Underlying Pathophysiological Mechanisms of Chronic Pain Resolution in Association with Connectivity in Brain

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Abstract

Background and aims: Chronic pain resolution is important but poorly understood. Here, we investigated the pathophysiological mechanisms underlying chronic low back pain (LBP) resolution with the focus on the inflammatory pathways. We performed a transcriptome-wide analysis in peripheral immune cells associated with connectivity in the brain of 32 participants with chronic LBP, followed by 14 weeks of physical exercise.

Methods: The blood samples were collected from 32 participants experiencing LBP who underwent 14 weeks of physical exercise. The first blood draw was done after two weeks of low-intensity exercise, and the second was after the last exercise session. Pain ratings were collected two weeks before the start of the program and then two weeks after the end of the study. To determine the success of the exercise intervention, the "persistent" and "improved" statuses were assigned to the participants based on the difference in the 7-day average pain intensity score. The DESeq2 and fgsea programs have been used for transcriptomics analysis.

Results: We found that participants with improved pain over the study span had a higher number of significantly differentially expressed genes (101) than persistent participants (0). At the end of the study, most genes (71) showed higher expression levels in the persistent pain group, whereas 14 genes showed higher expression levels in the improved pain group. Inflammatory pathways decreased in expression over time in the improved pain group but increased in the persistent pain group. At the end of the study, neutrophils and leukocyte activation pathways were significantly higher in the persistent pain group. In blood transcriptomics and brain imaging data correlation analysis, we identified many significantly correlated pathways with brain and neural development, mitochondria-related, blood vessel development, inflammatory, stress-related immune and synaptic pathways as major significant groups.

Conclusion: Our results suggest that active biological processes underlie pain resolution in chronic LBP patients, and active down-regulation of inflammation is a significant contributor, which is underlined by the reduction of leukocytes' activation, mostly macrophage, neutrophil, and possibly mast cell cells. These changes in blood transcriptomics possibly contribute to the improvement of the brain functions during the exercise as we found blood transcriptomics positively correlated with brain connectivity, those changes showed to underly the pain resolution during exercise.

Résumé

Contexte et objectifs: La résolution de la douleur chronique est importante mais mal comprise. Ici, nous avons étudié les mécanismes physiopathologiques sous-jacents à la résolution des lombalgies chroniques (LBP). Nous avons effectué une analyse à l'échelle du transcriptome des cellules immunitaires périphériques associées à la connectivité dans le cerveau de 3 participants atteints de lombalgie chronique, suivie de 14 semaines d'exercice physique.

Méthodes: Les échantillons de sang ont été prélevés auprès de 32 participants souffrant de lombalgie qui ont subi 14 semaines d'exercice physique. La première prise de sang a été effectuée après deux semaines d'exercices de faible intensité et la seconde après la dernière séance d'exercices. Les évaluations de la douleur ont été collectées deux semaines avant le début du programme, puis deux semaines après la fin de l'étude. Pour déterminer le succès de l'intervention d'exercice, les statuts « persistant » et « amélioré » ont été attribués aux participants en fonction de la différence du score moyen d'intensité de la douleur sur 7 jours. Les programmes DESeq2 et fgsea ont été utilisés pour la transcriptomique et l'analyse d'association.

Résultats: Nous avons constaté que les participants qui ont amélioré leur douleur au cours de l'étude avaient un nombre plus élevé de gènes exprimés de manière significativement différentielle (101) que les participants persistants (0). La plupart des gènes (71) présentaient des niveaux d'expression plus élevés dans le groupe de douleur persistante, tandis que 14 gènes présentaient des niveaux d'expression plus élevés dans le groupe de douleur améliorée. L'expression des voies inflammatoires a diminué au fil du temps dans le groupe de douleur améliorée, mais a augmenté dans le groupe de douleur persistante. Les voies d'activation des neutrophiles et des leucocytes étaient significativement plus élevées dans le groupe des douleurs persistantes. Dans l'analyse de corrélation des données de transcriptomique sanguine et

d’imagerie cérébrale, nous avons identifié le développement cérébral et neuronal, celui lié aux mitochondries, le développement des vaisseaux sanguins, les voies immunitaires et synaptiques inflammatoires liées au stress comme principaux groupes importants.

Conclusion: Nos résultats suggèrent que des processus biologiques actifs sont à l'origine de la résolution de la douleur chez les patients lombalgiques chroniques, et qu'une régulation négative active de l'inflammation y contribue de manière significative, ce qui est souligné par la réduction de l'activation des leucocytes, principalement des macrophages, des neutrophiles et éventuellement des mastocytes. . L’analyse de corrélation des données de transcriptomique sanguine et d’imagerie cérébrale a révélé sept voies significativement positivement corrélées à la connectivité cérébrale.

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List of Abbreviations

LBP — Low Back Pain

CLBP – Chronic Low Back Pain

PAR-Q – Physical Activity Readiness Questionnaire

HUNT – Norwegian Nord-Trøndelag Health Study

GRCh38 – Genome Reference Consortium human genome build 38

DESeq2 – Differential Expression of Sequences

TPM – Transcripts Per Million

GO – Gene Ontology

BMI — Body Mass Index

FDR — False Discovery Rate

CNS — Central Nervous System

NES — Normalized Enrichment Score

mPFC — Medial Prefrontal Cortex

DWI — Diffusion Weighted Imaging

rsfMRI — Resting-state functional MRI

TR – Repetition Time

TE – Echo Time

FA – Flip Angle

AQDC — Association Québécoise de la Douleur Chronique

CRIUGM — Centre de recherche de L'Institut Universitaire de gériatrie de Montréal

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Acknowledgments

First, I would like to thank my supervisor, Luda Diatchenko. I would not be able to develop as much without her professional and personal input. I did not only expand my scientific knowledge, but also she served as a great example of how to build communication with people, co-workers, and the team. I am also very thankful for her editorial input on all my work during the Master's program, including this Thesis.

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Of course, last but not least, I would like to thank my parents for being so supportive during that journey and allowing me to answer the main question I had before coming here (Masters). "Would I like to pursue and push further or take some time to try my chance somewhere outside the academia?" Thankfully, for everybody mentioned previously, I have an answer to that question. Thank you!

Contribution of Authors

All chapters of the thesis were written by me, Ivan Chumakov, Masters student from Dr. Luda Diatchenko lab, except “Functional MRI” and “Structural MRI” chapters in Materials and Methods section. “Functional MRI” and “Structural MRI” chapters in Materials and Methods section were written by Mathieu Roy’s student Erika Gentile.

Introduction

Pain serves an adaptive survival function, an inner warning system pointing to harm or a treatment. "An unpleasant sensory and emotional experience associated with or resembling that associated with actual or potential tissue damage" is how the International Association for the Study of Pain defined pain (Raja et al., 2020). Pain can linger long after a trauma or inflammatory event and is not always a sign of tissue damage (Katz & Melzack, 1999). *Chronic pain* is a medical condition that persists longer than it can be protective. This pain diminishes people's quality of life and increases medical expenses (Gatchel et al., 2014). The costs to society of managing pain and lost productivity due to chronic pain are high. As people age, pain becomes more common; in the UK, up to 62% of people over 75 reports having ongoing pain complaints (Fayaz et al., 2016). Strangely enough, though, older adults are underrepresented in pain clinics and pain management programs (Kee et al., 1998). Older adults' reluctance to seek pain treatment may be explained by negative attitudes toward pain treatment, particularly about perceived ineffectiveness, worries about unfavourable side effects, and addiction. It may also stem from the belief that pain is inevitable as one ages and that pain complaints are not as severe as other comorbidities (Makris et al., 2015).

Many treatments for low back pain have anti-inflammatory function alongside elucidating some activity in the immune system and are based on nonsteroidal anti-inflammatory drugs, acetaminophen, and corticosteroids, though none of them have shown remarkable effectiveness (Chou, 2010). Moreover, some of them have substantial side effects. While drugs have shown minimal effectiveness, exercise is the current first-line treatment prescribed to patients with chronic pain.

A population-based analysis of 4219 participants in the HUNT study revealed that people who engage in moderate physical activity report lower levels of musculoskeletal pain (Landmark et al., 2011, 2013). In a similar vein, men who engage in more physical activity during their leisure time are less likely to experience chronic pelvic pain (Zhang et al., 2015), and women who engage in more physical activity during their leisure time are less likely to experience low back pain during pregnancy (Mogren, 2005). Therefore, physical activity lowers the risk of developing chronic pain, whereas physical inactivity may be a risk factor. Physical exercise regimens have been found to improve pain and disability following meta-analyses consistently (Owen et al., 2020; Searle et al., 2015a, 2015b; Van Middelkoop et al., 2010), and most clinical guidelines worldwide now recommend it (Oliveira et al., 2018). Physical activity seems safe, even at high intensities, which is a significant benefit of exercise therapy (Ansuategui Echeita et al., 2022; Verbrugghe et al., 2019).

Furthermore, it has been demonstrated that several mental and cardio-respiratory health disorders coexist with CLBP (Von Korff et al., 2005). Numerous studies have demonstrated how crucial it is to maintain a high level of cardiorespiratory fitness (possibly through physical exercise) to reduce cardiovascular disease risk (Myers et al., 2015). Similarly, it has been demonstrated that physical activity positively impacts both symptomatic (Béland et al., 2020; Owen et al., 2020) and general mental health (Hoffman & Hoffman, 2007). Therefore, considering its potential positive impact on symptoms specific to low back pain, physical exercise therapy appears to be a key strategy in managing CLBP.

Patients with CLBP are currently prescribed various exercises (Owen et al., 2020). Likewise, a diverse range of rationales is employed to support the inclusion of CLBP patients in physical activity programs (Wood et al., 2021). Strengthening of the muscles and increased cardiorespiratory fitness are common causes. Resistance training (Steele et al., 2020) and aerobic training (Bruehl et al., 2020) can be used to achieve these two goals. Different training modalities are known to induce distinct adaptations, though there may be some overlap. To maximize force production capacity, resistance training, for example, causes neural and muscular adaptations (Damas et al., 2015; Hortobágyi et al., 2021; Suchomel et al., 2018). Conversely, aerobic exercise improves endurance performance by causing changes in the cardiovascular and muscular systems (Hellsten & Nyberg, 2015; MacInnis & Gibala, 2017). High-intensity multimodal exercise training programs, including both resistance and aerobic exercises, can potentially reduce pain and disability associated with CLBP while also offering overall benefits (Verbrugghe et al., 2019). Preliminary research indicates that patients with CLBP may think that movements require more work (Coppieters et al., 2021), limiting their ability to engage in physical activities (Marcora, 2016). Although pain itself may distort their perception of effort (Norbury et al., 2022), their diminished physical capacities and unfamiliarity with physical activity may also be factors. A multimodal approach seems particularly beneficial given the muscular (Steele et al., 2020) and cardiorespiratory (Van Der Velde & Mierau, 2000) deconditioning in patients with CLBP.

Recently, exercise therapy has made pain intensity reduction a top priority for treatment (Wood et al., 2021). Thankfully, various exercises seem to reduce pain (Owen et al., 2020). Nonetheless, according to Chou, Deyo, Friedly, Skelly, Hashimoto, and Owen, treatment effects are still low

to moderate. According to earlier research, exercise intensity may not have been high enough to produce the best possible improvement in CLBP patients (Searle et al., 2015a, 2015b; Van Middelkoop et al., 2010). Patients with CLBP seem to benefit more from higher exercise demands (Bruehl et al., 2020; Verbrugghe et al., 2019). Another hypothesis is that clinical trials are conducted for far too little (about eight weeks on average) (Owen et al., 2020). It is currently unclear if participants in CLBP treatment experience a maximum reduction in pain or if more extended exercise training regimens could provide even more pain relief. The prevalent use of pre- and post-intervention summary statistics (Bruehl et al., 2020; Verbrugghe et al., 2019) is most likely the cause of the current gap, essentially intervening in a mystery.

Research on the underlying mechanisms of CLBP persistence has been conducted. It has been suggested that the central nervous system (CNS) may be primarily responsible for maintaining or exacerbating chronic low back pain (CLBP) since the spinal pathology of the majority of patients (~85%) (Chou, 2010; Jarvik & Deyo, 2002) does not fully account for the persistence of the pain (Clauw, 2015). A cluster of centrally mediated symptoms, including fatigue, sleep disorders, mood disturbances, decreased central pain inhibition, and increased central pain facilitation, would appear as the hallmark of this central amplification process. According to a popular theory of chronic pain today, the inflammation accompanying pain suppresses the striatal dopaminergic system—the reward system that encourages actions involving an energy expenditure or taking a chance. This leads to "sickness behaviours" (Maier & Watkins, 1998) that promote healing by increasing pain and minimizing energy expenditure. While these reactions might be evolutionary advantageous in the short term following an injury or illness, they turn maladaptive when they persist past the point of healing, which appears to be the case with chronic pain. Physical training

can counteract this maladaptive behaviour by reducing sedentariness and increasing energy expenditure. As of right now, several studies have demonstrated that CLBP is linked to decreased volumes of the hippocampus and amygdala as well as altered connectivity of subcortical limbic structures, including the nucleus accumbens, hippocampus, and amygdala (Apkarian & Reckziegel, 2019; Baliki et al., 2012; Vachon-Presseau et al., 2016). Hippocampal volume (Erickson et al., 2011; Van Praag, 2008; Voss et al., 2013), perfusion (Pereira et al., 2007), function (Stillman et al., 2016), and connectivity between cortical and limbic structures (Herold et al., 2019) have all been demonstrated to increase with physical training.

Additionally, research indicates that exercise may start a complicated chain reaction of cytokines that eventually lowers the amounts of pro-inflammatory molecules in the blood (Cotman et al., 2007; Petersen & Pedersen, 2005). It is conceivable that decreased systemic levels of pro-inflammatory molecules may mediate the effects of physical training on the central nervous system (CNS), given the detrimental effects of systemic inflammation on the CNS (Ren et al., 2011; Treadway et al., 2019). Although there have been advances in understanding social, psychological, and brain processes contributing to developing or resolving chronic low back pain, underlying molecular mechanisms are still poorly understood.

Neuronal and non-neuronal cells mediate chronic pain, which is considered as a neuroinflammatory disorder (Ramesh et al., 2018). Neutrophils, monocytes, and T cells are examples of circulating immune cells drawn to tissue damage and inflammation areas. They also frequently penetrate the central and peripheral nervous systems (Kavelaars & Heijnen, 2021; Scholz & Woolf, 2007). When these cells are activated, various inflammatory mediators such as

lipids, proteases, and cytokines/chemokines are expressed. These mediators regulate pain directly on peripheral sensory or central second-order neurons and indirectly on other immune or local cells. It is believed that the existence of these activated immune and glial, either centrally or peripherally, adds to the chronic pain state's persistence. (Mifflin & Kerr, 2014; Newman et al., 2015; Chapman & Vierck, 2017).

Prior research on transcriptomic analysis and human genetic association has demonstrated that the nervous and immune systems interact intricately in the pathophysiology of chronic pain (Freidin et al., 2019; Ramesh et al., 2018). Specifically, Ramesh et al. (2018) investigated the contribution of endocannabinoid gene expression and genotype on low back pain susceptibility and chronicity. Their findings revealed significant upregulation of cannabinoid type 2 (CNR2) mRNA in all low back pain (LBP) patients, while fatty acid amide hydrolase (FAAH) mRNA and transient receptor potential cation channel subfamily V member 1 (TRPV1) mRNA were significantly upregulated in chronic LBP (cLBP) patients compared to controls. Furthermore, Freidin et al. (2019) conducted a large genome-wide association study, identifying genetic loci associated with back pain and its risk factors, including psychological components of pain perception and processing. These studies collectively emphasize the complex genetic architecture underlying chronic pain, implicating both neurological and immunological pathways, thereby supporting the need for further investigation into the molecular mechanisms involved. Therefore, the project's objective was to investigate the molecular pathophysiological mechanisms underlying the improvement of chronic low back pain (LBP) following exercise through a transcriptome-wide analysis of the peripheral immune cells and their association with brain connectivity. We hypothesized that the pain improvement in low back pain patients would

be associated with the down-regulation of inflammatory pathways and consecutively with brain connectivity measures.

Materials and Methods

Overview:

The participants were naive to the gym equipment, and their bodies were deconditioned; therefore, two weeks were dedicated to gym equipment education through low-intensity training. Afterward, subjects participated in a 12-week high-intensity physical exercise training program, three days per week, one hour per session, one-on-one with a kinesiologist. The participant's level of physical fitness and ability to engage in physical activity was assessed with the Physical Activity Readiness Questionnaire (PAR-Q, 2002 version) (Thomas et al., 1992). If the participant answered "yes" to at least 1 of the seven questions, he or she was directed to the study's physician for a physical examination.

Participants:

Fifty-seven participants (mean [SD]; 44 females, 23 males, age: 46.7 [14.2], height: 1.67 [0.09] cm, weight: 76.3 [15.4] kg, body mass index: 27.4 [4.97] kg.m-2) volunteered to participate. All participants met the criteria for CLBP by the proposed Canadian minimum dataset for CLBP research, which defines CLBP as back pain that has persisted for at least three months or resulted in pain for at least half of the days in the past six months. Participants were excluded if they had taken analgesic injections or infiltrations within the last six months, were undergoing cancer therapy, or had a self-reported pain of less than four over the past week at the enrolment (0 = no pain, 10 = intolerable pain). Participants had no known severe neurological or psychiatric

disorder or significant cardiorespiratory or musculoskeletal problems preventing them from participating in physical training. Out of 57 total participants 25 were excluded from the final analysis for several reasons, either for not having either phenotypic or transcriptomic data, or having first reported pain less than 4, which did not satisfy inclusion criteria. To define the success of the intervention, the statuses of "improved" and "persistent" pain group were assigned to all 32 remaining particpants based on the pain score difference of at least two between the first and second-time points. If the change was negative one, zero or positive, meaning the report of pain decreased insignificantly, increased or stayed the same over the course of the study, the status persistent pain group was assigned to participants. However, if the change was negative and more than one in magnitude, meaning the pain reduced, the status of improved pain group was assigned to the participants, making it 15 participants who improved and 17 who stayed persistent.

Grouping:

To define the success of the intervention, the statuses of "improved" and "persistent" pain group were assigned based on the pain score difference of at least two between the first and second-time points. If the change was negative one, zero or positive, meaning the report of pain decreased insignificantly, increased, or stayed the same over the course of the study, the status persistent pain group was assigned to participants. However, if the change was negative and more than one in magnitude, meaning the pain reduced, the status of improved pain group was assigned to the participants, making it 15participants who improved and 17 who stayed persistent.

Training:

All participants with CLBP underwent a 14-week multimodal exercise regimen. Tests involving maximal capacities were measured only after two weeks of low-intensity familiarization with the exercises and have been used to calibrate the intensity of the exercises for the remaining 12 weeks. Blood draws were taken before (23 ml) the first and last high-intensity physical training session on week three and week twelve to examine the long-term effects of exercise. Physical and psychosocial outcomes have been measured a week before the start of the training program and a week directly after its completion.

RNA Seq Sequencing:

Following the manufacturer's recommendation, four ml of blood samples were collected into PAXgene Blood RNA Tube (BD Biosciences, JN, USA). Tubes were then stored at −80°C. Total RNA was extracted from the blood using the PAXgene Blood RNA Kit protocol (BD Biosciences, JN, USA). Quality measurements for total RNA were performed using a Bioanalyzer and Nanodrop. A total of 64 RNA samples (500 ng) were sequenced by Genome Quebec (Montreal, Canada) using the Illumina NovaSeq technology. The samples corresponded to the 32 participants with available pain phenotypes and brain imaging data from two time points. Each sequenced sample presented an RNA integrity number value of at least 6. The sequencing was paired-end with an average read length of 100 nucleotides.

RNA Seq Analysis:

The deep-sequencing reads were mapped using STAR version 2.7.3a, with gene expression quantitation derived using the "–quantMode GeneCounts" option (Dobin et al., 2013). Reads

were mapped on human genome version GRCh38, with Ensembl gene annotations version 103 (Yates et al., 2019). The globin gene (ENSG00000244734) was removed for downstream analyses since the samples originated from blood. Gene expression in TPM units was estimated using TPMCalculator version 0.0.3 (Vera Alvarez et al., 2019).

The quality of the sequencing was confirmed by the high mapping rate of reads on the human genome (mean 99.9%) and by the average length of mapped reads (mean 194 nucleotides out of a total read length of 202).

Differential expression of genes was assessed using moderated statistical tests implemented in the R statistical package DESeq2 (Love et al., 2014). Each test was performed with the following covariables: sex, age, and body mass index (BMI), the fact of pain medication intake in the past three months as possible confounding factors for pain. Pathway enrichment scores were estimated using "fgsea", with statistical significance assessed using a fast permutation scheme (Korotkevich et al., 2016). That allowed us to perform gene set enrichment analysis and study pathways trajectories in time between the two pain groups. Slope, which measures the rate of change in the variable on the y-axis (pathways test statistic in improved pain group) as variable on x-axis changes (pathways test statistics in a persistent pain group) allowed us to calculate the difference of biological pathways' activity between two groups. Yellow dots mark inflammatory pathways. Test statistics were calculated based on the following formula: sign of the enrichment score (-1 or +1) multiplied by absolute value of qnorm (p-value divided by 2). Where qnorm considers percentile of normal distribution, mean and standard deviation. That way test statistic value gives an idea of the directionality of the pathways as well as magnitude of its expression.

Functional differences were assessed for selected pathways in Gene Ontology's (GO) biological processes, overarching under "inflammatory response" (GO: 0006954) and "leukocyte activation" (GO:0045321). Enrichment scores served as indicators of positive or negative association between variables in each test. The false discovery rate (FDR) of 10% was used to correct for multiple testing because tests are not independent.

Structural MRI:

Diffusion-weighted imaging (DWI) enables the creation of a map of the diffusion process of molecules, mainly water, in biological tissues. Molecular diffusion in brain tissues reflects interactions with macromolecules, fibers, and membranes. Using this approach, details about white matter tract architecture can be studied. The acquisition parameters for the multi-shell echo planar images were: b-values = 300, 1000 and 2000, diffusion directions = 108, voxel size = 2 x 2 x 2 mm, TR = 2200 ms, TE = 71.0 ms, acceleration factor = 2, base resolution = 110, slices = 57, field of view = 220 mm, interleaved multislice mode, strong fat suppression and encoding direction posterior to anterior. A separate b0 image was acquired in the anterior-to-posterior encoding direction.

Functional MRI:

Resting-state functional MRI (rsfMRI) enables the evaluation of relative regional functional fluctuations that occur in a resting state, a state when the participant is not performing an explicit task. As such, the participants were asked to keep their eyes open and remain as still as possible during data acquisition. This resting brain state condition was observed through changes in the Blood Oxygen Level-Dependent signal. The acquisition parameters for the T2*-weighted

multiband accelerated echo-planar images are: voxel size = 3 x 3 x 3 mm, TR = 785 ms, TE = 30 ms, FA = 54°, acceleration factor = 2, base resolution = 64, slices = 42, field of view = 192 mm, encoding direction anterior to posterior, interleaved multislice mode, number of volumes = 755 with a total acquisition time of 9min and 53s.

Results

Differential Gene Expression:

We assessed genome-wide transcriptomics differences in a cohort of 32 low back pain patients over time of the exercise between the first blood collection time point, two weeks after the start of exercise (T1), and the second blood collection time point, after the last exercise session on week 14th (T2). We observed a substantial difference in gene expression over the time of exercise in gene expression (Fig. 1A). There has been observed 102 genes significantly differentially down-regulated and 120 genes significantly differentially up-regulated at T2 compared with T1.

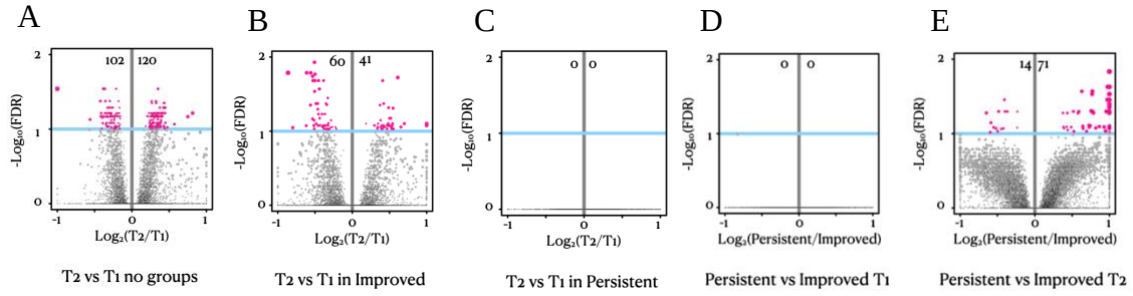

Fig 1. ***Differential Expression of Genes in the Study Contrasts.***

The volcano plot shows statistical significance (y-axis) as a function of fold change in gene expression (x-axis); each dot is a gene. (A) – Differences in gene expression in patients over the time of exercise. (B, C) - Difference in gene expression over time in the improved pain group (B) or the persistent pain group (C), (D, E) – Difference in gene expression between groups at the first time point T1 (D) and at the second time point T2 (E). Genes that would end up outside of the plot are squeezed inside. The vertical grey line indicates null fold change. Genes reaching statistical significance at the FDR 10% level (blue horizontal line) are highlighted in pink. Numbers in the middle along the central line indicate counts of significantly differentially expressed genes that are down-regulated or up-regulated.

We then analyzed the transcriptomics changes over the time of exercise separately in two groups: the one with improved pain throughout the exercise and the one with persistent pain. We identified a total of 101 significantly differentially expressed genes in the improved pain group with about equal amount of down-regulated (60) and up-regulated (41) genes (Fig. 1B). At the same time, there were zero genes significantly differentially expressed genes observed in the persistent pain group over time (Fig. 1C).

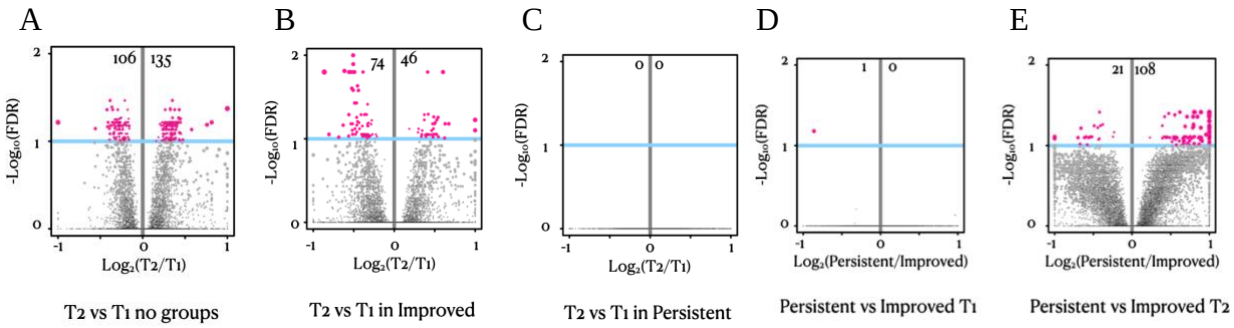

Fig 2.**Differential Expression of Genes Corrected for Drug Usage in the Study Contrasts.** *The volcano plot shows statistical significance (y-axis) as a function of fold change in gene expression (x-axis); each dot is a gene. (A) – Differences in gene expression in patients over the time of exercise. (B, C) - Difference in gene expression over time in the improved pain group (B) or the persistent pain group (C), (D, E) – Difference in gene expression between groups at the first time point T1 (D) and at the second time point T2 (E). Genes that would end up outside of the plot are squeezed inside. The vertical grey line indicates null fold change. Genes reaching statistical significance at the FDR 10% level (blue horizontal line) are highlighted in pink. Numbers in bold indicate counts of significantly differentially expressed genes that are down-regulated or up-regulated.*

At the T1 snapshot, we did not detect any significantly differentially expressed genes between the two groups (Fig. 1D), while at the second time point, the changes started to appear. Towards

the second time point, an increase in the number of significantly differentially expressed genes was observed between the groups. Most of the genes showed higher expression levels in the group with persistent pain (71) versus only 14 genes that showed higher expression levels in the group with improved pain (Fig. 1E). Importantly, when we introduced drug usage as a covariate in the study contrasts analyses, our conclusions regarding the pattern of differential gene expression remained the same (Fig. 2).

Taking into consideration that the fractions of cell types may differ between individuals biassing readings and counts of individual genes, we tested differences between cell type fractions both in within groups comparisons over time and between groups at a specific time point. The results of the analysis did not elicit any significant results indicating that cell type fractions did not affect the gene counts.

Pathways Trajectories in Time Between Two Pain Groups:

We found many biological pathways differentially expressed at a genome-wide level, even in the comparisons where no individual genome-wide significant differentially expressed genes were identified. This can occur when a substantial number of genes of the same pathway change expression in the same direction.

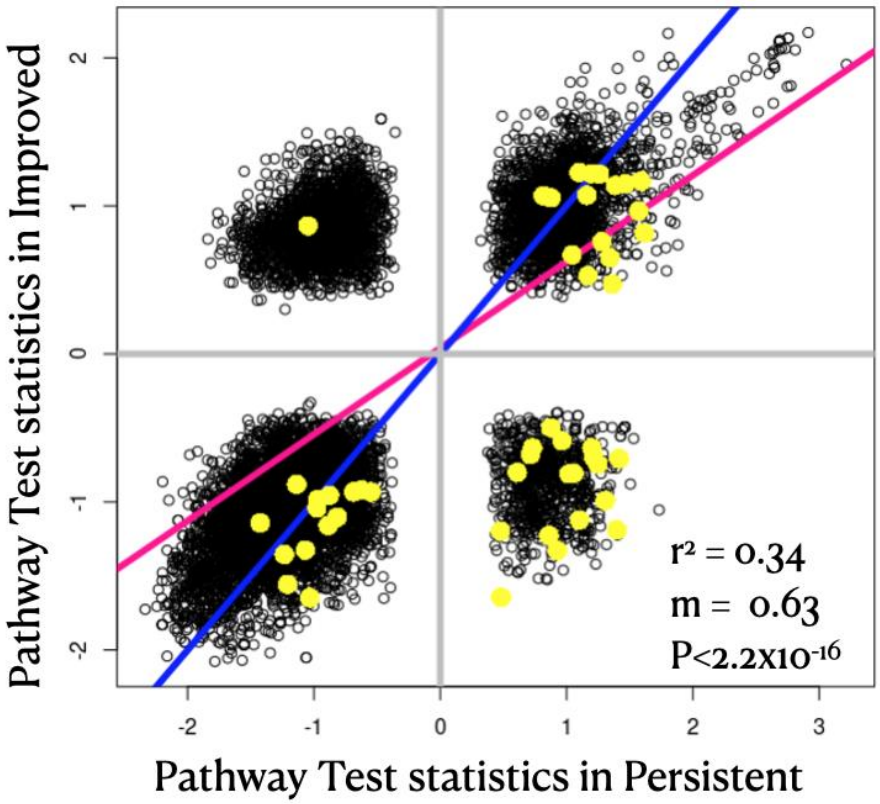

Fig. 3 ***Correlation of Pathway Trajectories in Time Between the Improved and Persistent Pain Groups***

Each dot is a pathway. Pathways coordinates are in test statistics space, obtained from pathway analyses by fgsea. The pink line was obtained from linear regression of the data, whereas the slanted blue line was from the theoretical expectation of equal trajectories. Yellow dots represent inflammatory pathways. Percent variance explained (Pearson's r^2), slope (m), and P-value of regression are reported.

We first tested the overall correlation in the biological pathways' activity over time between the improved pain and persistent pain patients' group. We found that the majority of transcriptional changes over time have a positive correlation (Fig. 3) (slope = +0.63, $P < 2.2 \times 10^{-16}$, $r^2 = 0.34$). There was a clear difference between groups in the magnitude of the response. The improved pain group's response intensity was about 63% smaller than that of the persistent pain group, calculated using the rate of change based on the slope. Most of the pathways, located at the top right and bottom left corners, have similar directionality reflecting the biological responses of both groups to the exercise. Nevertheless, pathways at the top left and bottom right quadrants of the plot are presented in Figure 3, indicating some pathways with opposite directions. We marked all inflammatory pathways in the correlation plot of the biological pathways activity over time between the improved pain and persistent pain patients' group (Fig. 3, highlighted in yellow). The inflammatory pathways are abundantly enriched in the anticorrelated cluster characterized by biological pathways downregulated overtime by the improved pain group and up-regulated persistent group, which are located at the bottom right quadrant (Fig. 3).

Inflammatory and Leukocyte Cell Activation Pathway Analysis:

We next focused on the differences between the improved and persistent pain groups for the Inflammatory and Leukocyte Cell Activation biological pathways. Throughout the exercise, pathways analysis shows that there is overall down-regulation of the inflammatory response pathways (Fig. 4A). Looking more precisely at the specific groups and time points, we found that patients from the improved pain group had inflammatory response pathways downregulated over the time of exercises (Fig. 4B) when the persistent pain group did not show any significant differences (Fig. 4C). Also, our analysis comparing both groups at a specific time point showed

that there are three inflammatory pathways up-regulated in the persistent pain group at T1 and five at T2 (Fig. 4D-E), consistent with more efficient down-regulation of inflammatory pathways in the improved pain group over the time of exercise.

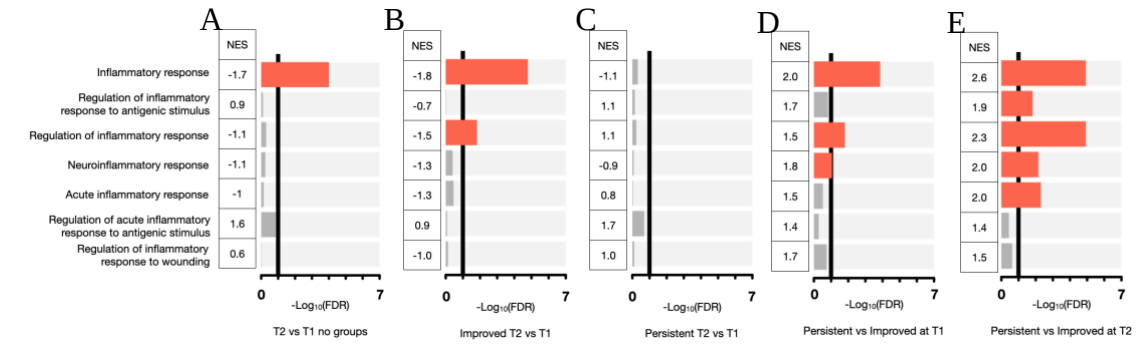

Fig. 4 ***Functional Difference in the Inflammatory Pathways Between the Improved and Persistent Pain Groups.***

The bar plots show FDR-corrected significance level of expression (x-axis) for each pathway; each bar is a subpathway of" inflammatory response" (GO: 0006954). (A) - Differentially expressed pathways over the time of exercise. (B, C) - Differentially expressed pathways over the time of exercise for improved pain group (B) or persistent pain group (C). (D, E) - Differentially expressed pathways between the persistent and improved pain group at the first time point T1 (D) and the second time point T2 (E). Statistically significant pathways at the FDR 10% level are highlighted in orange. The NES column shows normalized enrichment scores for every pathway.

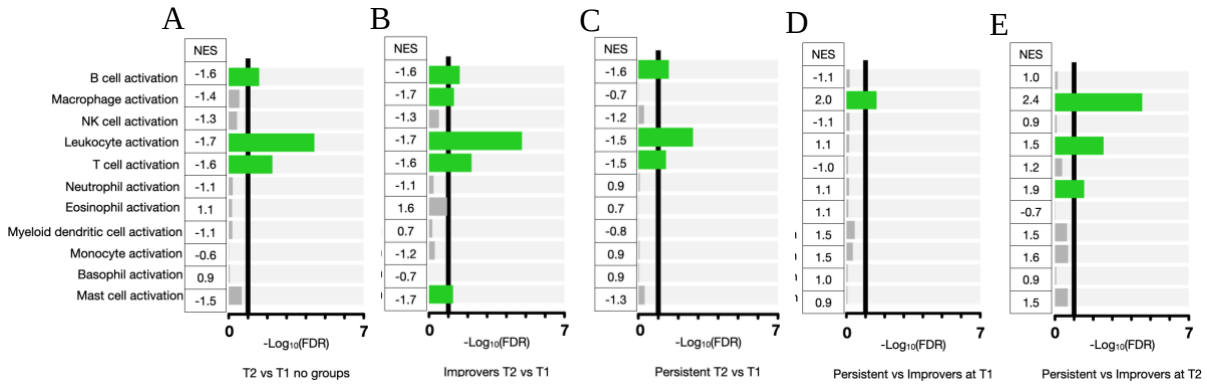

Fig. 5 ***Functional Difference in the Leukocyte Cell Activation Pathways Between the Improved and Persistent Pain Groups.***

The bar plots show FDR-corrected significance level of expression (x-axis) for each pathway; each bar is a subpathway of "leukocyte activation" (GO:0045321). (A) - Differentially expressed pathways over the time of exercise. (B, C) - Differentially expressed pathways over the time of exercise for improved pain group (B) or persistent pain group (C). (D, E) - Differentially expressed pathways between the persistent and improved pain group at the first time point T1 (D) and the second time point T2 (E). Statistically significant pathways at the FDR 10% level are highlighted in green. The NES column shows normalized enrichment scores for every pathway.

When analyzing blood cell activation pathways, we found that all detected significantly different cell activation pathways had negative enrichment scores. (Fig. 5A-C). However, in the improved pain group, we detected five significantly differentially expressed pathways with higher normalized enrichment scores in comparison with the persistent pain group, which had only three over time of the exercise (NES, Fig. 5B, C). Furthermore, the macrophage activation was the only cell activation pathway significantly differentially expressed in comparison between the persistent pain group with the improved group at T1 (Fig. 5D), and at T2, there were three significantly differentially expressed pathways (Fig. 5E). At T2, neutrophils and leukocyte activation pathways were also significantly higher in persistent pain group in comparison with improved pain group (Fig. 5D, E). Notably, when we used drug usage as a covariate in the study

contrasts analyses, our conclusions regarding the pattern of the changes of inflammatory and cell activation biological pathways remained the same (Fig. 6 and 7).

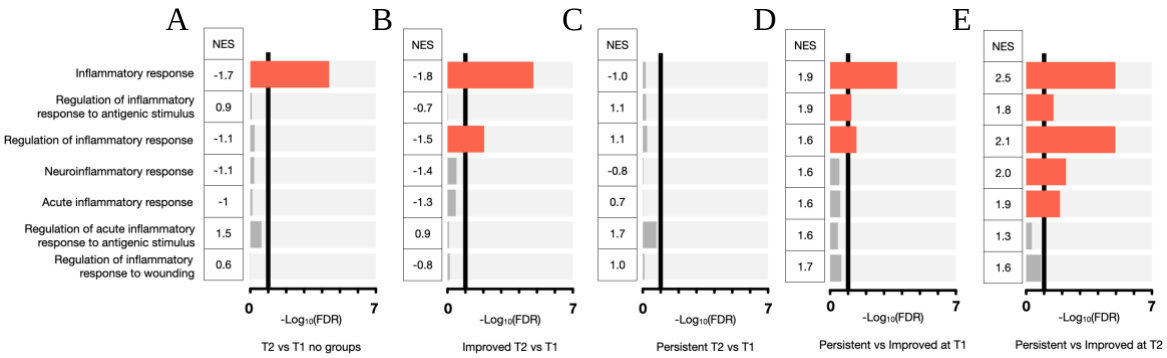

Fig. 6 **Functional Difference Corrected for Drug Usage in the Inflammatory Pathways Between the Improved and Persistent Pain Groups.**

The bar plots show FDR-corrected significance level of expression (x-axis) for each pathway; each bar is a subpathway of "inflammatory response" (GO: 0006954). (A) - Differentially expressed pathways over the time of exercise. (B, C) - Differentially expressed pathways over the time of exercise for improved pain group (B) or persistent pain group (C). (D, E) - Differentially expressed pathways between the persistent and improved pain group at the first time point T1 (D) and the second time point T2 (E). Statistically significant pathways at the FDR 10% level are highlighted in orange. The NES column shows normalized enrichment scores for every pathway.

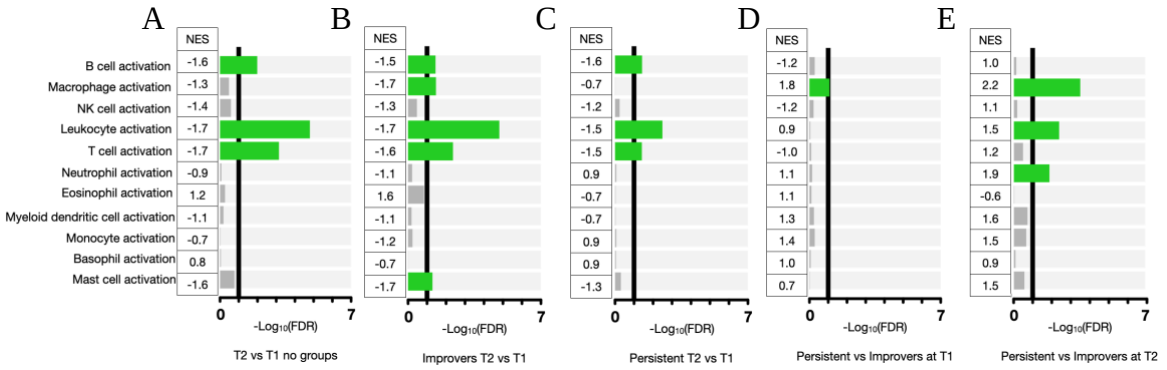

Fig. 7 **Functional Difference Corrected for Drug Usage in the Cell Activation Pathways Between the Improved and Persistent Pain Groups.**

The bar plots show FDR-corrected significance level of expression (x-axis) for each pathway; each bar is a subpathway of "leukocyte activation" (GO:0045321). (A) - Differentially expressed pathways over the time of exercise. (B, C) - Differentially expressed pathways over the time of exercise for improved pain group (B) or persistent pain group (C). (D, E) - Differentially expressed pathways between the persistent and improved pain group at the first time point T1 (D) and the second time point T2 (E). Statistically significant pathways at the FDR 10% level are highlighted in green. The NES column shows normalized enrichment scores for every pathway.

Transcriptomics Data in Association with Brain Phenotypes:

Genome-wide blood transcriptomics was assessed in participants right before the start of the 12 weeks of high-intensity exercise but after the two weeks of low-intensity exercise, and at the end of the 12-week high-intensity exercise training. Differential expression of genes in correlation with functional connectivity and structural connectivity between nucleus accumbens and medial prefrontal cortex was assessed using moderated statistical tests implemented in the R statistical package DESeq2. Pathway enrichment scores were estimated using "fgsea", with statistical significance assessed using a fast permutation scheme. Enrichment scores served as indicator of positive or negative association between variables in each test. At the level of gene expression, we found only two genes differentially expressed after correction for multiple comparisons (Table 1).

Differential expression of genes over time							
gene	baseMean	log2FoldChar	lfcSE	stat	pvalue	padj	GNam
ENSG00000165553	22.208	-3.613	0.774	-4.667	0.000003	0.036	NGB
ENSG00000112305	126.922	-0.359	0.080	-4.470	0.000008	0.046	SMAP1
ENSG00000166848	1315.804	-0.166	0.039	-4.234	0.000023	0.089	TERF2IP
ENSG00000198231	1346.493	-0.194	0.047	-4.151	0.000033	0.089	DDX42
ENSG00000176986	850.121	-0.324	0.080	-4.071	0.000047	0.089	SEC24C
ENSG00000188690	563.489	0.245	0.061	4.040	0.000053	0.089	UROS
ENSG00000163479	3580.898	0.314	0.078	4.016	0.000059	0.089	SSR2
ENSG00000070756	29246.557	-0.154	0.038	-4.012	0.000060	0.089	PABPC1
Association between change in pain and change in gene expression							
gene	baseMean	log2FoldChar	lfcSE	stat	pvalue	padj	GNam
ENSG00000062582	6.635	-1.769	0.518	-3.418	0.000631	1.000	MRPS24
ENSG00000264500	3.312	2.267	0.683	3.319	0.000905	1.000	MIR3124
ENSG00000125966	43.386	0.659	0.242	2.729	0.006347	1.000	MMP24
ENSG00000111641	13.212	0.766	0.286	2.680	0.007367	1.000	NOP2
ENSG00000204444	32.725	-0.606	0.226	-2.679	0.007382	1.000	APOM
ENSG00000220161	3.083	1.853	0.694	2.672	0.007532	1.000	LINC02076
ENSG00000134183	19.503	0.977	0.378	2.584	0.009764	1.000	GNAT2
ENSG00000272031	96.973	1.221	0.476	2.567	0.010259	1.000	ANKRD34A
Association between change in structural connectivity over time and change							
gene	baseMean	log2FoldChar	lfcSE	stat	pvalue	padj	GNam
ENSG00000112486	14.151	-16.836	5.209	-3.232	0.001230	1.000	CCR6
ENSG00000165912	2.778	36.703	13.282	2.763	0.005720	1.000	PACSIN3
ENSG00000199023	4.049	-28.875	10.528	-2.743	0.006093	1.000	MIR339
ENSG00000181322	8.976	-15.267	5.855	-2.607	0.009124	1.000	NME9
ENSG00000177710	6.808	23.034	8.945	2.575	0.010026	1.000	SLC35G5
ENSG00000283429	5.023	-21.487	8.406	-2.556	0.010583	1.000	MIR1244-3
ENSG00000259305	2.012	-35.181	13.807	-2.548	0.010830	1.000	ZHX1-C8orf76
ENSG00000260339	7.323	-18.604	7.375	-2.523	0.011652	1.000	HEXA-AS1
Association between change in functional connectivity over time and change							
gene	baseMean	log2FoldChar	lfcSE	stat	pvalue	padj	GNam
ENSG00000143476	14.456	-5.914	1.809	-3.269	0.001080	1.000	DTL
ENSG00000182324	15.598	-4.063	1.260	-3.226	0.001257	1.000	KCNJ14
ENSG00000232940	22.465	-3.624	1.129	-3.208	0.001336	1.000	HCG25
ENSG00000171116	1.865	13.325	4.306	3.095	0.001971	1.000	HSFX1
ENSG00000155367	22.461	3.295	1.156	2.852	0.004348	1.000	PPM1J
ENSG00000188739	13.811	-4.026	1.445	-2.786	0.005339	1.000	RBM34
ENSG00000166482	4.631	6.743	2.422	2.783	0.005378	1.000	MFAP4
ENSG00000259431	68.818	2.460	0.902	2.727	0.006399	1.000	THTPA

Table 1: ***Differentially Expressed Genes in Association with Brain-related Phenotypes.***

Table shows 4 contrasts with top 8 most significantly associated gene located on the top. Columns are: gene - gene's Ensemble ID; baseMean - the average number of reads mapped on the gene; log2FoldChange - the gene expression fold change in log2 units; lfcSE - the standard error of the fold change; stat - the test statistic; pvalue - the P-value associated with the fold

change; padj - the FDR-corrected P-value, adjusted for all genes (transcriptome-wide correction); Gnam - the gene's symbol.

Differential expression of pathways over time

pathway	desc	pval	padj	ES	NES	nMoreExtren	size
GO:0007005	mitochondrion organizat	1.48638E-07	0.000020	0.44	2.19	0	365
GO:0015980	energy derivation by ox	1.57819E-07	0.000020	0.50	2.36	0	206
GO:0045333	cellular respiration	1.61482E-07	0.000020	0.59	2.68	0	160
GO:0072522	purine-containing comp	1.61559E-07	0.000020	0.48	2.17	0	159
GO:0006164	purine nucleotide biosyn	1.62081E-07	0.000020	0.49	2.22	0	153
GO:0009199	ribonucleoside triphosph	1.62454E-07	0.000020	0.51	2.29	0	149
GO:0009144	purine nucleoside triphos	1.62549E-07	0.000020	0.50	2.27	0	148
GO:0009152	purine ribonucleotide bio	1.62549E-07	0.000020	0.49	2.20	0	148
GO:0009205	purine ribonucleoside tri	1.62929E-07	0.000020	0.51	2.29	0	144
GO:0009060	aerobic respiration	1.63415E-07	0.000020	0.61	2.72	0	139
GO:0022900	electron transport chain	1.63896E-07	0.000020	0.58	2.58	0	134
GO:0140053	mitochondrial gene exp	1.6427E-07	0.000020	0.59	2.63	0	130
GO:0046034	ATP metabolic process	1.64811E-07	0.000020	0.53	2.31	0	125

Association between change in pain and change in pathway expression

pathway	desc	pval	padj	ES	NES	nMoreExtren	size
GO:0006119	oxidative phosphorylatio	2.16309E-07	0.000086	-0.51	-2.23	0	107
GO:0140053	mitochondrial gene exp	2.17614E-07	0.000086	-0.51	-2.26	0	130
GO:0080134	regulation of response to	3.35069E-07	0.000110	0.29	1.55	1	994
GO:0010257	NADH dehydrogenase c	4.25448E-07	0.000124	-0.60	-2.29	1	55
GO:0032981	mitochondrial respirator	4.25448E-07	0.000124	-0.60	-2.29	1	55
GO:0032543	mitochondrial translatio	4.31879E-07	0.000124	-0.50	-2.13	1	102
GO:0007399	nervous system develop	5.02729E-07	0.000128	0.28	1.50	2	991
GO:0050767	regulation of neurogene	5.49031E-07	0.000128	0.42	1.92	2	182
GO:0009060	aerobic respiration	6.5468E-07	0.000150	-0.44	-2.01	2	139
GO:0022008	neurogenesis	6.95796E-07	0.000151	0.31	1.60	3	559
GO:0080135	regulation of cellular res	6.98568E-07	0.000151	0.31	1.60	3	522
GO:0045333	cellular respiration	8.77218E-07	0.000177	-0.42	-1.93	3	160
GO:0010975	regulation of neuron pro	9.12042E-07	0.000182	0.40	1.85	4	200

Association between change in structural connectivity over time and change in differential pathway expression

pathway	desc	pval	padj	ES	NES	nMoreExtren	size
GO:0140053	mitochondrial gene exp	1.96768E-07	0.000021	-0.47	-2.37	0	130
GO:0032543	mitochondrial translatio	1.97605E-07	0.000021	-0.46	-2.23	0	102
GO:0006909	phagocytosis	2.02903E-07	0.000021	0.43	2.15	0	117
GO:0071222	cellular response to lipop	2.0335E-07	0.000021	0.48	2.43	0	129
GO:0071219	cellular response to mole	2.03479E-07	0.000021	0.46	2.36	0	135
GO:0042742	defense response to bac	2.03629E-07	0.000021	0.43	2.23	0	141
GO:0045765	regulation of angiogenes	2.03964E-07	0.000021	0.40	2.07	0	154
GO:1901342	regulation of vasculatur	2.03986E-07	0.000021	0.39	2.05	0	155
GO:0042060	wound healing	2.04884E-07	0.000021	0.42	2.25	0	191
GO:0032496	response to lipopolysacc	2.05075E-07	0.000021	0.41	2.20	0	200
GO:0030099	myeloid cell differentiat	2.05077E-07	0.000021	0.39	2.12	0	199
GO:0002237	response to molecule of	2.05345E-07	0.000021	0.41	2.24	0	211
GO:1902105	regulation of leukocyte	2.0563E-07	0.000021	0.35	1.92	0	224

Association between change in functional connectivity over time and change in differential pathway expression

pathway	desc	pval	padj	ES	NES	nMoreExtren	size
GO:0080134	regulation of response to	1.31633E-07	0.000218	0.31	1.58	0	994
GO:0032101	regulation of response to	1.39035E-07	0.000218	0.32	1.59	0	655
GO:0031347	regulation of defense res	1.43541E-07	0.000218	0.34	1.64	0	504
GO:1901342	regulation of vasculatur	1.60922E-07	0.000218	0.48	2.07	0	155
GO:0045765	regulation of angiogenes	1.61006E-07	0.000218	0.48	2.06	0	154
GO:0002682	regulation of immune sy	2.64002E-07	0.000325	0.30	1.52	1	974
GO:0001944	vasculature developmer	3.10427E-07	0.000350	0.41	1.86	1	239
GO:0031349	positive regulation of de	1.07207E-06	0.000704	0.39	1.79	6	276
GO:0001568	blood vessel developmer	1.09133E-06	0.000704	0.40	1.82	6	228
GO:0002221	pattern recognition rece	1.1777E-06	0.000718	0.54	2.10	6	79
GO:0062197	cellular response to cher	2.69814E-06	0.001341	0.42	1.83	16	185
GO:0048585	negative regulation of re	4.9062E-06	0.002013	0.28	1.44	36	942
GO:0048514	blood vessel morphogen	8.23544E-06	0.002656	0.40	1.79	51	190

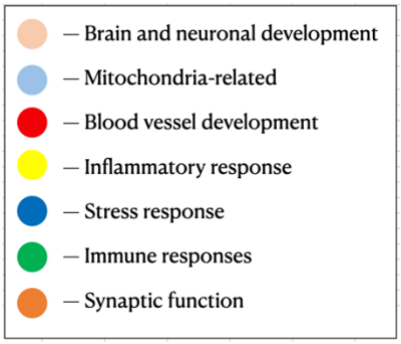

Table 2: ***Differentially Expressed Pathways in Association with Brain-related Phenotypes.*** *Table shows 4 contrasts with top 13 most significantly associated pathways located on the top. Columns are: pathway - Gene Ontology term ID; desc - GO description of the pathway; pval - the P-value associated with the enrichment score; padj - the FDR-corrected P-value, adjusted for all pathways (transcriptome-wide correction); ES - enrichment score for a given pathway; NES - normalized enrichment score, adjusted for gene-set sizes; nMoreExtreme - number of times a random gene set had a more extreme enrichment score value; size - number of genes within gene set which was tested for enrichment; leadingEdge - specific genes within the leading edge.*

However, at the level of biological pathways, many significant results were observed (Fig. 8A-D, Table 2). The differential pathways expression analysis over the time of exercise showed that the majority of significantly differentially regulated pathways (1069 vs 131) were downregulated over time (Fig. 8A). Furthermore, there was significant evidence that changes in pathways' expression were mostly positively associated with pain change (355 positively associated pathways vs 81 negatively), changes in structural connectivity (726 positively associated pathways vs 83 negatively), and changes in functional connectivity (247 positively associated pathways vs 32 negatively) (Fig. 8B-D, Table 2). Among major categories of correlated biological pathways, we found those that pertained to brain and neural development, blood vessel development, inflammatory response, stress response, immune responses, and synaptic function. Interestingly, pathways related to the mitochondria and energy showed the opposite direction compared to other pathways. They were negatively associated with changes in pain structural and functional connectivity (Fig. 8B-D), while the rest of the pathways showed a

positive association, and they were upregulated over the time of exercise while most of the pathways were downregulated (Fig. 8A).

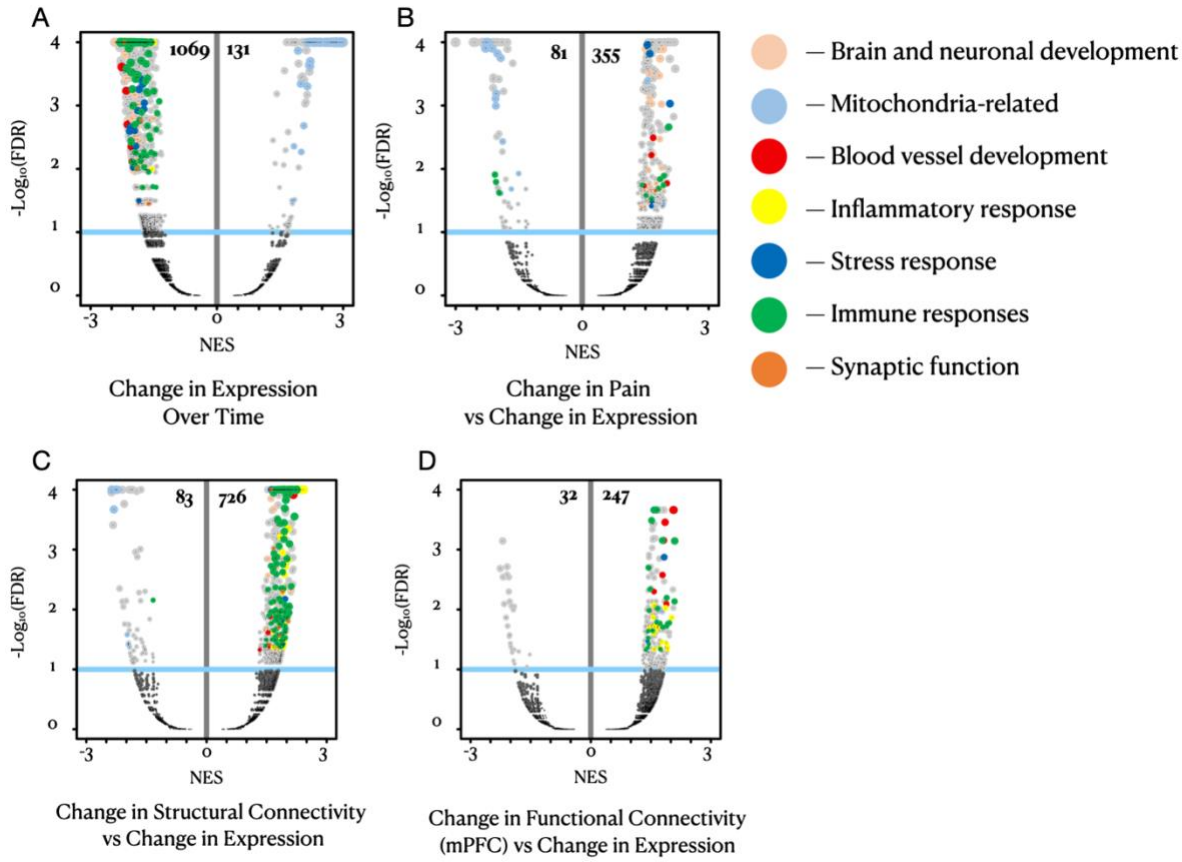

Figure 8: ***Association of Differentially Expressed Pathways with Brain- and Pain-Related Phenotypes.*** *Volcano plots show FDR-adjusted statistical significance (y-axis) as a function of normalized enrichment score (x-axis); each dot is a pathway. Pathways that would end up outside of the plot were squeezed inside. Vertical grey lines indicate null enrichment scores. Horizontal blue lines indicate the threshold for statistical significance at the FDR 10% level. Pathways reaching statistical significance at the FDR 10% level are highlighted in light grey. Numbers in bold indicate counts of significantly differentially expressed pathways that are down-regulated (at the top to the left of the grey middle line) or up-regulated (at the top to the right of the grey middle line). Figure legend shows color coding for different groups of pathways. A -*

Differential expression of pathways over time, B - Association between change in pain and change in gene expression, C - Association between change in structural connectivity over time and change in differential pathway expression, D - Association between change in functional connectivity over time and change in differential pathway expression.

Discussion

Current treatments for the management of CLBP, as well as other chronic pain conditions, are inadequate. Physical exercise is one of the few reliable interventions to aid in the management of chronic pain. However, the mechanisms underlying its therapeutic effects are unknown. Therefore, the study aimed to understand better pathophysiological mechanisms underlying pain improvement in chronic low back pain patients and look for an association between immune processes related to pain resolution and brain connectivity.

The results from gene expression analysis identified many significantly differentially expressed genes between the first and second-time points of blood collection. The result suggested that transcriptomics changes occur after inducing the exercise intervention. These results lead us further to explore differentially expressed genes in specific contrasts. We identified a significant difference between the number of significantly differentially expressed genes in the improved and persistent pain groups over time. The improved pain group had significantly more differentially expressed genes over time than the persistent pain group. These observations suggest that more transcriptional activity occurs in the improved pain group compared to the persistent pain group over time of the exercise training. At the first time point (T1) snapshot, we did not detect any significantly differentially expressed genes between the two groups,

suggesting similar transcriptional status at the gene level between groups. In contrast, the changes started to appear at the second time point (T2), yielding an increase in the number of significantly differentially expressed genes between the groups. The analysis suggested that although both pain groups have similar transcriptional states at the beginning of the exercise, their transcriptional states deviate at the end of the exercise course in association with their pain status.

We next analyzed biological pathways instead of the individual differentially expressed genes to find overarching pathways or groups of pathways associated with pain resolution. The overall correlation in the biological pathways' activity over time between the improved pain and persistent pain patients' group yielded an observation that the majority of transcriptional changes are positively correlated between the two groups, indicating that all individuals displayed similar biological responses and pathways at large, regardless of the pain outcome. However, based on the correlation slope, the improved pain group's response intensity was much smaller than that of the persistent pain group. The results from the analysis suggest that most of the pathways have similar directionality, reflecting the similar biological responses of both groups to the exercise. Nevertheless, some pathways are less abundant but present in opposite directions. Inspired by my Parisien et al. paper on the transition from acute to chronic pain state, we specifically analyzed the inflammatory pathways. One of the interesting findings was that inflammatory pathways were among those which are anti-correlated between two groups. Together, these results suggest that the directionality of some pathways and the magnitude of change in expression are essential for chronic pain improvement.

We next decided to focus on the leukocyte cell activation pathways and inflammatory pathways more closely. Our results suggest that there is overall downregulation of inflammatory response pathways throughout exercises. Considering our previous observation, we suggest that both groups downregulated inflammatory pathways through improved pain group patients downregulate them faster and more effectively than the persistent pain group (Parisien et al., 2022). Looking more precisely at the specific groups and time points, we found that patients from the improved pain group had inflammatory response pathways downregulated over the time of exercises when the persistent pain group did not show any significant differences supporting our hypothesis. At the second time, almost twice as many differentially expressed pathways were observed compared to the first time in snapshot contrasts between groups, suggesting the difference in inflammatory response activation levels only increased over time.

When analyzing the changes in the blood cell activation pathways over the time of exercise, we found that all detected significantly different cell activation pathways had negative enrichment scores, meaning they were downregulated over time in both pain groups. However, more pathways were downregulated in the improved pain group and with the higher normalized enrichment scores compared to the persistent pain group. Furthermore, the macrophage activation was already higher in the persistent pain group compared to the improved group at T1 and at T2 this difference between persistent and improved pain groups further increased. At T2, neutrophils and leukocyte activation pathways were also significantly higher in the persistent pain group than in the improved pain group. These observations suggest that the downregulation of inflammation associated with pain resolution in low back pain patients during exercise is

underlined by the reduction of leukocytes' activation, mostly macrophage, neutrophil, and possibly mast cell cells.

After studying the changes on the transcriptomic level, we decided to put things into perspective with brain connectivity changes. In line with previously reported by Herold et al., in this cohort the reduction in functional and structural connectivity was highly positively correlated with pain resolution. After associating changes in structural and functional connectivity between the nucleus accumbence and medial prefrontal cortex with pain changes and expression changes, we were able to pick major categories of correlated biological pathways; we found those that pertained to brain and neural development, mitochondria-related function, blood vessel development, inflammatory response, stress response, immune responses, and synaptic function. The majority of the groups of pathways were positively associated with brain connectivity and downregulated over time of the exercise. However, mitochondria and energy-related pathways were negatively associated with brain connectivity and upregulated over time, which might be explained by a stronger association of those pathways with exercise rather than pain resolution. The association results suggest that when structural and functional connectivity is downregulated, the major groups of pathways are also downregulated. Our results suggested a positive correlation between brain connectivity and blood transcriptomic activity at the pathway level.

There have been some limitations to the study design, which must be attended in the further studies. The major one was that the first blood draw, which was used later for sequencing, was done after two weeks of familiarization exercise. The two weeks of familiarization exercise

could cause changes in the activation of peripheral immune cells, making interpreting the results more challenging. The second limitation which should be noted is that average pain ratings were collected a week prior to the start of any physical activity. At the start of the physical exercise intervention (a week later), some people had pain scores lower than 4 (the original exclusion criteria), making room for improvement very small. Thirdly, the study lacked controls or people who had given blood and had exercised but did not have pain or those who had pain but did not do exercise. Having those would significantly improve the analysis of patients at the baseline and make the interpretation of the results easier. Last but not least, the sample size of 32 people is quite small and if increased in the future studies could lead to new discoveries or at least reduce the chance of committing type two error (false negative).

Conclusion and Future Directions

The results of our transcriptomics analyses of the cohort of LPB patients undergoing physical exercise provided few insights. First, it indicated that the relative abundance and magnitude of changes in active biological processes significantly contribute to pain improvement over physical exercise time. Second, we found a downregulation of inflammatory pathways over time driven by long-term exercise associated with pain improvement, consistent with previous research done by Parisien et al. on the transition from acute to chronic pain state. The faster and more efficient downregulation of inflammatory pathways is needed for successful pain resolution. Further, a more detailed analysis of specific groups of inflammatory and leukocyte activation pathways suggested that the downregulation of inflammation associated with pain resolution in low back pain patients during exercise is underlined by the reduction of leukocytes' activation, mostly macrophage, neutrophil, and possibly mast cell cells. Subsequent analysis of the correlation between blood transcriptomics and brain imaging data revealed that brain and neural

development, blood vessel development, inflammatory response, stress response, immune responses, and synaptic function are the major groups significantly positively correlated with brain structural and functional connectivity. Mitochondria-related function is the only group negatively correlated with brain structural and functional connectivity.

Future directions should test the causality between transcriptomics and brain connectivity and study more specific pathways from groups of pathways discovered here, which might serve as a novel therapeutic target for the treatment of low back pain. Prospects should include replicating the study, taking into consideration limitations mentioned previously. Also, a potential avenue would be to validate the study's results in the animal models and analyze the pathways and cell type activations discovered here. Since animal pain models allow more manipulations, they might be further focused on cell type fractions and how changes in those might elicit significant differential immune responses.

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