Expression Analysis of a Pro-Inflammatory Pathway in Neuropathic Pain

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INTRODUCTION

Polyneuropathy **(PNP)** is a term to describe generalized diseases of the peripheral nervous system. In some neuropathies such as small fiber neuropathy (SFN), pain is located to the skin distally in the leg, and the proximal leg is pain free, therefore this is a good model to study the local versus systemic role of an immune dysbalance [1].

This dysbalance may be triggered by the release of pro-inflammatory molecules, that can activate immune cells through the recognition of surface receptors, such as **TRPV1** or **TLR4**. Activation of TRPV1 triggers a strong influx of calcium, activating **SIRT1** through a calcium dependent protein kinase [2,3].

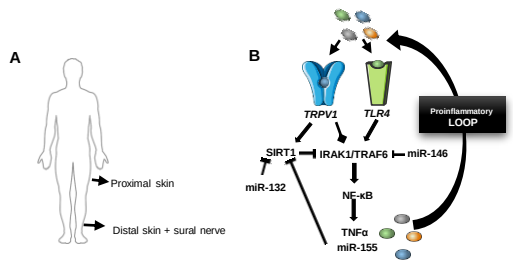
Induction of these two receptors upregulates a **NF-κB** mediated pathway, via **IRAK1/TRAF6,** secreting pro-inflammatory mediators, including **TNFα** and microRNA-155 [4,5].

MicroRNAs like **miR-146, miR-132 and miR-155** are small pieces of RNA that might modulate this pathway [6-8].

The secretion of pro-inflammatory cytokines, such as TNFα, can activate the same receptors, creating a **pro-inflammatory loop**.

The study of this pro-inflammatory loop in patients with PNP may elucidate whether the inflammatory state of the tissue plays a role in pain and nerve degeneration and regeneration

FIGURE 1. Graphical abstract of the samples and hypothesis



A) Illustration of the samples collected in this project. B) Proposed pro-inflammatory pathway taking place in patients with painful polyneuropathies.

METHODS

Experiments were performed on sural nerves and whole skin biopsies from the lower leg and the upper thigh of 67 informed patients with PNP (Table 1).

Samples were collected and stored in RNAlater at -80°C and RNA was isolated following the miRNeasy minikit for nerve samples and microkit for skin samples.

microRNA cDNA synthesis was performed with 5 ng of sample using miRCURY LNA Universal cDNA synthesis kit. mRNA cDNA synthesis was performed with 250 ng of sample.

miCDNA RT-qPCR was performed using miRcury LNA SYBR Green Master mix and ROX reference. mCDNA RT-qPCR was performed using TaqManAssay endogenous control (VIC-MGB) and TaqManAssay target gene (FAM-MGB).

TABLE 1. Cohort of patients

	Number of patients
Sural nerve	67
Non inflammatory neuropathy	26
No Pain (NRS=0)	7
Mild Pain (1≤NRS≤3)	9
Severe Pain (NRS≥4)	10
Inflammatory neuropathy	41
No Pain (NRS=0)	10
Mild Pain (1≤NRS≤3)	13
Severe Pain (NRS≥4)	18
Skin	39
Non inflammatory neuropathy	17
No Pain (NRS=0)	3
Mild Pain (1≤NRS≤3)	5
Severe Pain (NRS≥4)	9
Inflammatory neuropathy	22
No Pain (NRS=0)	4
Mild Pain (1≤NRS≤3)	6
Severe Pain (NRS≥4)	12

Control and target genes analyzed by RT-PCR are described in Table 2.

The results were analyzed with SPSS and visualized with GraphPad Prism 7. Significance was considered when the p value was lower than 0.05.

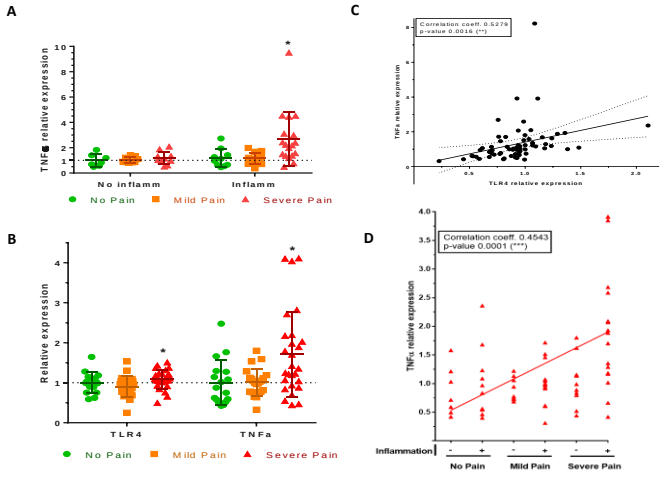
TABLE 2. RT-qPCR control and target genes.

mRNA	Control (VIC)	Target gene (FAM)
Nerve & Skin		
	RPL13a	TLR4
		TRPV1
		SIRT1
		TNFα

microRNA	Control	Target gene
Nerve		
	5S + U6	hsa-miR-146a-5p
		hsa-miR-132-3p
		hsa-miR-155-5p
Skin		
	5S + Snord44	hsa-miR-146a-5p
		hsa-miR-132-3p
		hsa-miR-155-5p

RESULTS IN NERVE

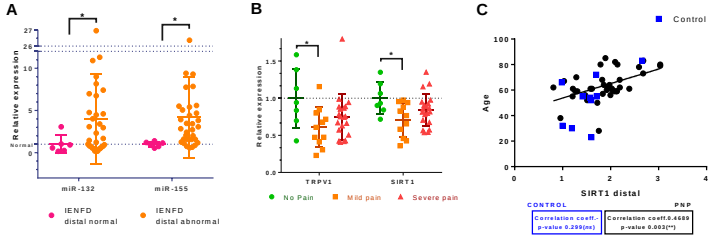
FIGURE 2. Gene expression of pro-inflammatory components in sural nerve.



A) and B) show the TNFα and TLR4 relative expression between patients with no, mild and severe pain. In A) the cohort is divided in patients with an inflammatory or non inflammatory neuropathy. Data normalized to patients with no pain and no inflammation. B) shows the correlation between the expression of TNFα and TLR4 and D) between TNFα and the severity of the disease. Both correlation have a p-value<0.05. * when 0.01<p-value<0.05.

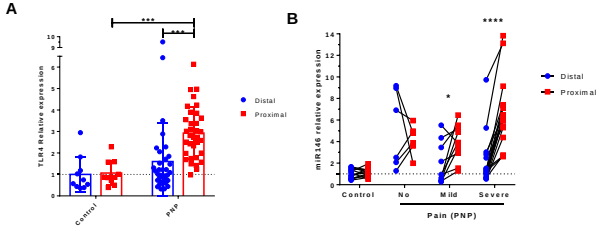
RESULTS IN SKIN

FIGURE 3. Gene expression of pro-inflammatory components in distal skin



A) Relative expression of miR132 and miR155 between patients with a normal and abnormal intraepidermal nerve fiber density (IENFD). Data is normalized to patients with a normal IENFD. B) Relative expression of SIRT1 and TRPV1 between patients with no, mild and severe pain. Data is normalized to patients with no pain. C) Positive correlation between the expression of SIRT1 in distal skin and the age of the patient. * when 0.01<p-value<0.05.

FIGURE 4. Gene expression of pro-inflammatory components in skin between distal and proximal regions.



A) Relative expression between patients with PNP and healthy controls. B) Expression of miR146 between distal and proximal skin in patients with no, mild and severe pain in comparison to healthy controls. Data normalized to healthy controls. *** when 0.0001<p-value<0.001, **** when p-value<0.0001.

CONCLUSIONS

In nerve (Fig.2), we saw an upregulation of TLR4 and TNFα in patients with inflammation and severe pain (A, B). Furthermore, we found a correlation between TLR4 and TNFα (C), and between TNFα and the severity of the disease (D).These results indicate that an activation of the inflammatory pathway might be involved in the development of pain.

In distal skin (Fig. 3), we observe an upregulation of miR132 and miR155 in patients with a reduced IENFD (A). These microRNAs can modulate the expression of SIRT1, therefore it might explain the downregulation of SIRT1 and TRPV1 that we find in patients with pain, in comparison to patients without pain (B). Considering the involvement of SIRT1 in the NF-κB mediated pathway, this suggests that an upregulation of this pro-inflammatory loop might be involved in the loss of nerve fibers and the development of pain. Furthermore, SIRT1 modulation might be influenced by age in patients with PNP, since we find a correlation between both.

When we compared distal and proximal skin (Fig.4), we can see an upregulation of TLR4 in both regions in patients with PNP in comparison to healthy controls, suggesting an involvement of TLR4 in the development of the disease. Furthermore, we found a downregulation of miR146 in the distal region in comparison to proximal, in patients with mild and severe pain, indicating an upregulation of the IRAK1-TRAF6 complex and the NF-κB mediated pathway.

Our results suggest that an activation of the proposed pro-inflammatory loop might contribute to the intraepidermal nerve fiber degeneration and the development of pain in patients with PNP

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ABBREVIATIONS: IENFD, intrapidermal nerve fiber density; **IRAK1**, interleukin-1 receptor-associated kinase 1; **miR**, microRNA; **NF-κB** , nuclear factor kappa-light-chain-enhancer of activated B cells; **NRS**, numeric pain rating scale; **PNP,** polyneuropathy; **SFN**, Small fiber neuropathy; **SIRT1**, sirtuin1; **TLR4**, toll-like receptor 4; **TNFα**, tumor necrosis factor-α; **TRAF6**, TNF receptor associated factor 6; **TRPV1**, transient receptor potential cation channel V1

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