflammatory phenotype.

These data have multiple implications and can open a series of opportunities. Firstly, we suggest that targeting this mode of
neuron–macrophage communication could prove to be a promising and innovative analgesic strategy and provide an alternative to current treatments of pain following nerve trauma, which show limited efficacy at present. Secondly, specific elements of the exosome cargo could be targeted and we provide here an example of such approach centered on miR-21-5p. Finally, the possibility of delivering exosomes in a tissue-specific manner may spare essential neuron–macrophage communication, providing a potential therapy with limited side effects. We note that preparations of exosomes derived from dendritic cells have entered clinical trials for immunotherapy in cancer patients and both production and characterization of clinical-grade exosome products are being actively pursued. The application of this technology could be translated to the treatment of neuropathic pain.

Methods

Animals. Experiments were carried out in 8–12-week-old male C57BL/6 mice according to the United Kingdom Animals (Scientific Procedures) Act 1986, and following the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. Adult TRPV1 KO mice and WT control littermates were kindly donated by Stuart Bevan, King’s College London. Animals were housed with ad libitum access to food and water and maintained on a 12 h light/dark cycle. Experimental study groups were randomized and blinded.

Fig. 10 Conditional deletion of miR-21 in sensory neurons prevents the development of mechanical hypersensitivity and is associated with polarization of macrophages toward an anti-inflammatory phenotype. a Downregulation of miR-21-5p levels in DRG of miR-21 cKO compared to WT littermate mice. Data are expressed as means ± S.E.M., n = 5 mice for each group. #P < 0.05 compared to WT, Student’s t-test. b Effect of miR-21 deletion in DRG sensory neurons on the development of mechanical hypersensitivity following SNI. Data are presented as 50% of paw withdrawal thresholds (PWT); means ± S.E.M., n = 10 mice. #P < 0.05, ##P < 0.01, compared to WT ipsilateral, two-way ANOVA followed by Tukey test. c Bar charts represent absolute number of leukocyte in DRG. d Representative scatterplots of immune cells sorted from pools of ipsilateral L4 and L5 DRG obtained from WT mice or miR-21 cKO mice, on day-7 post SNI injury. Numbers in gates refer to the percentage of positive cells for each specific marker. e Number of macrophages, f M1 macrophages (g) and M2 macrophages. Statistical analysis was performed on data from two independent experiments. Data are expressed as means ± S.E.M., n = 4 for each group. *P < 0.05, **P < 0.01, and ***P < 0.001, one-way ANOVA, post hoc Bonferroni.
Generation of sensory neuron-specific miR-21 null mice. The mouse line miR-21, with a KO first conditional allele, was sourced from the Jackson Laboratory (accession number 36060). Mice were bred at Embl and DRG-Deleter mice were subsequently with Avil-Cre driver mice for conditional ablation in DRG27. The geno-typing strategy of the line has been described previously27,28. Conditional KO and WT littermates (8–10-week-old males) were used. Procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were maintained at the EMBL, Mouse Biology Unit, Monterotondo, Italy, in accordance with Italian legislation (Art. 9, 27 January 1992, no 116) under licence from the Italian Ministry of Health.

Behavioral testing. Static mechanical withdrawal thresholds were assessed by application of calibrated von Frey monofilaments (0.02–1.0 g) to the hind paw plantar surface. Testing started with the application of a 0.07 g filament and each paw was cycled between application and removing stimulus intensity, until a withdrawal response was achieved or application of 1.0 g filament failed to induce a response, in order to avoid tissue damage. The 50% paw withdrawal threshold (PWT) was determined by increasing or decreasing stimulus intensity, and evaluated using Dixon’s “up–down” method. Experiments were performed blind.

Induction of neuropathy. Mice received a SNL21 under isoflurane anesthesia. Briefly, the skin and muscle of the left thigh were incised to expose the sciatic nerve and its three terminal branches. The common peroneal and tibial nerves were tightly ligated and the distal nerve stump was removed, while the sural nerve was left intact. In sham-injured mice, sciatic nerve was exposed but not ligated or excised. PWTs were examined prior to and daily from days 2 to 7 after surgery.

Intrathecal and subcutaneous delivery of oligomers. Intrathecal cannula catheterization was performed on the same day of SNL surgery. Under anesthesia a small laminectomy was made over the thoracic spinal cord40. A polyethylene catheter (Alzet, Charles River Ltd, UK) was inserted under the dura mater in the lumbar enlargement and attached to a subcutaneous osmotic pump (Alzet 1007D, Charles River Ltd, UK). For subcutaneous oligomer administration pumps were not connected to the cannula. An LNA-based miR-21-5p inhibitor and scrambled control oligor were custom-made as fluorescein amide (FAM)-labeled compounds by Exiqon (Denmark). Sequences are reported in Supplementary Table 1. The oligomers were mixed (1:5 v/v) with 1-Fect in vivo transfection reagent (Neuromics, 2B Scientific, UK) and delivered for 7 days at 12 pmol/day. At the end of treatments, catheter and pump were checked to ascertain efficient delivery.

In situ hybridization and immunohistochemistry. Sections (10 μm) were obtained from fresh frozen L4 and L5 DRG using a cryostat (Leica CM3050, UK) and mounted onto Superfrost Plus slides (Thermo Scientific, UK). Cryosections were fixed in 4% paraformaldehyde (PFA) and treated with proteinase K (5 μg/mL; Sigma, UK). The sections were acetylated with 1.5% triethanolamine/0.25% acetic anhydride (1:3 v/v) for 10 min, air-dried in a hybridization chamber and baked at 80 °C for 1 h. Hybridization was performed with digoxigenin (DIG)-labeled probe complementary to mouse miR-21 (0.5 pmol, LNA miRCURY probe; Exiqon). Scrambled probes were used as controls. Probe oligonucleotides were hybridized to sections with 1 μg mouse anti-DIG horseradish peroxidase antibody (1:500, Abcam, UK). After in situ hybridization signals were enhanced using a tyramide amplification system labeled with Alexa-488 (Invitrogen, UK). Following hybridization, sections were incubated with a mouse anti-GFAP (1:400, Millipore), followed by an anti-rabbit Alexa Fluor (10 μm) were taken using a cryostat (Bright Instruments) and thaw-mounted onto Superfrost Plus slides (Thermo Scientific, UK) and mounted onto Superfrost Plus slides (Thermo Scientific, UK). Cryosections were fixed in 4% paraformaldehyde (PFA) and treated with proteinase K (5 μg/mL; Sigma, UK). The sections were acetylated with 1.5% triethanolamine/0.25% acetic anhydride (1:3 v/v) for 10 min, air-dried in a hybridization chamber and baked at 80 °C for 1 h. Hybridization was performed with digoxigenin (DIG)-labeled probe complementary to mouse miR-21 (0.5 pmol, LNA miRCURY probe; Exiqon). Scrambled probes were used as controls. Probe oligonucleotides were hybridized to sections with 1 μg mouse anti-DIG horseradish peroxidase antibody (1:500, Abcam, UK). After in situ hybridization signals were enhanced using a tyramide amplification system labeled with Alexa-488 (Invitrogen, UK). Following hybridization, sections were incubated with a mouse anti-GFAP (1:400, Millipore), followed by an anti-rabbit Alexa Fluor 685 secondary antibody (1:1000, Invitrogen). The immunoreactivity was visualized using a Zeiss LSM710 confocal microscope and images were acquired using the LSM software (Zeiss, UK). Positive cells were quantified in areas of 25 μm x 10 μm² with ImageJ software (version 1.46r, Wayne Rasband, National Institutes of Health, Bethesda, MD). At least four sections from three mice per group were used. For flow cytometry analysis, L4 and L5 DRG pooled from six samples were resuspended in F-12 Nutrient Mixture (Ham; Gibco) supplemented with dispase (3 mg/ml, Roche), collagenase type IV (0.1%, Worthington), and DNAseI (200 U/ml, Roche). Cells were triturated in MEM Nutrient Mixture F-12 Ham (Sigma–Aldrich) containing 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco; DRG medium). Cell suspensions were then centrifuged at 800 RPM for 5 min and pellets resuspended in fresh medium (1.5% BSA in HBSS, Gibco).

Primary cultures of dissociated DRG neurons. DRG were collected and placed into F-12 Nutrient Mixture (Ham; Gibco) supplemented with 0.1% collagenase type IV (Worthington). Thereafter, DRG were triturated and cell suspensions centrifuged at 600 RPM for 6 min. Pellets were resuspended in fresh DRG medium supplemented with NGE (20ng/ml) and plated on poly-L-ornithine (100 μg/ml, Sigma–Aldrich)-pre-coated glass coverslips. Cultures (10,000–22,500 cells/well) were incubated at 37 °C for 24 h. In the experiments where miR-21 was overexpressed, cultured neurons were transduced with a green fluorescent protein (control) or miR-21 lentiviral vector24 and were incubated at 37 °C for 72 h.

Primary cultures of pure sensory neurons. Non-neuronal cells were separated from DRG with TrypLE (Life Technologies) and 0.1% trypsin (Gibco) by centrifugation through a 70-μm filter and centrifuged for 8 min at 1000 RPM. Pellets were washed, resuspended, and incubated in MACS buffer containing 0.5% w/v BSA and non-neuronal biotin antibody cocktail for 5 min on ice. After wash, cells were incubated with anti-biotin microbeads (Miltenyi Biotech) and purified through LD columns in the QuadroMACS separator. The eluates were centrifuged at 1000 RPM for 8 min and pellets resuspended in DRG medium. Plates were coated with 1:10 serum in phosphate-buffered saline (PBS). Dishes were followed by 1:100 Alexa Fluor 488 goat anti-rat (BD Bioscience) in F-12. Approximately 8000 cells/well were incubated at 37 °C for 24 h.

On the experimental day, neurons were either incubated with 0.001% DMSO or caspacin (1 μM) for 25 min and 3 h or KCl (25 and 50 mM) for 3 h. After stimulation, an aliquot of the supernatants was collected for LDH cytotoxicity assay (Pierce, Thermofisher) and the remaining volume centrifuged at 13,000 g at 4 °C for 2 min to remove apoptotic bodies and cell debris. Thereafter, exosomes were isolated by ultracentrifugation (100,000 g), while cells were collected for Dicer immunoprecipitation, western blot analysis, and real-time PCR.

EV isolation and analysis. Pure neuron culture media were centrifuged at 13,000 g for 2 min at 4 °C to remove apoptotic bodies and cell debris. Supernatants were incubated with CellTrace® CFSE dye (1:1000, Invitrogen) for 10 min on ice. Then, samples were ultracentrifuged at 100,000 g at 4 °C for 1 h. EVs deposited at the bottom of each tube were subjected to ImageStream® analysis or incubated with macrophages for uptake experiments. Acquisition on the ImageStream® MKIII was conducted at slow flow rate and x60 magnification, with the “Remove Beads” option switched off. The 488 nm laser was set at 200 mW, the side scatter laser was set at 70 mW, and CFSE fluorescence was detected in channel 2. Data are expressed as vesicles/ml (after ensuring stabilization of flow rate). EV size distribution analysis was performed using an NS300 Nanoparticle Tracker with 488 nm scatter laser and high-sensitivity camera (Malvern Instruments Ltd, Malvern, UK). For each sample, a 350 μl particle scatter was recorded three times on each of four different flow conditions (arbitrary speed 50) at camera level 16 and analysis threshold 5, using the NTA 3.2 acquisition and analysis software.

Primary macrophage in culture. Macrophages were obtained by lavage of the peritoneal cavity with 1% penicillin/streptomycin sterile saline, plated, and allowed to adhere. Thereafter, non-adherent cells were removed by washing and adherent macrophages covered with red fluorescent microbeads (1 μm, 0.1% w/v, Molecular Probes) and were washed to remove non-adherent cells under flow conditions (arbitrary speed 50) at camera level 16 and analysis threshold 5, using the NTA 3.2 acquisition and analysis software.

To measure specific binding, macrophages were washed with PBS and resuspended in fresh medium (1.5% BSA in HBSS, Gibco) without FBS. For the competitive inhibition assay, macrophages were preincubated with the competitive ligand (diluted 1:10–1:50) for 15 min prior to labeling with fluorescent microbeads (Zeiss), and transfection efficacy (%) after 48 h was calculated using the following formula for the correct transfection efficiency as described:

\[ \text{Transfection efficacy (%) after 48 h} = \left( \frac{D - E}{ \text{V0} } \right) \times 100 \]
template sample was diluted to 5 ng/μl. SYBR Green PCR for miRs 21-5p, Let7b-5p, 124-3p, and 134-5p was performed using ExiLENT (2010). Small RNAs were isolated and miR levels detected by quantitative polymerase chain reaction (qPCR).

For flow cytometry analysis, plated cells were detached with Ca2+/Mg2+ free phosphate buffer saline containing EDTA (10 mM), centrifuged at 2,000 RPM for 8 min at 4 °C, and pellets were then resuspended in 1.5% BSA in 1× HBSS. TNF-α and IL-6 levels were quantified in the culture media of macrophages using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems®).

Imaging neuron-derived vesicles’ incubation with macrophages. Adherent peritoneal macrophages (2 × 106 cells/well) were cultured for 24 h at 37 °C and then incubated with CFSE-stained EVs derived from pure DRG neurons for further 1 h. Cells were then washed and resuspended in buffer (10 mM EDTA in DPBS supplemented with 3% FBS) containing anti-mouse-FITC F4/80 (1:1200, ebioscience) for 30 min. After fixation (BD Bioscience fixation buffer) and centrifugation, cells were acquired on the ImageStream M® MKII, at slow flow rate and x60 magnification, with the 488 nm laser set at 200 mW, the 633 nm laser set at 100 mW, and the side scatter laser set at 3.7 mW. F4/80-APC fluorescence was detected on channel 11, EV CFSE fluorescence was detected on channel 2, brightfield images were acquired on channels 1 and 9, and side scatter was detected on channel 6. Appropriate single fluorochrome controls were used for compensation, and macrophages treated with unbleached vesicles were used as a fluorescence reference minus one gating control for CFSE positive. Single macrophages were gated based on expression and total cell area and aspect ratio. Data are expressed as percentage of macrophages positive for CFSE and median fluorescence intensity of all singlet macrophages.

Real-time PCR. Intracellular miRNAs and Dicer mRNA levels in cultured DRG: Neurons were lysed with a mirVana RNA Isolation Kit (Ambion). Total and small RNA-enriched fractions were isolated and RNA- eluted using RNase-free RNase. Real-time PCR are expressed as percentage of macrophages positive for CFSE and median fluorescence intensity of all singlet macrophages.

Flow cytometry analyses. Isolated DRG neurons and peritoneal macrophages were resuspended in HBSS plus 1.5% BSA. An aliquot of cell suspension was used for counting to derive the absolute number of cells in each sample. Cells were stained on ice for 20 min with anti-mouse CD16/CD32 (Clone 2.4G2, BD Biosciences) to block Fc receptors, followed by incubation with a mix of fluorochrome-conjugated anti-mouse antibodies: CD45.1-Pacific Blue™ (Clone 30-F11, BioLe- gend), F4/80 PE (Clone RM6-1, ebioscience), CD11b-APC (Clone M1/70, ebioscience), CD206-PE-Cy7 (Clone C068C2, BioLegend), and CD11c-APC eFluor780™ (Clone N418, ebioscience). After washes, cells were diluted in flow buffer and run through a LSRII® Fortessa™ cell analyzer (BD Bioscience). Samples were analyzed with FlowJo software (Tree Start, Ashland, OR, USA).

Genome-wide microarray analysis of sorted macrophages. Macrophages (F4/80+ CD11b+ 2–5000 cells) were sorted from a pool of ipsilateral L4/L5 DRG of SNI WT and miR-21 KO using a FACS Aria II sorter (BD Bioscience). Total RNA was prepared from the cell lysate. Each condition was represented by independently collected biological triplicates. Labeled cell extracts were processed for microarray analysis using the WT Pico Amplification kit (Thermofisher) and hybridized to Affymetrix Mouse 430V2 Arrays. Mass pre-processed data were generated in Expression Console (Thermofisher) and analyzed for differential gene expression using Transcript Analysis Console (Thermofisher) with a P value cutoff < 0.05 and two-fold change filter applied. Statistically significant differentially expressed gene list associated with each condition was further annotated and interrogated using MetaCore software (Reuter). The data are available from the Gene Expression Omnibus (GEO) repository system with the accession number GSE104270.

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Author contributions

Conceptualization, R.S. and M.M.; methodology, R.S., K.M., V.V., H.A.-A., L.-F.W., T.P., J.G., H.R.J., M.P., J.K., I.C., P.H., and D.C.; investigation, R.S., K.M., J.K., L.C., P.H., and M.M.; writing—original draft, M.M.; writing—review and editing, K.M., R.S., M.P., and M.M.; funding acquisition, M.M. and P.H.; resources, T.P., J.L., and P.M.; supervision, L.-F.W., P.H., M.P., and M.M.

Additional information

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Competing interests: J.L. and P.M. are employees of Exiqon A/S. The remaining authors declare no competing financial interests.

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