Original Experimental

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# Association between systemic inflammation and experimental pain sensitivity in subjects with pain and painless neuropathy after traumatic nerve injuries

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**Abstract**

**Objectives:** Peripheral neuropathies that occur secondary to nerve injuries may be painful or painless, and including a low-grade inflammation and pro-inflammatory cytokines associated with both regeneration and damage of peripheral nerve cells and fibers. Currently, there are no validated methods that can distinguished between neuropathic pain and painless neuropathy. The aim of this study was to search for proinflammatory and anti-inflammatory proteins associated with pain and experimental pain sensitivity in subjects with surgeon-verified nerve injuries in the upper extremities.
**Methods:** One hundred and thirty-one subjects [69 with neuropathic pain, NP; 62 with painless neuropathy, nP] underwent a conditioned pain modulation (CPM) test that included a cold pressor task (CPT) conducted with the non-injured hand submerged in cold water (4 °C) until pain was intolerable. CPM was assessed by pain ratings to pressure stimuli before and after applying the CPT. Efficient CPM effect was defined as the ability of the individual's CS to inhibit at least 29% of pain (eCPM). The subjects were assigned to one of two subgroups: pain sensitive (PS) and pain tolerant (PT) after the time they could tolerate their hand in cold water (PS<40 s and PT=60 s) . Plasma samples were analyzed for 92 proteins incorporated in the inflammation panel using multiplex Protein Extension Array Technology (PEA). Differentially expressed proteins were investigated using both univariate and multivariate analysis (principal component analysis-PCA and orthogonal partial least-squares discriminant analysis-OPLS-DA).
**Results:** Significant differences in all protein levels were found between PS and PT subgroups (CV-ANOVA p<0.001), but not between NP and nP groups (p=0.09) or between inefficient CPM (iCPM) and eCPM (p=0.53) subgroups. Several top proteins associated with NP could be detected using multivariate regression analysis such as stromelysin 2 (MMPs), interleukin-2 receptor subunit beta (IL2RB), chemokine (C-X-C motif) ligand 3 (CXCL3), fibroblast growth factor 5 (FGF5), chemokine (C-C motif) ligand 28 (CCL28), CCL25, CCL11, hepatocyte growth factor (HGF), interleukin 4 (IL4), IL13. After adjusting for multiple testing, none of these proteins correlated significantly with pain. Higher levels of CCL20 (p=0.049) and CUB domain-containing protein (CDCP-1; p=0.047) were found to correlate significantly with cold pain sensitivity. CDCP-1 was highly associated with both PS and iCPM (p=0.042).
**Conclusions:** No significant alterations in systemic proteins were found comparing subjects with neuropathic pain and painless neuropathy. An expression of predominant proinflammatory proteins was associated with experimental cold pain sensitivity in both subjects with pain and painless neuropathy. One these proteins, CDC-1 acted as "molecular fingerprint" overlapping both CPM and CPT. This observation might have implications for the study of pain in general and should be addressed in more detail in future experiments.

**Keywords:** cold pressor task; conditioned pain modulation; experimental pain; inflammation; neuropathic pain; proteomic profile.

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## Introduction

Peripheral neuropathies, which occur secondary to a wide range of pathologies [1] may be painful or painless and may

be associated with neuroinflammation as a possible mechanism in both regeneration and injury of peripheral nerve cells and fibers [1]. Interestingly, earlier studies could not detect differences between sensory profiles of patients with neuropathic pain and neuropathy without pain [2–5]. Mounting evidence suggests that neuroinflammation resulting from neuro-glial and neuro-immune interactions not only serves as a driving force for chronic pain [6], including neuropathic [2], but also is implicated in other neurological and psychiatric diseases [6]. The concept of microglial activation has been extensively studied in relation to neuropathic pain models in the laboratory [7–9] and in human studies [10–13]. Low-grade systemic inflammation induced in healthy individuals with low doses of lipopolysaccharide (LPS) was associated with increased pain sensitivity positively correlated with plasma interleukin-6 (IL-6) and interleukin-8 (IL-8) levels and impaired descending pain modulatory systems [14, 15]. Changes in inflammation-related biomarkers have been found to be associated with various responses to experimental stimuli [16–18], but also with cold pain tolerance and pain thresholds [19] in healthy volunteers. We have previously demonstrated that except for prolonged time of after sensation (time from maximum pain intensity following cold experimental stimuli until the pain intensity decreased and the subjects became pain free) in subjects with neuropathic pain, no other differences in endogenous pain modulation were found comparing individuals with painful and painless neuropathy [5]. The key question why some patients are moderately affected, having no pain after a traumatic nerve injury, while others having a similar nerve lesion develop debilitating chronic neuropathic pain is still not yet understood. In an effort to further address this question, we aimed to analyse the association of inflammatory-related proteins to neuropathic pain compared to non-painful neuropathy, as well as associations between inflammation biomarkers and conditioned pain modulation (CPM) and cold pain tolerance. Instead of only analyzing relatively few substances at a time, we simultaneously measured multiple neuro-inflammatory biomarkers relevant for the pathophysiology of neuropathic pain using a multiplex proximity extension assay (PEA) panel, allowing detection of more complex patterns of change. Protein expressions of the subjects with definite neuropathic pain after nerve lesions in upper extremities were compared with those resulted from samples collected from a group of subjects with neuropathy without pain. These analyses were performed on a published cohort of subjects [5].

# Methods

The study followed the ethical guidelines from the Helsinki Declaration (as amended in 2013) and has been approved by the Regional Ethics Committee (Project identity: ICONSS, Dnr: 2015/265; NCT03174665 for Clinical trial organization). Informed consent has been obtained from all individuals included in this study.

### Subjects recruitment-patients with painful neuropathy

Questionnaires about pain intensity, previous medication, and the Self-Administered Leeds Assessment of Neuropathic Signs and Symptoms (S-LANSS questionnaire) were sent 2016 to 1051 patients admitted to the Hand Surgery Clinic, Uppsala, Sweden with a diagnosis of traumatic nerve injury in the upper extremities. Seven hundred and six patients returned the questionnaire (response rate of 67.1%). Of the 669 patients who underwent surgical treatment, 337 (50.3%) suffered persistent pain after surgery and 346 patients (51%) had no pain. Seventy-three patients with pain and seventy-three subjects without pain, who underwent nerve suture surgery, were invited to participate in a follow-up study [5]. The questionnaires were repeated on the day of examination. The inclusion criteria for the participants were as follows: age ≥ 18 years, pain more than 50 mm on a 100 mm Visual Analogue Scale (VAS), no acute illness or diseases that might influence laboratory performance. The exclusion criteria were as follows: presence of polyneuropathy, diabetes mellitus, peripheral vascular disease, history of malignant disease, chronic alcohol consumption. The participants with pain and S-LANSS≥12 (indicating predominantly neuropathic pain) [20] were recruited for the group with neuropathic pain. S-LANSS has a sensitivity of 82 to 91% and a specificity of 80 to 94%, compared to clinical diagnosis to indicate neuropathic pain [21]. The confirming sensory impairment on examination of the somatosensory system with pain in the innervation territory of a previous intraoperatively verified injured nerve, strongly indicated a diagnosis of "definite neuropathic pain" for all the subjects with pain [22, 23]. All the subjects had a definite traumatic nerve lesion, seen by the surgeon intraoperatively and the confirmatory tests for a nerve lesion, as required for this diagnosis according to the present definition, were carried out.

**Subjects with neuropathy without pain:** Seventy-three subjects without pain but with previously defined injuries and nerve repairs were recruited from the previous study [24]. They were pre-screened by the same questionnaires and repeated once more on the day of examination and had the same exclusion criteria as in the pain subject group. They had weekly average pain < 20 mm on a 100 mm VAS and S-LANSS<12.

Eligibility for all participants was determined only after completion of a health history questionnaire, interview about pain intensity, and a routine clinical bedside neurological examination. All participants were asked to refrain from any pain medication for at least 12 h before the experimental session.

## Procedures

All participants were informed about the test program before (by telephone) and after arrival in the laboratory. The participants attended a single appointment. All sessions followed the same procedure and were performed by the same trained examiner who read from a standardized instruction protocol when performing CPM.

## Clinical assessment

The participants completed extensive questionnaires, which included sociodemographic data, education level, work status, family and medical history, time from operation. Body mass index was calculated using the formula weight/height$^2$ (kg/m$^2$). Baseline brachial resting blood pressure was examined before the experiment was started.

## Pain assessment and clinical examination

Participants were asked to rate their mean clinical pain over the past week on VAS (0–100). During clinical examination of the somatosensory system in the neuropathic painful area, touch was tested with a camel-hair brush (0.5 Somedic, Sweden), pain with a sharp tooth pick, and cold and warm temperature stimuli with warm (40 °C) and cold (25 °C) rollers (Senselab Rolltemp, Somedic). The contralateral uninjured side served as within subject control.

## Conditioned pain modulation

The current investigator has described the CPM paradigm previously [5]. It involved tourniquet pressure test stimulus (TS) applied to one leg, before and after thermal conditioning stimulus (CS) conducted by immersing the uninjured hand in 4 °C cold water (Figure 1).

**Test stimulus (TS):** The TS was delivered by a tourniquet applied midcalf on the leg corresponding to the non-injured arm and inflated from 60 to 100 mm Hg above the systolic blood pressure (typically 220–250 mm Hg) until the pain intensity reported by the subject was over 50 on a 0–100 mm VAS. The test-stimulus (TS) was applied for a duration of 120 s before (TS1) and after (TS2) the conditioning stimulus (CS) at the same pressure.

**Conditioning stimulus (CS):** The conditioning stimuli were given by having the subjects immerse their non-injured hand up to the wrist in a cold-water bath at 4 °C cooled by a refrigerated water circulator (Somedic, 2015, Sweden) for maximally 1 min. Cold is a reliable CS [25] and was applied immediately after the subject became pain free after TS1, and ended when the subject withdrew the hand from the cold-water bath, or maximally for 1 min. The water level was set at height of 30 cm and maintained at a constant temperature to keep the stimulated area consistent.

Time in the cold-water bath (Time CS), and time until the pain intensity decreased and the subjects became pain free (Time off) after removing the hand from cold water were expressed by the area under the curve ($AUC_{CS}$, $AUC_{time\ off}$). Immediately after the subjects became pain free after the conditioning stimulus, an identical test-stimulus (TS2) was repeated. The subject was instructed to rate continuously the pain intensity level of both the test stimulus and conditioning stimulus with the eVAS slider until they became pain free. They could discontinue the trial at any time if they could not tolerate the painful pressure (120 s) or cold water stimuli (60 s). The subjects who could keep the hand in cold water for a period of time of 60 s were labeled accordingly pain tolerant (PT) and those who took their hand out under 40 s were

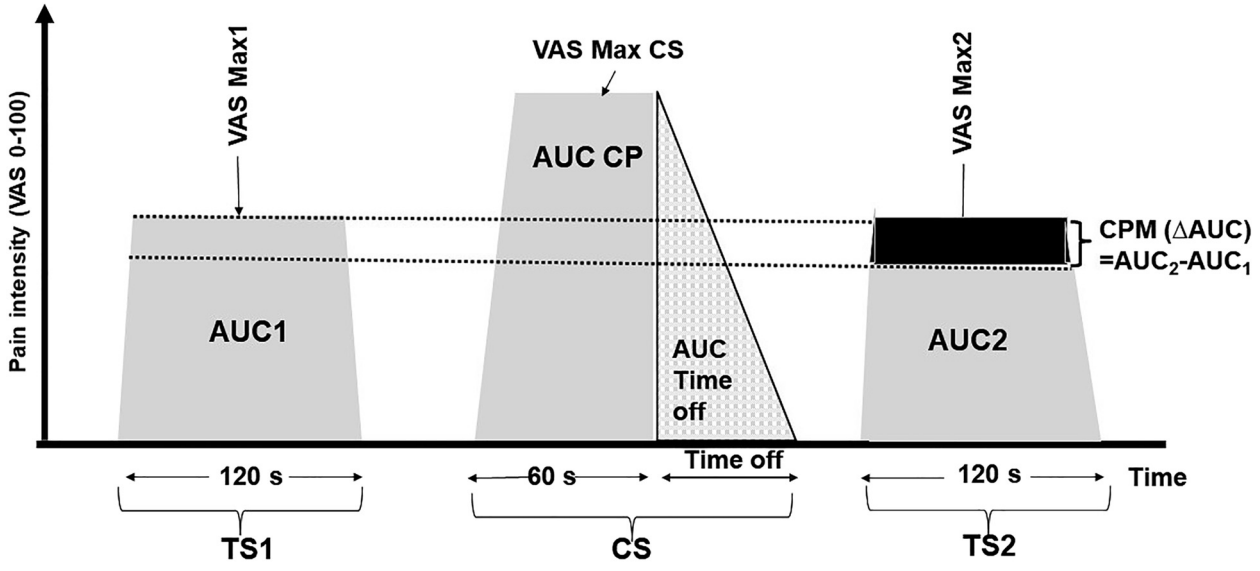


**Figure 1:** Timeline showing CPM stimuli administration.
TS=test stimulus (pressure pain); CS=conditioning stimulus (cold water); TS1=pressure pain ratings during the first test stimulus (120 s) and returns to baseline; TS2=pressure pain ratings during the second test stimulus (120 s) and return to baseline; CS=pain ratings during conditioning stimulus (60 s) and return to baseline; time off time to return to baseline after CS; AUC1=area under the curve as resulted from pain rating over time during TS1; AUCCS area under the curve during CS; AUC2=area under the curve as resulted from pain ratings over time during TS2.

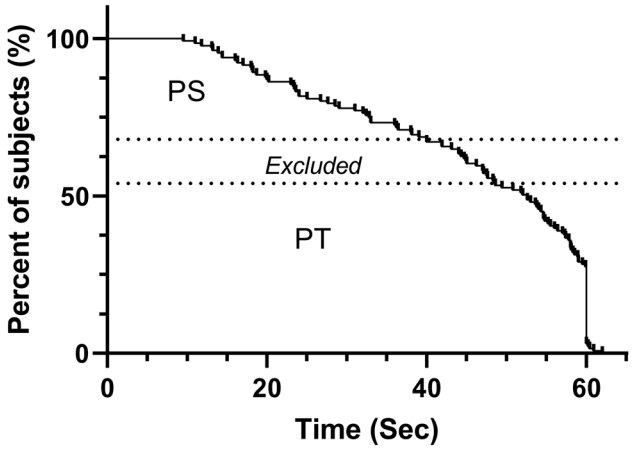


**Figure 2:** Kaplan–Meier curve displaying proportion of pain tolerant (PT) and pain sensitive (PS) subjects to cold pressor task. The *horizontal axis* represents the time of follow-up starting from the time when subjects immersed their hands in cold water while the *vertical axis* represents the estimated percent of subjects. Each vertical tick represents a subject.

labeled pain sensitive individuals (PS) [26], (Figure 2). The subjects who took their hands out from cold water after 40 s and before 59 s, "a middle group of unclear responders" were not included in the analysis. Demographic, endogenous pain modulation, life quality and psychologic variables were compared between PT and PS subjects.

**Calculation of conditioned pain modulation:** To quantify **CPM**, the deviation of pain ratings from the set point was continuously recorded and summed over time to produce an area under the curve (AUC) value. From the start point of the first test stimulus forward, this dependent variable (AUC) of the VAS response over time was calculated for both test stimulus ($AUC_1$, $AUC_2$) and conditioning stimulus ($AUC_{CS}$). Thus, **CPM** was calculated as the difference in area under the curve of pain rating responses between the last test stimulus after the CS and the test stimulus before CS ($\Delta AUC = AUC_2 - AUC_1$). The **CPM effect (%CPM)** is defined as the percent change of the pain intensity evoked by the TS induced before and after the CS. The formula usually used is as follows: the CPM effect change was (TS1 pain subtracted from TS2 pain) divided by TS1 pain or [(TS2 pain – TS1 pain)/TS1 pain] × 100. The percentage of CPM (%CPM) = $\Delta AUC \times 100/AUC_1$. The CPM effect varies from pain inhibition to facilitation. Therefore, negative CPM scores indicate pain inhibition, positive CPM scores indicate pain facilitation and zero indicates no effect. Efficient CPM effect was defined as the ability of the individual's CS to inhibit pain by at least 29% of pain [27].

## Questionnaires

The subjects completed questionnaires prior to coming to the study such as S-LANSS and the following three questionnaires were completed when the participants came to the experiment.

**Quality of life:** Quality of life was measured at the start of the study with the 36-Item Short-Form Health Survey (RAND-36), a health survey that consists of eight items investigating physical and mental status [28].

**Depression and anxiety:** The Hospital Anxiety and Depression Scale (HADS) is a psychometric questionnaire specifically developed for non-psychiatric patients to identify the grade of anxiety disorder and/or depression. It consists of two subscales, anxiety and depression. The total score for each domain was calculated as the sum of the respective 7 items (ranging from 0–21), with normal values (0–7), borderline cases [8–10] and pathological values [11–21, 29].

**QuickDASH (disability of shoulder, arm and hand):** QuickDash is a short, reliable, and valid measure of physical function and symptoms related to upper limb musculoskeletal disorders by shortening the full, thirty-item DASH (Disabilities of the Arm, Shoulder and Hand) Outcome Measure [30].

## Measures of inflammatory biomarkers in blood

**Blood sampling:** Blood samples (20 mL) were collected from the antecubital vein from all participants in Li-heparin PST™ tubes (367886, Becton-Dickinson, Franklin Lakes, NJ, USA). The tubes were centrifuged within 30 min at 2400 g for 5 min at room temperature, and the plasma was transferred to aliquot tubes and frozen at –70 °C in a registered Biobank until further analysis.

**Protein analysis with proximity extension assay (PEA):** The multiplex Protein Extension Array Technology (PEA) was conducted using Proseek Multiplex Inflammation I (Olink Bioscience, Uppsala, Sweden) according to the manufacturer's instructions. The antibody based 92-plex-panel of inflammation-related proteins (PEA, Proseek Inflammation, Olink, Uppsala) was chosen based on previous studies originating from experiments performed by our group in patients with neuropathic pain [10, 31, 32] (detailed description in Supplemental material). All samples were analyzed on the same occasion, using the same batch of reagents.

## Statistics

**2.8.1 Univariate statistics analysis (UNA):** Standard traditional univariate statistics was performed with IBM SPSS Statistic version 19.0.0.1, GraphPad Prism 8 and SAS version 9.4 (SAS Institute, Inc). The level of significance was set at a p-value < 0.05. Descriptive statistics are presented as means and standard deviations for continuous variables and absolute numbers and percentages for categorical variables. For continuous variables, the Mann Whitney U test was used. As this was an overlapping cohort study no *a priori* power calculation was performed. To compare CPM, "Time off" between subjects with pain and painless neuropathy, sensitive (S) and tolerant (T) subjects and to compare differences between the subjects in the same group, a two-way ANOVA (group and side) was performed. A *post hoc* unpaired t-test was performed for between group comparisons and a *post hoc* paired t-test for within group comparisons. To assess correlation between endogenous pain modulation and biomarkers, Spearman's rank correlation test (two-tailed) was used. For PEA the data were exported from the Biomark reader and normalized using Olink Wizard for GenEx software. A five parameter log-logistic function was fitted to the standard curve measurements, after outliers had been removed.

The LOD was defined as the protein concentration in the fitted standard curve that corresponded to the PCR cycle threshold $m_{\text{Ctblank}} - 2s_{\text{Ctblan}}$ where $m_{\text{Ctblan}}$ and $s_{\text{Ctblan}}$ denote the mean and the standard deviation for threshold cycle (Ct) for the blank, respectively. The Ct is the threshold cycle, the real time PCR fractional cycle, where fluorescence reaches a preset threshold. The data used for further statistical analysis were in normalized protein expression units (NPX) on $\log_2$ scale where a high value corresponds to a high protein concentration. The Ct values are normally distributed allowing the use of t-test for comparison between groups. Ct values for each protein were compared between males and females using a two-sample t-test. Multiple testing approach amplifies the probability of false positive findings [33]. To reduce the risk of false discoveries caused by multiple testing (unadjusted p-values), a Benjamini–Hochberg false discovery rate (FDR) method was applied [34] to adjust the p-values (adjusted p-values). FDR used in the calculations was set at cut-off of 0.05. A linear regression adjusting for subject sex and BMI as potential covariates was used for all proteins after t-test adjusting for multiple testing according to Benjamini and Hochberg. The use of this method of alpha-adjustment for multiple comparisons reduces the significance value to very stringent levels and increases the chances of a Type 2 error (false negative error; falsely accepting the null hypothesis) [33]. To handle the limitations of the statistical analysis discussed previously, both univariate and multivariate data analysis (MVDA) were used in the study. Several previous studies investigated proteomics in pain research have used the same methods for multivariate analysis [35–37].

**Multivariate data analysis (MVDA):** MDVA was applied using principal component analysis (PCA) and multivariate regression analysis (orthogonal partial least squares analysis OPLS) modelling using SIMCA P+ (version 17, Sartorius Stedim Biotech, Umeå, Sweden). PCA was used to review the data set and detect potentially multivariate outliers (using score plots in combination with Hotelling's T2 which identifies strong outliers and distance to model in X-space which identifies moderate outliers). All observations were included and all variables were log transformed before the statistical analyses. The loading plot reported the multivariate relationships between variables. Variables with high loadings were considered significant. OPLS-DA was used to compare and investigate whether any of the quantified proteins could discriminate between the groups with neuropathic pain (NP) and painless neuropathy (nP), subgroups pain sensitive (PS) and pain tolerant (PT) and between subjects with inefficient (iCPM) and efficient (eCPM) conditioned pain modulation. The importance of the variables was measured as a Variable Influence on Projection (VIP). VIP≥1.3 was considered significant. Coefficients were used to note the direction of the relationship (positive or negative correlation). The OPLS-DA analysis was performed in two steps. In the first step we included all the proteins with VIP ≥1.0 and >95% jackknifed confidence interval and then we included proteins with VIP ≥ 1.3 in a new regression model which is presented in the results. The table presenting data contains p(corr) for each significant protein (an absolute p(corr) >0.4–0.5 was considered significant). In the same table, $R^2$ (goodness of fit), $Q^2$ (goodness of prediction) and Analysis of Variance of Cross-Validated predictive residuals (CV-ANOVA) used to assess model reliability, were also computed. The statistical significance for the observed class separation in the OPLS-DA models was measured by calculating the CV-ANOVA p-values as a tuning method, applying a cut-off of $p<0.05$.

# Results

## Clinical symptoms and subjects' characteristics with neuropathic pain or painless neuropathy

Of the 73 subjects with neuropathic pain, four subjects were excluded from analysis: two of them had no pain at follow-up (n=2) and two for technical problems with CPM and the collection of blood samples. Eleven subjects were excluded from the group without pain from analysis because of the associated disease (n=3) and technical problems (n=8). Sixty-nine (31 females/38 males) subjects with neuropathic pain [av. age 48 years] and sixty-two (25 females/37 males) subjects with neuropathy without pain [av. age 49 years] after similar injury were compared. The characteristics for the participants are presented in Table 1. In the present study, a longer time from trauma and surgery was seen in the group without pain in comparison with the group with pain (p=0.03). Almost all NP subjects were classified as ASA physical status 1 or 2; only 4 subjects in the NP group and 6 subjects in the non-pain group were ASA 3. In the group with pain, only 6 subjects used analgesic/anti-inflammatory medications and none in the group without pain. As expected, the subjects with neuropathic pain rated significantly higher pain intensities ($p<0.0001$), had significant reductions of the physical component of RAND-36 scores ($p<0.0001$) and a higher degree of disability (Quick DASH, $p<0.0001$) than non-painful controls. No difference was observed between the experimental groups with pain and without pain for either anxiety or depression scores measured with the HADS questionnaire (Table 1).

## Clinical symptoms and characteristics of the subjects with cold pain tolerance (PT), cold pain sensitive (PS), subjects with inefficient CPM (CPMi) and efficient CPM (CPMe)

Forty-three (23 females) subjects were cold pain sensitive (32% PS) [av. age 50 years] and sixty-nine (24 females) subjects were cold pain tolerant (52% PT) [av. age 50 years]. In the cold pain sensitive subgroup was 19 subjects with NP and in the pain tolerant subgroup was 36 subjects with NP. No differences were seen between PT and PS subgroups or between those with CPMi and CPMe (Table 1). Nineteen subjects who took their hands from the cold water >40 s to ≤59 min were excluded from protein analysis of the subgroups (Figure 2). It was difficult to interpret these group as

**Table 1:** Sociodemographic and clinical characteristics of the subjects included in the study and characteristics of the subgroups of cold pain sensitive (PS) and tolerant subjects (PT) and of the subgroups with efficient (eCPM) and inefficient (iCPM) conditioned pain modulation.

| | | NP (n=69) | nP (n=62) | Group diff | PS (n=43) | PT (n=69) | Group diff | CPMe (n=52) | CPMi (n=26) | Group diff |
|---|---|---|---|---|---|---|---|---|---|---|
| **Age, (y)** | Median [IQR] | 50 [28–63] | 49 [27–65] | 0.443 | 50 [33–61] | 59 [38–61] | 0.273 | 46 [35–53] | 53 (20–85) | 0.107 |
| **Gender** | Male female | 38 (55%) | 38 (61%) | 0.545 | 21 (48%) | 45 (65%) | 0.487 | 44 (84%) | 16 (61%) | 0.109 |
| | (N%) | 31 (45%) | 25 (39%) | | 22 (52%) | 24 (35%) | | 22 (16%) | 10 (38%) | |
| **Pain/no pain (N)** | | | | | 19 (44%) | 36 (53%) | 0.333 | 24 (46%) | 13 (50%) | 0.458 |
| | | | | | 24 (55%) | 33 (47%) | | 28 (54%) | 13 (50%) | |
| **Pain duration (y)** | Mean ± SD | 2.3 ± 2.5 | 3.4 ± 1.5 | ***0.031*** | 2.7 ± 2.5 | 2.3 ± 1.5 | 0.987 | 2.1 ± 0.6 | 2.7 ± 1.8 | 0.378 |
| **BMI (kg/m$^2$)** | Median [IQR] | 25 [23–29] | 26 [24–28] | 0.397 | 26 [25–29] | 27 [23–29] | 0.983 | 26 [17–32] | 26 [19–38] | 0.989 |
| **ASA** | | | | 0.642 | | | 0.072 | | | 0.257 |
| | I | 35 (50%) | 31 (50%) | | 24 (55%) | 25 (36%) | | 25 (48%) | 14 (53%) | |
| | II | 30 (43%) | 26 (41%) | | 16 (37%) | 40 (57%) | | 25 (48%) | 12 (46%) | |
| | III | 4 (7%) | 6 (9%) | | 3 (6.9%) | 3 (7%) | | 2 (4%) | 1 (3.8%) | |
| **Pain medication** | After the operation | 44 (63%) | 25 (40%) | ***0.020*** | 23 (53%) | 24 (34%) | 0.098 | 30 (57%) | 17 (65%) | 0.653 |
| **Actual medication** | Opioids | 2 (2.8%) | 0 | ***0.011*** | 2 (4.6%) | 0 | 0.471 | 2 (3.8%) | 2 (7.6%) | 0.976 |
| | Tricyclics/ duloxetine | 3 (4.3%) | 2 (3.2%) | | 2 (4.6%) | 3 (4.3%) | | 4 (7.6%) | 2 (7.6%) | |
| | Gabapentinoids | 4 (5.7%) | 0 | | 2 (4.6%) | 2 (2.8%) | | 3 (5.7%) | 1 (3.8%) | |
| | Paracetamol | 4 (5.7%) | 0 | | 2 (4.6%) | 2 (2.8%) | | 3 (5.7%) | 3 (11%) | |
| | COX-inhibitors | 5 (7.2%) | 0 | | 3 (6.9%) | 2 (2.8%) | | 4 (7.6%) | 1 (3.8%) | |
| **Employment (n)** | | | | 0.954 | | | 0.876 | | | 0.785 |
| | Employed | 49 (71%) | 50 (80%) | | 35 (77%) | 51 (74%) | | 30 (57%) | 15 (56%) | |
| | Retired | 10 (14%) | 8 (13%) | | 5 (11%) | 10 (14%) | | 7 (33%) | 6 (23%) | |
| | Unable to work | 9 (13%) | 2 (3.2%) | | 2 (3.6%) | 7 (10%) | | 4 (7.6%) | 4 (15%) | |
| | Other | 1 (1.4%) | 2 (3.2%) | | 1 (2.3%) | 1 (1.4%) | | 1 (1.9%) | 1 (3.8%) | |
| **Nerve injury (n)** | | | | 0.643 | | | 0.745 | | | 0.568 |
| | Digital nerves total (M, U, R) | 31 (5, 10, 15) (44%) | 41 (17, 8, 15) (66%) | | 20 (9, 10, 5) (46%) | 29 (19, 5, 5) (42%) | | 25 (13, 7, 5) (48%) | 15 (10, 2, 3) (56%) | |
| | Median | 9 (13%) | 3 (4.8%) | | 10 (23%) | 2 (2.8%) | | 7 (13%) | 3 (11%) | |
| | Ulnar | 6 (8.6%) | 1 (1.6%) | | 5 (11%) | 2 (2.8%) | | 5 (9.5%) | 3 (11%) | |
| | Radial | 3 (3.3%) | 4 (6.4%) | | 3 (6.9%) | 3 (4.3%) | | 2 (3.8%) | 2 (7.6%) | |
| | Multiple nerves | 20 (30%) | 13 (21%) | | 19 (44%) | 12 (17%) | | 18 (34%) | 9 (34%) | |
| **Reoperation (n)** | | 11 (16%) | 4 (6.4%) | NS | 9 (20%) | 6 (8.6%) | NS | 7 (13%) | 6 (23%) | NS |
| **Dominant hand (right) (n)** | Right | 67 (97%) | 54 (87%) | NS | 40 (93%) | 65 (94%) | NS | 49 (94%) | 23 (88%) | NS |
| **Injury site (right) (n)** | Right | 28 (40%) | 27 (43%) | NS | 21 (43%) | 32(46%) | | 25 (48%) | 13 (61%) | NS |
| **Pain intensity (VAS 0–100 mm)** (median, range) | Maximum last week | 65 (0–80) | 0 | ***<0.0001*** | 50 (0–70) | 44 (40–80) | 0.978 | 35 (0–100) | 50 (0–80) | 0.302 |
| | Minimum last week | 17 (0–30) | 0 | ***<0.0001*** | 0 (0–60) | 0 (0–60) | 0.989 | 0 (0–20) | 23 (20–40) | ***0.007*** |
| | Average last week | 41 (21–75) | 0 | ***<0.0001*** | 20 (0–60) | 47 (30–70) | 0.614 | 20 (0–22) | 40 (0–60) | 0.062 |
| | Current | 32 (23–60) | 0 | ***<0.0001*** | 20 (0–60) | 40 (20–70) | 0.654 | 25 (0–30) | 35 (0–37) | 0.654 |
| **Other chronic pain (n)** | | 20 (28%) | 21 (33%) | 0.420 | 10 (23%) | 10 (14%) | 0.638 | 11 (25%) | 8 (30%) | |
| | Joint pain | 18 (26%) | 16 (25%) | | 9 (20%) | 5 (9.4%) | | 4 (7.6%) | 3 (11%) | |
| | Low back pain | 12 (17%) | 6 (9%) | | 1 (2.3%) | 1 (1.4%) | | 5 (9.6%) | 3 (11%) | |
| | Headache | 4 (5.7%) | 2 (3.2%) | | 0 | 0 | | 1 (1.9%) | 0 | |
| | Other | 9 (14%) | 1 (1.6%) | | 2 (3.6%) | 5 (9.4%) | | 1 (1.9%) | 2 (7.6%) | |

**Table 1:** (continued)

| | | NP (n=69) | nP (n=62) | Group diff | PS (n=43) | PT (n=69) | Group diff | CPMe (n=52) | CPMi (n=26) | Group diff |
|---|---|---|---|---|---|---|---|---|---|---|
| **LANSS part A** | Median (range) | 11 [9–16] | 0 (0–3) | ***<0.0001*** | 6.5 (0–16) | 5.5 (0–16) | 0.878 | 8 (0–10) | 4 (0–13) | 0.956 |
| **LANSS part B** | Median (range) | 6 (6–8) | 3 (0–3) | ***<0.0001*** | 5.6 (3–8) | 5.5 (3–8) | 0.450 | 8 (3–8) | 8 (2–8) | |
| **Bedside examination** | | | | ***<0.0001*** | | | 0.978 | | | 0.978 |
| **Loss of function** (n) | Touch | 35 (50%) | 37 (59%) | 0.242 | 28 (65%) | 34 (49%) | 0.243 | 27 (51%) | 15 (57%) | |
| | Pinprick | 47 (68%) | 46 (76%) | 0.336 | 27 (62%) | 43 (63%) | 0.976 | 28 (54%) | 13 (61%) | |
| | Warm | 40 (57%) | 38 (61%) | ***0.003*** | 24 (55%) | 41 (59%) | 0.567 | 32 (61%) | 14 (53%) | |
| | Cold | 33 (47%) | 29 (46%) | 0.981 | 20 (46%) | 32 (46%) | 0.976 | 26 (42%) | 12 (46%) | |
| **Gain of function** (n) | Touch | 53 (76%) | 23 (37%) | ***<0.0001*** | 26 (60%) | 38 (55%) | 0.546 | 30 (57%) | 15 (57%) | |
| | Pinprick | 25 (36%) | 3 (4.8%) | ***0.0009*** | 12 (27%) | 16 (23%) | 0.567 | 12 (23%) | 7 (26%) | |
| | Warm | 25 (36%) | 12 (19%) | ***<0.0001*** | 12 (27%) | 22 (31%) | 0.247 | 14 (26%) | 8 (30%) | |
| | Cold | 33 (47%) | 12 (19%) | ***<0.0001*** | 10 (29%) | 20 (28%) | 0.356 | 16 (30%) | 8 (30%) | |
| **HADS anxiety** (n) | | | | 0.133 | | | 0.459 | | | 0.998 |
| 0–7 | No anxiety | 47 (68%) | 50 (80%) | | 32 (74%) | 52 (75%) | | 35 (67%) | 22 (84%) | |
| 8–10 | Mild anxiety | 12 (17%) | 8 (12%) | | 7(16%) | 10 (14%) | | 12 (23%) | 2 (7.6%) | |
| ≥11–21 | Severe anxiety | 10 (14%) | 4 (6.4%) | | 4 (9,3%) | 6 (8.6%) | | 5 (9.5%) | 2 (7.6%) | |
| **HADS depression** (n) | | | | 0.143 | | | 0.987 | | | 0.991 |
| 0–7 | No depression | 60 (86%) | 53 (85%) | | 38 (88%) | 63 (91%) | | 49 (94%) | 20 (76%) | |
| 8–10 | Mild depression | 6 (8.6%) | 7 (11%) | | 4 (9.3%) | 8 (11%) | | 1 (1.9%) | 6 (23%) | |
| ≥11–21 | Severe depression | 3 (4.3%) | 2 (3.2%) | | 1 (2.3%) | 4 (5.7%) | | 1 (1.9%) | 1 (3.8%) | |
| **QuickDASH** (Mean ± SD) | | 34 ± 22 | 7.6 ± 12 | ***<0.001*** | *22* ± 24 | 20 ± 20 | 0.699 | *17* ± 19 | *28* ± 26 | 0.951 |
| **RAND-36** (Mean ± SD) | PF | 77 ± 20 | 89 ± 17 | ***<0.0001*** | 80 ± 10 | 84 ± 18 | 0.965 | 87 ± 15 | 77 ± 24 | 0.953 |
| | RP | 53 ± 37 | 83 ± 29 | ***<0.0001*** | 64 ± 13 | 69 ± 39 | 0.978 | 71 ± 31 | 58 ± 37 | 0.938 |
| | BP | 52 ± 22 | 83 ± 23 | ***<0.0001*** | 65 ± 26 | 67 ± 27 | 0.944 | 71 ± 35 | 59 ± 39 | 0.955 |
| | GH | 67 ± 25 | 74 ± 19 | 0.149 | 65 ± 23 | 72 ± 21 | 0.943 | 71 ± 21 | 66 ± 23 | 0.978 |
| *Physical component RAND-36* | | 251 ± 85 | 330 ± 69 | ***<0.0001*** | 277 ± 65 | 297 ± 55 | 0.938 | 232 ± 85 | 257 ± 70 | 0.938 |
| | MH | 77 ± 21 | 79 ± 18 | 0.663 | 78 ± 20 | 78 ± 18 | 0.994 | 78 ± 18 | 78 ± 19 | 0.991 |
| | RE | 68 ± 38 | 83 ± 32 | 0.098 | 78 ± 27 | 76 ± 35 | 0.987 | 76 ± 35 | 67 ± 36 | 0.997 |
| | SF | 79 ± 26 | 87 ± 22 | **0.040** | 83 ± 30 | 84 ± 24 | 0.954 | 82 ± 35 | 80 ± 27 | 0.963 |
| | VT | 62 ± 24 | 64 ± 20 | 0.429 | 63 ± 23 | 69 ± 12 | 0.783 | 63 ± 19 | 59 ± 25 | 0.997 |
| *Mental health component RAND-36* | | 285 ± 99 | 313 ± 81 | 0.195 | 278 ± 40 | 310 ± 51 | 0.936 | 310 ± 51 | 294 ± 40 | 0.938 |

Data presented as n (%), mean ± SD, or median [IQR 25th, 75th percentile]. NP=Neuropathic pain; CS=control subjects; NS=non-significant; BMI=body mass index; ASA=American Society of Anesthesiologist's Physical Status Classification System; Pain VAS=Average weekly pain on a visual analogue scale (0–100, worst=100); LANSS=Leeds Assessment Signs and Symptoms Scale; HADS=hospital anxiety and depression scale (0–21, worst=21 for each subscale); Quick Dash=disabilities of the arm, shoulder and hand score; RAND-36=36 item health survey; PF=physical function; RP=physical role/function; BP=body pain; GH=general health; MH=mental health; RE=emotional role/function; VT=vitality. Independent Samples Mann Whitney U test was used for the between group comparisons. p-value < 0.05 was considered significant. Statistically significant differences between the groups are indicated in bold.

## Endogenous pain modulation

We discussed in a previous article [5] that a CPM effect >29% indicating a significant analgesic response during CPM [27] was seen in 28 (40%) of the subjects with neuropathic pain and 24 (39%) subjects with neuropathy without pain. These 52 participants were included in the subgroup with efficient CPM (CPMe). Except for a longer time after

sensation (time off, p=0.04) in the group with neuropathic pain, no significant differences in CPM and CPM effect were seen between these two groups. Inefficient CPM (CPMi) was seen in 26 subjects (13 of them with neuropathic pain). Non responders (CPMn; n=53 subjects) or the participants with CPM effect between ≤29% and 0, were not included in the protein analysis of the subgroup CPMi and CPMe. Significant differences between CPMi and CPMe subgroups were seen in relation with CPM effect (p<0.001), but also longer time of after sensation (time off) in the CPMi subgroup (p=0.004).

Duration in bath was longer in tolerant subjects (PT; 60 s) in comparison with sensitive subjects (PS; 26 s) (p<0.0001). The CPM and CPM effect did not differ between PT and PS subjects (Table 2).

## Proteomic profiling of subjects with neuropathic pain and subjects with neuropathy without pain

All of the 92 proteins incorporated in the panel were in the detection range and were included in the analysis. PCA for neuropathic pain and neuropathy without pain indicated no clear difference in the measured protein levels of the 92 proteins incorporated in the panel (Figure 3). OPLS-DA was performed. The NP/nP model parameters of (R2X) and (R2Y) were 0.41 and 0.24 respectively and (Q2) was 0.30. This showed that a 41% variation explained 30% of the difference between the groups. However, (Q2) was smaller than 0.5 and statistically cross verification of CV-ANOVA

**Table 2:** Differences in endogenous pain modulation between the subjects with neuropathic pain (NP) and nonpainful neuropathy (nP), between subgroups with pain tolerance (PT) and pain sensitive (PS) and between subgroups of subjects with efficient CPM (CPMe) and inefficient CPM (CPMi)

| Group | Mean SD | $AUC_1$ | $AUC_2$ | Δ-AUC | %CPM effect | VAS max1 | VAS max2 | Time off (cold) (sec) | VAS Max CS (cold) | $AUC_{CS}$ (cold) | AUC time off | Duration CS (s) | Duration in bath (s) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1. NP (n=69)** | Mean | 6148 | 4742 | −1450 | −22 | 67 | 57 | 43 | 87 | 4785 | 2169 | 89 | 47 |
| | ±SD | 3221 | 3011 | 2245 | 32 | 29 | 29 | 46 | 17 | 2533 | 1803 | 49 | 17 |
| **2. nP (n=62)** | Mean | 6359 | 5273 | −1086 | −20 | 64 | 53 | 28 | 84 | 3991 | 1532 | 74 | 46 |
| | ±SD | 2982 | 3272 | 2014 | 30 | 23 | 30 | 16 | 22 | 1868 | 981 | 25 | 17 |
| | ***p*** | 0.081 | 0.185 | 0.373 | 0.644 | 0.436 | 0.263 | **0.041** | 0.461 | 0.137 | **0.042** | 0.052 | 0.576 |
| **3. PT (n=69)** | Mean | 6130 | 4670 | −1369 | −22 | 51 | 40 | 32 | 84 | 5195 | 1905 | 91 | 60 |
| | ±SD | 2911 | 2879 | 1998 | 28 | 32 | 23 | 18 | 16 | 2194 | 1651 | 20 | 6 |
| **4. PS (n=43)** | Mean | 6232 | 5145 | −2348 | −18 | 61 | 51 | 38 | 90 | 3284 | 1789 | 65 | 26 |
| | ±SD | 3396 | 3383 | 2326 | 32 | 27 | 28 | 53 | 12 | 2027 | 1238 | 57 | 10 |
| | ***p*** | 0.292 | 0.926 | 0.332 | 0.974 | 0.954 | 0.926 | 0.972 | 0.976 | **<0.0001** | 0.435 | **0.001** | **<0.0001** |
| **5. CPMe (n=52)** | Mean | 5872 | 3249 | −2623 | −50 | 49 | 25 | 29 | 83 | 4234 | 1659 | 77 | 48 |
| | ±SD | 3917 | 2251 | 2241 | 19 | 24 | 16 | 15 | 23 | 2270 | 1100 | 26 | 16 |
| **6. CPMi (n=26)** | Mean | 5816 | 6483 | 666 | 19 | 52 | 60 | 48 | 87 | 4331 | 2188 | 86 | 37 |
| | ±SD | 3019 | 3362 | 1410 | 19 | 24 | 25 | 63 | 18 | 2583 | 2013 | 67 | 17 |
| | ***p*** | 0.761 | **<0.0001** | **<0.0001** | **<0.0001** | 0.988 | **<0.001** | **0.004** | 0.984 | 0.654 | **0.004** | 0.961 | 0.957 |

Data presented as n (%), mean ± SD, or median [IQR 25th, 75th percentile].

AUC1 area under the curve test 1; AUC2 area under the curve test 2; CPM=Δ-AUC=$AUC_2 - AUC_1$; %CPM=$[(AVG_2 - AVG_1)/AVG_1 \times 100]$; VAS max1=maximum visual analog scale test 1; Time off is time from maximum pain intensity to 0 after conditioning stimulus; AUC time off=area under the curve from maximum pain intensity over time until the subjects became pain free; VAS max CS is the maximum pain intensity on visual analog scale; $AUC_{CS}$ area under the curve=pain ratings in time under CS; duration CS (sec) total time of conditioning stimulus; Duration in bath-seconds for hand in bath. Statistically significant differences between the groups are indicated in bold.

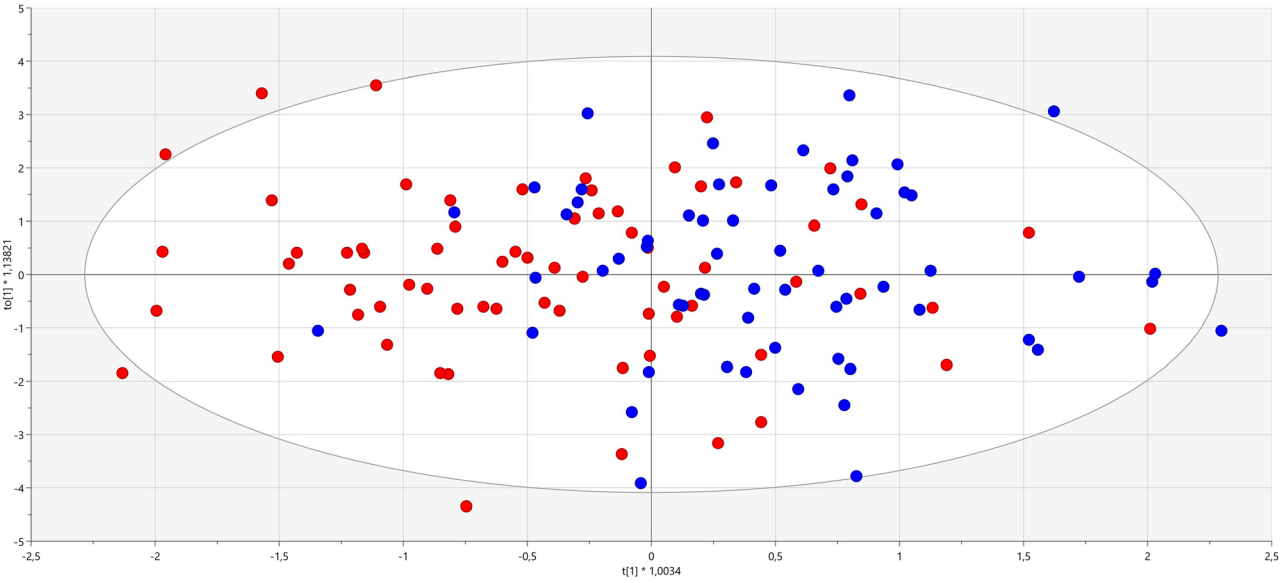


**Figure 3:** Principal component analysis (PCA) plot of protein data that characterizes the trends exhibited by the proteomic profiles of the subjects with neuropathic pain (NP, red) and neuropathy without pain (nP, blue). Each dot represents a sample and each color represents the type of the sample. There was no clear difference between groups.

(p=0.09) demonstrated no significant effect. A total of 20 proteins with VIP>1 are presented in Table 3. Among them, eleven proteins had VIP over 1.3. Significant alterations of proteins associated with neuropathic pain at unadjusted univariate analysis were matrix metalloproteinase or stromelysin 2 (MMPs; p=0.01) which was associated with pain and fibroblast growth factor 5 (FGF5; p=0.34) that was negatively associated with pain. Other top proteins identified with MVDA as associated with pain were interleukin-2 receptor subunit beta (IL2RB), chemokine (C-X-C motif) ligand 3 (CXCL3). Chemokine (C-C motif) ligand 28 (CCL28), CCL25, CCL11, hepatocyte growth factor (HGF), interleukin 4 (IL4), Il-13 had negative p(corr) signs indicating negative association with pain (Table 3). Unfortunately, no protein alterations and no differences related to the entire groups of pain and painless subjects were found after adjustment with Benjamini–Hochberg false discovery rate and adjustment for age and BMI (Table 4).

## Protein level in subjects with inefficient and efficient CPM

PCA for CPMi och CPMe indicated no clear difference in the measured protein levels of the 92 proteins incorporated in the panel (Figure 4). OPLS-DA was performed, but the results failed to indicate a good predictive model (Q2 was 0.07 and average cumulative R2 of *X* and *Y* of this model was 0.25 and 0.48). Cross-verification indicated no significance (CV-ANOVA with p=0.9). When these analyses were re-run using the proteins with VIP>1.3, no significant regressions were obtained for the changes in CPM (R2=0.15, Q2=0.02, CV-ANOVA p-value=0.53) (Table 3). Eighteen proteins had VIP>1.3 at MVDA (Table 3): chemokine (C-X-C motif) ligand 9 (CXCL9) and chemokine (C-X-C motif) ligand 1 (CXCL1). CDCP1 and CXCL9 had p (corr) positive signs and were associated with CPMi. Efficient CPM was associated with CXCL-1. Only CUB domain-containing protein (CDCP1; p=0.04) was found to be significant after adjustment for multiple testing according to Benjamini and Hochberg. No differences between subgroups with iCPM and eCPM in the protein expressions as determined by a t-test and adjusting for multiple testing could be demonstrated (Table 4).

## Proteomic profile of the cold pain tolerant (PT) and pain sensitive (PS) subjects

Statistically significant differences were seen in the protein levels between cold pressor sensitive and tolerant subjects as determined by univariate analysis (unadjusted analysis p=0.02 and adjusted for multiple testing according to Benjamini and Hochberg and for sex and BMI as potential covariates; p=0.03). PCA revealed a separation of the PT and PS groups (Figure 5). OPLS-DA indicated a good predictive model (Q2 was 0.71 and average cumulative R2 of *X*

**Table 3:** Top protein results from t-test of patients with neuropathic pain and subjects without pain, cold pain tolerant (PT) and cold pain sensitive (PS) and subjects with inefficient (iCPM) and efficient CPM (eCPM).

| | | | | Adjusted for multiple testing (MT) | | | | | Adjusted for sex and BMI and MT | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Nr | Protein | Pain (NP) | no pain (nP) | Estimated difference | CI Low | CI high | Unadjusted p-Value | Adjusted p-Value | Estimated difference | Unadjusted p-Value | Adjusted p-Value |
| 1. | **MMP10** | 9.475 | 9.182 | 0.293 | 0.07 | 0.07 | **0.010** | 0.944 | 0.286 | **0.010** | 0.985 |
| 2. | **FGF-5** | 1.237 | 1.310 | −0.073 | −0.15 | 0.01 | **0.034** | 0.976 | −0.078 | **0.034** | 0.985 |
| 3. | IL-2RB | 1.661 | 1.556 | 0.105 | −0.01 | 0.22 | 0.095 | 0.976 | −0.153 | 0.063 | 0.985 |
| 4. | CCL28 | 2.850 | 2.992 | −0.141 | −0.30 | 0.02 | 0.096 | 0.976 | −0.107 | 0.099 | 0.985 |
| 5. | IL4 | 0.355 | 0.505 | −0.150 | −0.34 | 0.04 | 0.128 | 0.976 | −0.148 | 0.131 | 0.985 |

| | | | | Adjusted for multiple testing (MT) | | | | | Adjusted for sex and BMI and MT | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Nr | Protein | Sensitive (PS) | Tolerant (PT) | Estimated difference | CI Low | CI high | Unadjusted p-Value | Adjusted p-Value | Estimated difference | Unadjusted p-Value | Adjusted p-Value |
| 1. | **CDCP1** | 2.982 | 2.621 | 0.306 | −0.33 | 0.05 | **0.003** | 0.030 | 0.306 | **0.032** | **0.049** |
| 2. | **TGF-α** | 3.032 | 2.901 | 0.131 | −0.24 | 0.22 | **0.040** | 0.403 | 0.131 | 0.381 | 0.307 |
| 3. | **CCL20** | 8.065 | 7.675 | 0.301 | −0.69 | −0.08 | **0.005** | 0.025 | 0.390 | **0.021** | **0.047** |
| 4. | MCP-1 | 11.54 | 11.34 | 0.195 | −0.37 | −0.11 | 0.062 | 0.275 | 0.195 | 0.302 | 0.579 |
| 5. | **HGF** | 8.669 | 8.493 | 0.197 | −0.34 | −0.22 | **0.037** | 0.172 | 0.176 | 0.327 | 0.477 |
| 6. | **CCL25** | 6.498 | 6.297 | 0.230 | −0.43 | −0.33 | **0.045** | 0.232 | 0.230 | 0.367 | 0.767 |
| 7. | **MCP-3** | 2.343 | 2.146 | 0.197 | 0.07 | 0.08 | **0.042** | 0.347 | 0.321 | 0.387 | 0.653 |
| 8. | **Ftl3l** | 9.272 | 9.176 | 0.196 | −0.38 | −0.07 | **0.013** | 0.128 | 0.196 | 0.273 | 0.756 |
| 9. | **IL7** | 3.280 | 3.409 | −0,126 | −0.02 | −0.13 | **0.008** | 0.238 | −0.128 | 0.403 | 0.765 |
| 10. | **TRANCE** | 4.795 | 5.046 | −0.249 | −0.02 | 0.05 | **0.051** | 0.252 | −0.234 | 0.051 | 0.256 |
| 11. | **IL17C** | 3.250 | 3.058 | 0.221 | −0.52 | −0.08 | **0.008** | 0.051 | 0.231 | 0.082 | 0.345 |

| | | | | Adjusted for multiple testing (MT) | | | | | Adjusted for sex and BMI and MT | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Nr | Protein | eCPM | iCPM | Estimated difference | CI Low | CI high | Unadjusted p-Value | Adjusted p-Value | Estimated difference | Unadjusted p-Value | Adjusted p-Value |
| 1 | **CDCP1** | 2.602 | 3.038 | −0.424 | −0.20 | 0.08 | **0.01** | **0.010** | −0.233 | **0.032** | **0.042** |
| 2 | AXIN | 8.234 | 7.980 | 0.254 | 0.12 | 0.01 | 0.09 | 0.128 | 0.167 | 0.171 | 0.391 |
| 3 | **CXCL9** | 6.918 | 7.313 | −0.400 | 0.03 | 0.22 | **0.001** | 0.032 | 0.120 | 0.169 | 0.678 |
| 4 | **CXCL1** | 11.04 | 10.08 | 0.169 | 0.23 | 0.05 | **<0.001** | 0.238 | 0.010 | 0.338 | 0.609 |

In the first part of the table the p-values were adjusted for multiple testing (MT) according to Benjamini and Hochberg. In the last three columns the same proteins are adjusted for patients' sex and BMI as covariates with p-values adjusted for MT according to Benjamini and Hochberg. The confidence interval (CI) limits depicts the 95 percent confidence interval of the estimated difference between the two groups. In bold are the statistically significant differences between the groups.

and *Y* of this model was 0.95 and 0.88). Cross-verification indicated highly significant results (CV-ANOVA with p<0.001). A total of 9 proteins associated with cold pain sensitivity had VIP>1.3. Only two of these proteins topped the list of significant proteins associated with PS after adjusting for multiple testing according to Benjamini and Hochberg: CUB domain-containing protein (CDCP1; p=0.04) and chemokine (C–C motif) ligand 20 (CCL20; p=0.04). Several other protein alterations scrutinized with MVDA and found statistically significant at unadjusted p values comparing PT/PS subgroups were transforming growth factor alpha (TGF-α; p=0.04), chemokine (C–C motif) ligand 25 (CCL25; p=0.04), hepatocyte growth factor (HGF; p=0.03), monocyte chemotactic protein 3 (MCP-3; p=0.04), IL7 (p=0.008), IL17C (p=0.008) (Tables 3 and 4). Interestingly, using MVDA, eleven proteins were found among the top proteins, in both CPMi/CPMe and PS/PT subgroups (CDCP-1, SIRT2, AXIN, STAMBP, CXCL9, IL7, CCL25, CASP8, TGF-α, IL10RB, MCP-1). From these, expressions of CCL25 and TGF-α, were identified among the top 20 proteins in all groups and subgroups comparisons and CDCP1 was found to be significant associated with both PS and iCPM.

## Discussions

No significant differences between the groups with neuropathic pain and painless neuropathy regarding

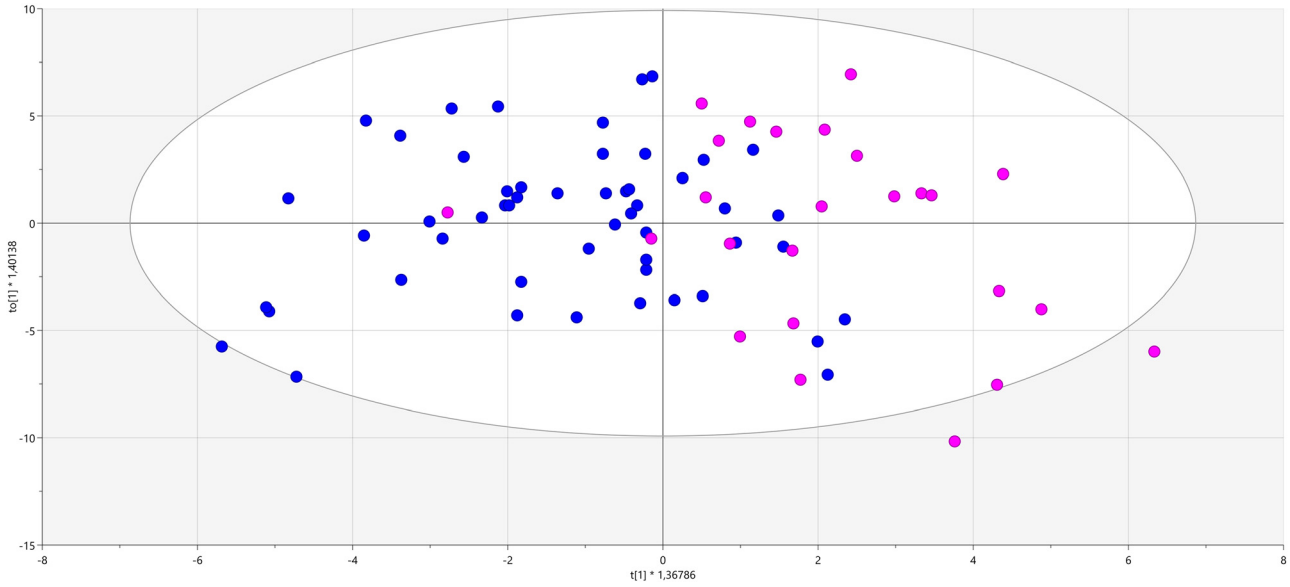


**Figure 4:** Principal component analysis (PCA) plot of protein data that characterizes the proteomic profiles of the subjects with efficient CPM (blue) and inefficient CPM (pink). Each dot represents a sample and each color represents the type of the sample. There was no clear difference between groups.

**Table 4:** Orthogonal partial least square (OPLS) regressions of changes in subjects with neuropathic pain (NP) and neuropathy without pain (nP), cold pain sensitive (PS), cold pain tolerant (CT), with efficient conditioned pain modulation (CPMe) and inefficient CPM (CPMi) using the values of the inflammatory substances as repressors (X-variables).

| | NP/nP | | | PS/PT | | | CPMi/CPMe | | |
|---|---|---|---|---|---|---|---|---|---|
| **No** | **Variables** | **VIP** | **p(corr)** | **Variables** | **VIP** | **p(corr)** | **Variables** | **VIP** | **p(corr)** |
| 1 | **MMP-10** | **2.82** | **0.61** | **CDCP-1** | **1.58** | **0.60** | **CDCP-1** | **1.78** | **0.60** |
| 2 | **FGF-5** | **2.77** | **−0.54** | **TGF-α** | **1.42** | **0.55** | **SIRT 2** | **1.70** | **−0.35** |
| 3 | **IL2RB** | **2.73** | **0.52** | **CCL20** | **1.42** | **0.54** | **AXIN** | **1,65** | **−0.35** |
| 4 | **CCL28** | **2.65** | **−0.51** | **CXCL9** | **1.37** | **0.53** | **STAMBP** | **1,60** | **−0.34** |
| 5 | **IL-4** | **2.03** | **−0.43** | **MCP-1** | **1.37** | **0.53** | **ST1A1** | **1.59** | **−0.34** |
| 6 | **CX3CL1** | **2.00** | **0.41** | **HGF** | **1.33** | **0.52** | **CXCL 9** | **1.59** | **0.34** |
| 7 | **CXCL11** | **1.90** | **0.37** | **CCL25** | **1.31** | **0.43** | **CXCL-1** | **1.57** | **−0.44** |
| 8 | **CCL25** | **1.45** | **−0.36** | **MCP-3** | **1.30** | **0.42** | **PDL-1** | **1.55** | **−0.33** |
| 9 | **CCL11** | **1.37** | **−0.36** | **Ftl3l** | **1.30** | **0.41** | **CD40** | **1.53** | **0.32** |
| 10 | **HGF** | **1.36** | **−0.35** | CCL11 | 1.13 | 0.40 | **TWEAK** | **1.49** | **−0.35** |
| 11 | **IL13** | **1.36** | **0.30** | TNFRSF9 | 1.23 | 0.38 | **CXCL5** | **1.49** | **−0.33** |
| 12 | PD-L1 | 1.28 | 0.29 | AXIN1 | 1.09 | −0.33 | **CASP8** | **1.48** | **−0.31** |
| 13 | GDNF | 1.26 | 0.29 | SIRT2 | 1.09 | −0.32 | **TGF-α** | **1.43** | **0.24** |
| 14 | IL-6 | 1.24 | 0.28 | IL7 | 1.06 | 0.41 | **MCP-1** | **1.38** | **0.21** |
| 15 | MMP-1 | 1.22 | 0.26 | TRANCE | 1.06 | 0.20 | **CD244** | **1.37** | **0.21** |
| 16 | SCF | 1.19 | 0.26 | IL17C | 1.06 | 0.19 | **IL7** | **1.34** | **0.21** |
| 17 | IL-8 | 1.10 | 0.19 | CASP8 | 1.06 | 0.18 | **LAPTGF β** | **1.32** | **−0.21** |
| 18 | CXCL5 | 1.10 | −0.15 | IL10RB | 1.04 | 0.18 | **4E-BP1** | **1.30** | **−0.21** |
| 19 | CCL3 | 1.08 | −0.15 | STAMBP | 1.04 | 0.17 | IL10RB | 1.29 | 0.10 |
| 20 | TGF-α | 1.07 | −0.15 | CSF 1 | 1.04 | 0.16 | CCL25 | 1.29 | 0.11 |
| | R2=0.41 | | | **R2=0.95** | | | R2=0.15 | | |
| | Q2=0.30 | | | **Q2=0.89** | | | Q2=0.02 | | |
| | CV ANOVA p=0.09 | | | **CV ANOVA p<0.001** | | | CV ANOVA p=0.53 | | |

The proteins with variable of importance (VIP) value exceeding 1 are shown in this table; p(corr), a positive sign indicates positive association (higher levels of cytokines/chemokynes) in the first subgroup of subjects. The three bottom rows report R2, Q2, CV-ANOVA (p-value). In bold are presented the proteins with VIP (variable influence of projection) above 1.3.

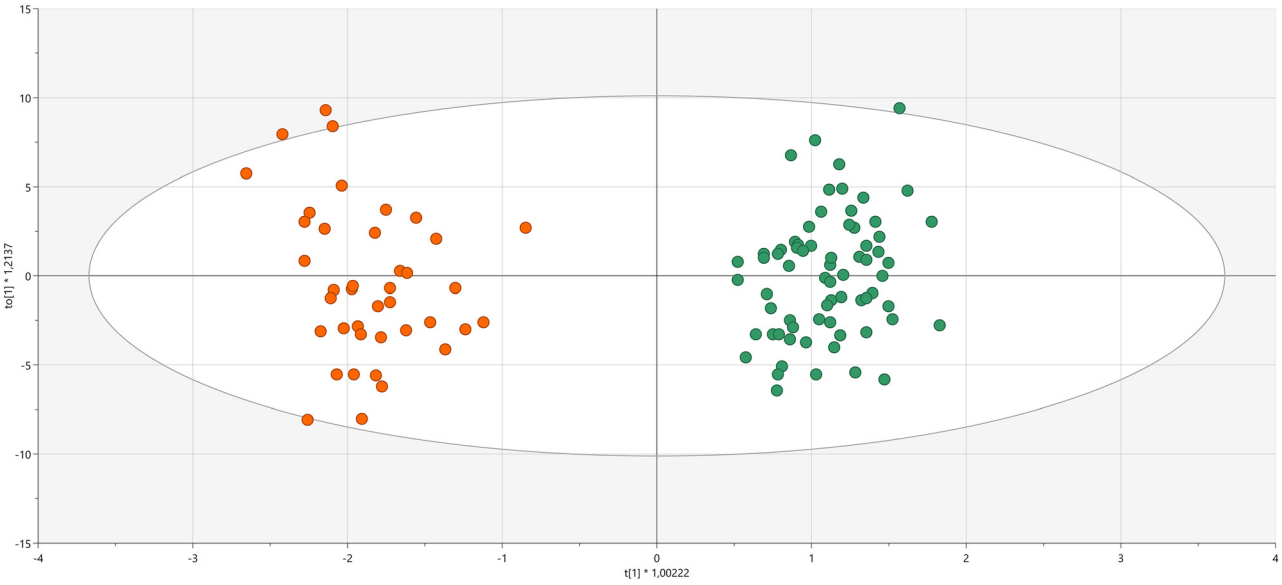


**Figure 5:** Principal component analysis (PCA) plot of protein data that characterize proteomic profiles of cold tolerant (PT) and sensitive (PS) subjects. Each subject is a dot of the studied cohort. There was clear difference between PT (green) and PS (orange). The 2 axes represent the 2 principle components of the model.

inflammatory markers could be seen. This result continues the list of negative studies demonstrating that the subjects who eventually developed neuropathic pain exhibited no other differences in comparison with painless subjects with neuropathy when assessed by quantitative sensory testing (QST) [2, 38–40] CPM, [4], and skin biopsies [2, 41]. A subgroup of subjects from both chronic neuropathic pain and neuropathy without pain groups with predominant proinflammatory protein profile demonstrated diminished tolerance to the cold pressor task. CDCP-1 and CCL20 was highly associated with cold pain intolerance and CDCP-1 with both pain cold sensitivity and inefficient CPM. To the best of our knowledge, this is the first study in which cold pain tolerance of subjects with pain or painless neuropathy was evaluated in relation with a proteomic profile.

## Subjects

Less than half of patients who undergo nerve repair after injury develop neuropathic pain. Injury of a major nerve, younger age and less time after trauma and surgery were predictors for pain after traumatic nerve injuries in the upper extremities in a previous study [24]. We tried to eliminate many confounders that could affect the comparison between subjects with pain and no pain. No differences in socio-demographic data and type of nerve injuries in upper extremities were seen. In addition, all of the subjects had a definite traumatic nerve lesion, described by the surgeon in detail, who had seen the injured nerve intraoperatively. Chronic pain is known to impair function and to decrease patients' quality of life. Patients with chronic neuropathic pain described in other clinical trials were most frequently recruited from pain clinics and likely to report low quality of life [42]. Contrary to this, none of the subjects recruited in this study had ever met a pain specialist or attended a pain clinic. Perhaps these characteristics were the reason behind the results obtained in this study where no signs of systemic inflammation have identified.

## Inflammation and pain

There is an expanding body of evidence linking inflammation with health and disease [43]. Although depression is one of the confounders confirmed as a proinflammatory state [44, 45], the majority of the participants (85%) in our study demonstrated no signs of depression. As described by Slavich [46], inflammatory processes are upregulated by interpersonal stressors involving social threat and adversity leading to an inflammatory phenotype that is seen in the overlap of depression with several somatic conditions such as chronic pain, metabolic syndrome, and obesity.

## Neuropathic pain and inflammation

Accumulating evidence has demonstrated that neuroinflammation contributes to initiation and maintenance of neuropathic pain [47] and certain proinflammatory

cytokines are elevated in neuropathic pain conditions [16, 48]. The potential proinflammatory biomarkers found in serum associated with neuropathic pain were IL-6 for lumbar radicular pain [49] and postherpetic neuralgia [50] and TNFα, IL-2 for painful neuropathy [13, 51]. The present study failed to identify any of these biomarkers as responsible for the difference between neuropathic pain and neuropathy without pain. The results are similar to a previous study indicating that TNF-α and IL-6 in serum were not different between patients with painful and painless neuropathies [52]. Contrary to evidence suggesting that low-grade inflammation and cytokine signaling are playing a critical role in neuropathic pain [53], the results are still conflicting with no difference in the systemic proteomic profile to distinguish clearly between painful or painless neuropathy. In patients with type 2 diabetes, deficits in systemic cytokines and chemokines were linked to polyneuropathy in general but not specifically to the painful or painless entity [54]. A high-inflammation subgroup with 14 inflammation-related proteins associated with neuropathy and higher pain intensity was found in another study using a multivariate analysis approach in order to investigate differences between neuropathic pain and neuropathy without pain in diabetes mellitus [37]. One can argue that with MVDA more proteins were picked up giving over-optimistic results. Univariate and multivariate analysis are not mutually exclusive [55]. Thus, in order to maximize the extraction of relevant information from metabolomics datasets both univariate and MVDA-analysis were implemented and presented in our study, but significant results were considered only after adjusting for multiple testing. Taken together all these proteomic studies delivered a huge number of proteins, some of them considered unique for certain types of neuropathy, but in fact a lot of overlapping proteins could be seen [56]. Changes in inflammation patterns were not only seen in neurotic pain, but also in many other types of chronic pain, e.g. fibromyalgia [57]. The patterns are similar, but we do not know if it is a general phenomenon, found in certain groups of chronic pain patients or is related with the onset of pain and comorbidities.

### Cold pressor test, conditioned pain modulation and inflammation

Interestingly, differences between the proteomic profile of cold-water tolerant and sensitive subjects were found. It is known that pro and anti-inflammatory biomarkers interact with each other in a balanced manner with pro-inflammatory cytokines being involved in chronic pain conditions and anti-inflammatory cytokines as having an antinociceptive effect. There is anecdotal evidence that cold-water adaptation is able to reduce the magnitude of pro-inflammatory triggers [58], reduced plasma stress hormones, and ACTH and cortisol response [58, 59]. One can argue that the cold pressor test is a painful stimulus which downregulates the acute immune response [18], but these changes occurred between 30 to 120 min post experimental testing, a longer time frame than in the present study where the blood was taken exactly after CPM [60]. Inflammation-related biomarkers were found to be associated with cold pain tolerance and pain thresholds in a previous cohort study of young individuals [19]. It was also demonstrated that pain tolerance to hand immersion in cold water was associated with lack of persistent pain 3 years after surgery [61] and pain hypersensitivity could predict pain and disability after low back surgery [61]. In a previous study exploring the relation between inflammation and the cold pressor task in healthy subjects, the protein with the strongest negative association with cold pain tolerance was C–C motif chemokine 28 (CCL28) [19]. In our study CDCP-1 was highly associated with both cold pain intolerance and inefficient CPM. Studies have shown that CDCP-1 has been implicated in cell adhesion and autoimmunity [62]. This protein modulates T cell responses upon activation and aberrant CDCP1-signaling is associated with inflammation [62, 63]. Thus, rather than demonstrating a direct link between chronic neuropathic pain and inflammation, we can postulate that the subjects with more inflammation would be more pain sensitive to the cold pressor test and probably to acute pain. Our studies on this cohort of subjects with chronic neuropathic pain and neuropathy without pain revealed that the sensitivity to acute pain initiated with experimental stimuli is separated from that of the persistent pain in individuals with no associated comorbidities.

## Methodological considerations and limitations

Strengths: We have investigated a group of patients who all have a verified nerve lesion, and two distict outcomes, neuropathic pain or no pain, in spite of having very similar lesions. Thus, all the pain patients have a definite neuropathic pain, and the control subjects belong to the same cohort. The investigated group also showed a low degree of co-morbidities, such as depression and anxiety, otherwise often seen in populations recruited from pain clinics.

This study has a number of limitations. First, an independent healthy control group without nerve injury was not included in the comparison between the groups. Although the study had clear selection criteria and tried to eliminate all the conditions that might affect inflammatory mediators [2], in both groups there were subjects with pains other than post-traumatic neuropathic pain such as joint pain, back pain, headache. No conclusion about whether CPM responses were correlated with clinical manifestations of chronic neuropathic pain could be reached [64]. Other studies might aim to identify specific subgroups of subjects with dysfunctional endogenous pain inhibition [65]. Patients with neuropathic pain have altered proteomic and neuropeptide constituents in cerebrospinal fluid (CSF) compared to controls [10]. It would also be useful in future studies to use both subject-matched CSF and blood samples.

## Conclusions

No significant alterations in systemic proteins were found comparing subjects with neuropathic pain and painless neuropathy. The results are still conflicting with no difference in the systemic proteomic profile to distinguish clearly between painful or painless neuropathy. Thus, our understanding of why some patients develop neuropathic pain and others not after a similar traumatic nerve injury remains inadequate. An expression of predominant proinflammatory proteins was associated with experimental cold pain responsivity in both subjects with pain and painless neuropathy. One these proteins, CDC-1 acted as "molecular fingerprint" found in experimental pain sensitivity explored with both CPM and CPT. Further studies are warranted to evaluate the clinical utility of this biomarker for deep phenotyping of subjects with pain and subgroups of subjects with cold pain sensitivity and inefficient CPM.

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