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INTERAÇÃO ENTRE AS VIAS DAS QUINURENINAS E DA TETRAIDROBIOPTERINA NA HIPERSENSIBILIDADE À DOR

Florianópolis
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Tese submetida ao Programa de Pós-graduação em Bioquímica da Universidade Federal de Santa Catarina e pela Instituição *Macquarie University* em regime de cotutela para a obtenção do título de Doutora em Bioquímica e *Biomedical Sciences*.
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ROLE OF KYNURENINE AND TETRAHYDROBIOPTERIN PATHWAYS IN PAIN HYPERSENSITIVITY

A thesis submitted in fulfilment of a Doctor of Philosophy (Biomedical Sciences and Biochemistry) by the Universidade Federal de Santa Catarina and Macquarie University under a cotutelle modality.
Supervisors: Prof. Dr. Alexandra Susana Latini (UFSC) and Prof. Dr. Gilles Guillemin (Macquarie University)

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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I dedicate this thesis to my beloved family: Margot, Moacir and Camila.

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“I don´t believe there would be any science at all without intuition”
Rita Levi Montalcini, Nobel Prize in Physiology or Medicine (1986)

RESUMO

A dor crônica é uma doença debilitante que apresenta um grande impacto socioeconômico, porém ainda sem tratamento eficiente e seguro. Um dos fatores que dificulta o sucesso do tratamento da dor crônica é a compreensão limitada dos mecanismos moleculares que a sustentam. Além disso, os recursos clínicos para a avaliação da intensidade e outras características da dor crônica são subjetivos e, portanto, dificultam diagnóstico e tratamento acurados para esta doença. Desta forma, recursos que permitam uma avaliação objetiva e que possam informar sobre mecanismos subjacentes à dor crônica são desejáveis. Múltiplas evidências associam a ativação do sistema imunológico como um componente da dor crônica. Dados da literatura demonstram que mediadores pró-inflamatórios são encontrados em concentrações elevadas em pacientes com dor crônica. Muitos destes mediadores perpetuam a resposta inflamatória pela ativação de vias metabólicas, incluindo as vias das quinureninas (KYN) e da tetraidrobiopterina (BH4), que geram metabólitos solúveis com potencial para aumentar a sensibilidade à dor. O objetivo geral desta tese foi explorar o potencial dos metabólitos relacionados às vias KYN e BH4 como biomarcadores de dor crônica. Esse objetivo foi explorado por meio da avaliação do perfil dos metabólitos e sua correlação com as características da dor crônica em três grupos de pacientes afetados por condições diferentes de dor crônica. Os dados resultantes desta pesquisa revelam que a BH4 e a razão entre KYN / triptofano foram direta ou indiretamente associados à intensidade da dor nos participantes com dor crônica. Além disso, metabólitos das vias KYN e BH4 foram correlacionados com características e sintomas de dor crônica, como depressão, estresse e cinesiofobia. Nossos dados mostraram uma redução de citocinas anti-inflamatórias e um aumento de mediadores pró-inflamatórios no plasma de indivíduos com dor crônica. Ainda, alguns dos metabólitos das vias KYN e BH4 foram positivamente correlacionados com marcadores pró-inflamatórios, sugerindo que os mediadores imunológicos induzem a ativação dessas vias metabólicas em condições de dor crônica. Coletivamente, os resultados desta tese sugerem que os metabólitos KYN e BH4 podem ser quantificados como marcadores de dor crônica em fluídos corporais humanos que apresentam baixo grau de invasividade. Baseados no conhecimento prévio da literatura e nos dados derivados do estudo aqui apresentado, sugerimos que as vias KYN e BH4 estejam envolvidas nos mecanismos subjacentes à dor crônica. Assim, metabólitos relacionados às vias KYN e BH4 podem ser propostos como marcadores de dor e também como ferramentas que informam sobre os mecanismos fisiopatológicos dessa condição. Os resultados desta tese podem informar estudos futuros e orientar a validação dos biomarcadores para dor crônica. Logo, nossos resultados podem auxiliar o diagnóstico, prognóstico e tratamento adequados da dor crônica.

Palavras-chave: Biomarcadores. Dor crônica. Dor neuropática. Dor inflamatória. Neuroinflamação. Quinureninas. Tetraidrobiopterina.

RESUMO EXPANDIDO

Introdução

A dor crônica é uma doença debilitante que apresenta um grande impacto socioeconômico, porém ainda sem tratamento eficiente e seguro. Um dos fatores que dificulta o sucesso do tratamento da dor crônica é a compreensão limitada dos mecanismos moleculares que a sustentam. Além disso, os recursos clínicos para a avaliação da intensidade e outras características da dor crônica são subjetivos e, portanto, dificultam diagnóstico e tratamento acurados para esta doença. Desta forma, recursos que permitam uma avaliação objetiva e que possam informar sobre mecanismos subjacentes à dor crônica são desejáveis. Múltiplas evidências associam a ativação do sistema imunológico como um componente da dor crônica. Dados da literatura demonstram que mediadores pró-inflamatórios são encontrados em concentrações elevadas em pacientes com dor crônica. Muitos destes mediadores perpetuam a resposta inflamatória pela ativação de vias metabólicas, incluindo as vias das quinureninas (KYN) e da tetraidrobiopterina (BH4), que geram metabólitos solúveis com potencial para aumentar a sensibilidade à dor.

Objetivos

O objetivo geral desta tese foi explorar o potencial dos metabólitos relacionados às vias das KYN e da BH4 como biomarcadores de dor crônica. Os objetivos específicos desta tese foram: *i*) Investigar o perfil de metabólitos das vias KYN e da BH4 em coortes de pacientes afetados por condições de dor crônica que possui a inflamação como parte do mecanismo fisiopatológico; *ii*) Examinar a existência de correlações entre os metabólitos das vias das KYN e da BH4 com as características da dor crônica.

Metodologia

Esses objetivos foram explorados por meio da avaliação do perfil dos metabólitos das vias das KYN e da BH4 e sua correlação com as características da dor crônica em três grupos de pacientes afetados por diferentes condições de dor crônica. Portanto, foram conduzidos três estudos de corte transversal que incluíram pacientes afetados por *i*) dor neuropática diabética, *ii*) síndrome complexa da dor regional e *iii*) dor abdominal relacionada à colite ulcerativa. Em cada um destes estudos foram avaliadas características clínicas da dor crônica nos participantes, incluindo a intensidade da dor, função emocional e características psicossociais relacionadas à dor (cinesiofobia, catastrofização e autoeficácia em relação à dor). Todas as avaliações clínicas foram realizadas por meio de escalas e questionários validados. Além disso, foram coletadas amostras de sangue e/ou urina para a avaliação do perfil dos metabólitos das vias das KYN e da BH4, bem como o perfil de marcadores inflamatórios nas amostras biológicas dos participantes. Todas as avaliações bioquímicas foram realizadas por meio de cromatografia líquida e gasosa, bem como por meio de ensaios imunoenzimáticos. Por fim, foram realizadas análises estatísticas pertinentes para verificar as associações entre os dados clínicos e bioquímicos, incluindo análises de inteligência artificial.

Resultados e Discussão

Os dados resultantes desta pesquisa foram organizados no formato de três artigos científicos. No primeiro artigo científico foram apresentadas as análises dos níveis de citocinas e metabólitos das KYN / BH4 no soro de indivíduos afetados por dor neuropática diabética (DNP). Foram verificados 1) níveis aumentados das citocinas pró-inflamatórias, GM-CSF e IL-8, no soro de pacientes com DNP; 2) Níveis séricos de citocinas pró-inflamatórias positivamente correlacionados com os metabólitos das KYN / BH4; 3) Níveis de neopterina e da razão KYN / triptofano (Trp) aumentados no soro de pacientes com DNP, sendo o último positivamente correlacionado com os escores de dor. Esses achados sugerem que a ativação inflamatória e das KYN / BH4 podem participar na fisiopatologia da dor neuropática como uma complicação da *diabetes mellitus*. Esses resultados são importantes para avançar a elucidação dos mecanismos responsáveis pelo desenvolvimento da dor neuropática, bem como fornecer possíveis biomarcadores que possam apoiar o diagnóstico e orientar o tratamento da DNP.

No segundo artigo científico foram apresentadas análises relacionadas ao estado imunológico e metabólico de indivíduos com a síndrome complexa da dor regional (CRPS). Foram observados 1) níveis diminuídos da citocina anti-inflamatória IL-37 e do aminoácido Trp no soro dos participantes com CRPS, bem como níveis elevados de GM-CSF em um subgrupo de participantes com CRPS. 2) Além disso, identificamos várias

correlações positivas entre metabólitos das vias KYN / BH4 com as características clínicas e psicológicas dos participantes com CRPS. Especificamente, *i*) a neopterina se correlacionou com os escores de depressão, *ii*) o ácido xanturênico se correlacionou com os escores de depressão e estresse, *iii*) a razão KYN / Trp se correlacionou com os escores de cinesiofobia; *iv*) a BH4 se correlacionou com os escores de dor em participantes com CRPS. 3) Finalmente, foram realizadas análises com recursos de aprendizado de máquina, as quais revelaram um conjunto de quatro variáveis binárias que representam biomarcadores capazes de distinguir pacientes com CRPS de indivíduos saudáveis. Esse conjunto de biomarcadores combinou IL-37 (baixo), GM-CSF (alto), número de células T reg (alto) e número de células T CD8 + (alto). Esses resultados podem informar estudos futuros e orientar a validação de biomarcadores com potencial de auxiliar no diagnóstico clínico da CRPS.

No terceiro artigo científico foram apresentadas análises do metabolismo da BH4 e características clínicas em pacientes com colite ulcerativa (UC). Foram verificados que 1) a dor abdominal é um sintoma prevalente durante a fase ativa (85,7%) e o período de remissão (47,8%) da UC. 2) A exacerbação do metabolismo da BH4 pode aumentar os níveis de serotonina e contribuir para a dor abdominal na UC. Especificamente, foram observados *i*) níveis elevados de BH4 na urina e plasma dos participantes com UC. *ii*) Os níveis de serotonina no plasma rico em plaquetas foram positivamente correlacionados com os níveis urinários de BH4 e com os escores de dor nos pacientes afetados por UC. Considerando que a enzima limitadora da biossíntese da serotonina é dependente do cofator BH4, é possível que a exacerbação do metabolismo da BH4 possa contribuir para o aumento da produção de serotonina. Por sua vez, os níveis aumentados de serotonina podem favorecer a hipersensibilidade do cólon e a dor abdominal em indivíduos afetados pela UC. Esses dados sugerem uma contribuição da exacerbação do metabolismo da BH4 para a dor abdominal relacionada à UC. Além disso, nossos resultados sugerem que a BH4 na urina pode ser um biomarcador pouco invasivo para indicar a presença e a gravidade da dor abdominal na UC.

De forma geral, os dados gerados como parte desta tese revelam que a BH4 e a razão entre KYN / Trp foram direta ou indiretamente associados à intensidade da dor nos participantes com dor crônica. Além disso, metabólitos das vias KYN e BH4 foram correlacionados com características e sintomas de dor crônica, como depressão, estresse e cinesiofobia. Ainda, alguns dos metabólitos das vias KYN e BH4 foram positivamente correlacionados com marcadores pró-inflamatórios, sugerindo que os mediadores inflamatórios induzem a ativação dessas vias metabólicas em condições de dor crônica.

Considerações Finais

Coletivamente, os resultados desta tese sugerem que os metabólitos das KYN e da BH4 podem ser quantificados como marcadores de dor crônica em fluídos corporais humanos que apresentam baixo grau de invasividade. Baseados no conhecimento prévio da literatura e nos dados derivados do estudo aqui apresentado, sugerimos que as vias das KYN e da BH4 estejam envolvidas nos mecanismos subjacentes à dor crônica. Assim, metabólitos relacionados às vias KYN / BH4 podem ser propostos como marcadores de dor crônica e também como ferramentas que informam sobre os mecanismos fisiopatológicos dessa condição. Os resultados desta tese podem informar estudos futuros e orientar a validação dos biomarcadores para dor crônica. Logo, estes resultados podem auxiliar o diagnóstico, prognóstico e tratamento adequados da dor crônica.

Palavras-chave: Biomarcadores. Dor crônica. Dor neuropática. Dor inflamatória. Neuroinflamação. Quinureninas. Tetraidrobiopterina.

ABSTRACT

Chronic pain is a debilitating disease with a devastating socioeconomic impact, yet no efficient and safe treatment is available. One explanation for the limited success of chronic pain management is the inadequate understanding of the molecular mechanisms that underpin chronic pain. Additionally, clinical subjective ratings to evaluate the intensity and other features of chronic pain have played a major role in poor diagnosis and treatment. Thus, objective assessment that can inform about the mechanism underlying chronic pain and support the management of this disease is desired. Current evidence supports immune system activation as a component of chronic pain. Pro-inflammatory mediators are known to be increased during chronic pain conditions. Many of them perpetuate the inflammatory scenario by activating metabolic pathways, including the kynurenine (KYN) and the tetrahydrobiopterin (BH4) pathways, which generate bioactive soluble metabolites with the potential to increase pain sensitivity. Thus, the overarching aim of this thesis was to explore the potential utility of KYN and BH4 pathways-related metabolites as biomarkers of chronic pain. This aim was explored through the evaluation of KYN and BH4 pathways profile and its correlation with chronic pain features in three different cohorts of patients with chronic pain. The arising data reveals that BH4 and the ratio KYN/ tryptophan were directly or indirectly associated with pain intensity across the cohort of chronic pain participants studied. Some of the KYN and BH4 pathways metabolites were correlated with chronic pain features and symptoms, such as depression, stress, and kinesiophobia. Our data showed a reduction of anti-inflammatory cytokines and an increase of pro-inflammatory mediators in the plasma of chronic pain-affected subjects. Additionally, some of the KYN and BH4 pathways metabolites were positively correlated with pro-inflammatory markers, suggesting that immune mediators drive activation of these pathways in chronic pain conditions. Collectively, the data generated in this thesis showed that KYN and BH4 metabolites can be quantified in human body fluids, in a low-invasive manner, as markers of chronic pain and its related features. Based on previous literature and data from our study, it is feasible that KYN and BH4 pathways are also involved in the mechanisms underlying chronic pain. Hence, KYN and BH4 pathways-related metabolites can be proposed as markers of pain that also inform about the pathophysiological mechanisms responsible for chronic pain. The results from this thesis can support future studies and guide the validation of potential biomarker sets for chronic pain that might assist in disease-modifying drugs development and guide the diagnosis, prognosis, and treatment of chronic pain.

Keywords: Biomarkers. Chronic pain. Neuropathic pain. Inflammatory pain. Neuroinflammation. Kynurenine. Tetrahydrobiopterin. Pain sensitization.

LIST OF FIGURES

CHAPTER 2

ORIGINAL ARTICLE: KYNURENINE AND TETRAHYDROBIOPTERIN PATHWAYS CROSSTALK IN PAIN HYPERSENSITIVITY

CHAPTER 3

ORIGINAL RESEARCH ARTICLE: KYNURENINE, TETRAHYDROBIOPTERIN AND CYTOKINE INFLAMMATORY BIOMARKERS IN INDIVIDUALS AFFECTED BY DIABETIC NEUROPATHIC PAIN

ORIGINAL RESEARCH ARTICLE: NOVEL IMMUNE BIOMARKERS IN COMPLEX REGIONAL PAIN SYNDROME

ORIGINAL RESEARCH ARTICLE: TETRAHYDROBIOPTERIN AS A KEY FACTOR PROMOTING COLITIS ABDOMINAL PAIN

LIST OF BOXES

CHAPTER 2

LIST OF TABLES

CHAPTER 2

CHAPTER 3

LIST OF ABBREVIATIONS

1-MT: 1-methyl-tryptophan
ABS: Australian Bureau of Statistics
AUC: Area under curve
BH4: Tetrahydrobiopterin
CDC: Center for Disease Control and Prevention
CNS: Central nervous system
CRP: C-reactive protein
CRPS: Complex Regional Pain Syndrome type 1
DNP: Diabetes Painful Neuropathy
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GTPCH: Guanosine triphosphate cyclohydrolase I
HV: Healthy volunteers
IASP: International Association for the Study of Pain
ICD: International Classification of Diseases
IDO1: Indoleamine 2,3-dioxygenase 1
IFN-γ: Interferon gamma
IL-37: Interleukin-37
IL-8: Interleukin-8
IL1-β: Interleukin-1beta
IMMPACT: Initiative on Methods, Measurement, and Pain Assessment in Clinical Trials
KYN: Kynurenine
NSAIDs: nonsteroidal anti-inflammatory drugs
SF-MPQ: Short-form McGill Pain Questionnaire
TPH: Tryptophan hydroxylase
TNF-α: Tumour necrosis factor alpha
Trp: Tryptophan
TRPA1: Transient receptor potential subfamily A member 1
TRPM8: Transient receptor potential cation channel subfamily M member 8
TRPV1: Transient receptor potential cation channel subfamily V member 1
UC: Ulcerative colitis
VAS: Visual Analogue Scale
WHO: World Health Organization
YLDs: Years lived with disability

TABLE OF CONTENT

LIST OF PUBLICATIONS, ABSTRACTS AND PRESENTATIONS ARISING FROM THIS THESIS

PEER-REVIEWED JOURNAL ARTICLES

(P1) Russo, M.; Georgius, P.; **Staats Pires A.;** Heng, B. R.; Allwright, M.; Guennewig, B.; Santarelli, D.; Bailey, D.; Fiore, N.; Tan, V.; Latini, A.; Guillemin, G.; Austin, P. Novel immune biomarkers in Complex Regional Pain Syndrome. Journal of Neuroimmunology. 2020.

(P2) **Staats Pires, A. C.;** Heng, B.; Tan, V. X.; Latini, A.; Russo, M. A.; Santarelli, D. M.; Bailey, D.; Wynne, K.; O'brien, J. A.; Guillemin, G.; Austin, P. Kynurenine, tetrahydrobiopterin and cytokine inflammatory biomarkers in individuals affected by diabetic neuropathic pain. **Frontiers in Neuroscience.** 2020.

(P3) **Staats Pires, A. C.**; Tan, V. X.; Heng, B.; Guillemin, G.; Latini, A. Kynurenine and Tetrahydrobiopterin Pathways Crosstalk in Pain Hypersensitivity. **Frontiers in Neuroscience.** 2020.

CONFERENCE PRESENTATIONS

2020 **Pires, A. C. S.** Kynurenine, Tetrahydrobiopterin, and Cytokine Inflammatory Biomarkers in Individuals Affected by Diabetic Neuropathic Pain. In: EnCouRage – Macquarie University, 2020, Sydney, Australia.

2019 **Pires, A. C. S.** Exacerbated BH4 metabolism in experimental colitis pain. In: NSW Cell & Developmental Biology Meeting, 2019, Sydney, Australia.

2019 **Pires, A. C. S.** Exacerbated BH4 metabolism in experimental colitis pain. In: Macquarie Neurodegeneration Meeting, 2019, Sydney, Australia.

2018 **Pires, A. C. S.** Exacerbated BH4 metabolism in experimental colitis pain. In: Current Topics in Biochemistry Meeting, 2018, Florianopolis - Brazil.

2018 **Pires, A. C. S.** Tetrahydrobiopterin Metabolism is Exacerbated in Experimental Colitis Pain. In: World Congress on Pain organized by the International Association for the Study of Pain, 2018, Boston - USA.

INVITED SEMINARS

2018 **Staats Pires, A. C.**, The role of kynurenine and tetrahydrobiopterin in pain hypersensitivity, Departamental seminar, Universidade Federal de Santa Catarina, Brazil, 2018

OTHER RELATED PUBLICATIONS DURING THE CANDIDATURE

PEER-REVIEWED JOURNAL ARTICLES

(P1) Leclerc, D.; **Staats Pires A.**; Guillemin, G.; Gilot, D. Detrimental activation of AhR pathway in cancer: an overview of therapeutic strategies. **Current Opinion in Immunology.** 2021.

(P2) Bhat, A.; **Staats Pires A.;** Tan, V.; Chidambaram S. B.; Guillemin, G. Effects of Sleep Deprivation on the Tryptophan Metabolism. **International Journal of Tryptophan Research**. 2020.

(P3) Latini A.; De Bortoli Da Silva L.; Da Luz Scheffer D.; **Pires A. C. S.**; De Matos F. J.; Nesi R. T.; Ghisoni K.; De Paula Martins R.; De Oliveira P. A.; Prediger R. D.; Ghersi M.; Gabach L.; Pérez M. F.; Rubiales-Barioglio S.; Raisman-Vozari R.; Mongeau R.; Lanfumey L.; Aguiar Jr, A. S. Tetrahydrobiopterin improves hippocampal nitric oxide-linked long-term memory. **Mol Genet Metab**. v.125, n.2, p104-111, 2018.

CHAPTER 1

PROBLEM STATEMENT & RESEARCH APPROACH

This chapter provides an overview of the thesis rationale, which summarizes the research problem and research gap faced in the field. Subsequently, the hypothesis and aims of this thesis are presented, followed by an overview of the research approach.

1 PROBLEM STATEMENT & RESEARCH APPROACH

1.1 PROBLEM STATEMENT

Acute pain is an early-warning protective system essential to detect and minimize contact with damaging or noxious stimuli (IASP, 2017). However, the neural circuit responsible for the pain experience can undergo maladaptive changes and result in persistent pain (Woolf & Salter, 2000). The pain that lasts or recurs for longer than 3 months is defined as chronic pain (IASP, 2019), which normally is beyond normal tissue healing time and thus loses the warning function of physiological acute pain.

Chronic pain is considered a debilitating and complex disease associated with physical and psychosocial disturbances, existing together and influencing one another (Bandura, 1978; Treede et al., 2019). The impact of chronic pain on people's life is substantial, affecting activities of daily living, social life, and work, with approximately 20 % of people with chronic pain unable to work due to pain (Andrew, Derry, Taylor, Straube, & Phillips, 2014).

Chronic pain is a growing public health concern responsible not only for considerable personal suffering worldwide but also for substantial costs to the nations. One in five people worldwide suffer from chronic pain (Gureje, Von Korff, Simon, & Gater, 1998) and a high prevalence of chronic pain is observed in both developed and emerging countries, including United States (20 %), Australia (20 %) and Brazil (40 %) (ABS, 2017; Dahlhamer et al., 2018; de Souza et al., 2017). The chronic pain-associated burden to the nations is mainly due to medical treatment, decreases in worker productivity, and quality of life. To put into perspective, the estimated total yearly costs of chronic pain in the United States and Australia are USD $ 635 billion and AUD $ 139.33 billion, respectively (Gaskin & Richard, 2012; Painaustralia, 2019). These costs are estimated to rise in the absence of changes to treatments and prevalence rates of chronic pain (Painaustralia, 2019).

Despite substantial costs associated, complete relief for chronic pain is uncommon due to efficacy limitations and adverse effects from the treatments available (Busse et al., 2018; Chu, Angst, & Clark, 2008). Accordingly, up to 50 % of people living with chronic pain are missing out on treatment that could satisfactorily improve their health and quality of life (de Souza et al., 2017). This reflects the deficient current pharmacological treatments for chronic pain that aim for palliative pain relief and not the cure of the disease (ICSI, 2009). In addition, long-term use of the most common analgesics is associated with serious adverse effects that need to be weighed against their indication (Chu et al., 2008). In the United States, for example,

the misuse of prescription opioids is the fastest growing form of drug misuse and is the leading cause of accidental overdose and mortality (CDC, 2020), a phenomenon termed the Opioid Epidemic (NIDA, 2020). These concerns, as well as the restricted efficacy, have resulted in some re-assessment and debate regarding practices surrounding chronic use of opioids (IASP, 2018).

Furthermore, the inherent subjectiveness of pain experience further complicates the advance of chronic pain management in both clinical and research fields. Specifically, the current gold standard assessment of pain is based on subjective self-reports, which have limited accuracy and utility under certain circumstances (Dworkin et al., 2005; Treede et al., 2019). For example, the reliability of self-reports can be affected by psychosocial factors and, thus, bias the assessment of outcomes from therapeutic interventions in clinical and research contexts (Campos et al., 2019; Sullivan et al., 2001). An emerging adjuvant in this matter is the identification of markers that inform about the pathophysiological mechanism responsible for chronic pain (Woolf, 2010). In this case, the measurement of biomarkers could inform about the cellular and molecular factors involved in the chronic pain of a given patient, allowing personalised pharmacotherapy recommendations and the evaluation of pharmacotherapy target engagement. This approach would improve our mechanistic understanding of chronic pain and open new avenues for individualized chronic pain medicine (Tracey, Woolf, & Andrews, 2019).

In the light of these challenges, current efforts have focused on identifying new therapeutic targets and biomarkers that selectively and specifically address chronic pain pathophysiological mechanisms (Latremoliere & Costigan, 2017; Tracey et al., 2019). However, the development of disease-modifying therapy for chronic pain is not a simple task given the complexity of this disease, and the incomplete understanding of the exact molecular mechanisms that underpin chronic pain (Woolf, American College of, & American Physiological, 2004). The current knowledge about chronic pain pathophysiology describes it as interactions of multiple pain generators and amplifiers from biopsychosocial nature, which affect a variety of body systems and biochemical pathways, all simultaneously interacting in the same patient (Bandura, 1978; Treede et al., 2019; Woolf et al., 2004).

Despite substantial advances in our understanding of the pathophysiology behind pain persistence, much remain to be elucidated (Woolf & Salter, 2000). The participation of inflammation as a component of chronic pain has been identified more than four decades ago (De Castro Costa, De Sutter, Gybels, & Van Hees, 1981); however, it is still unclear the exact molecular mechanisms triggered by inflammation that can contribute to the establishment chronic pain (McMahon, La Russa, & Bennett, 2015). Pro-inflammatory mediators are known

to be increased during chronic pain conditions (Austin & Moalem-Taylor, 2010; Chua et al., 2019; Sweitzer, Hickey, Rutkowski, Pahl, & DeLeo, 2002). Many of them perpetuate the inflammatory scenario by activating metabolic pathways, including the kynurenine (KYN) and the tetrahydrobiopterin (BH4) pathways (Guillemin, Smythe, Takikawa, & Brew, 2005; Werner et al., 1990), which are active generators of soluble compounds that can modulate neuronal activity and, therefore, pain sensitivity (Fujita et al., 2019; Latremoliere et al., 2015; Maganin et al., 2021).

Specifically, the rate-limiting enzymes of KYN and BH4 pathways (namely indoleamine 2,3-dioxygenase 1 [IDO1] and guanosine triphosphate cyclohydrolase I [GTPCH], respectively) are inducible and have their expression controlled by pro-inflammatory mediators, such as interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), and interleukin-1beta (IL1-β) (Guillemin et al., 2005; Werner et al., 1990). Of note, KYN and BH4 pathways were consistently activated in inflammatory and neuropathic preclinical models of chronic pain, where both rate-limiting enzymes, IDO1 and GTPCH, were upregulated in dendritic cells and macrophages that infiltrated tissue after injury (Latremoliere et al., 2015; Maganin et al., 2021). Increased levels of metabolites from KYN and BH4 pathways were associated with thermal and mechanical hypersensitivity, while the pharmacological inhibition of IDO1 and GTPCH attenuated hypersensitivity in preclinical models of chronic pain (Rojewska, Ciapala, Piotrowska, Makuch, & Mika, 2018; Tegeder et al., 2006).

There is an urgent need to address the broad problems caused by chronic pain. Overall, inadequate understanding of pathogenesis and difficulties with a reliable assessment of chronic pain are major problems delaying the development of effective and safe therapy for this disease (Woolf, 2010). Some of the key advances necessary to a more successful chronic pain management are, therefore, *i)* to improve the understanding of the mechanisms that generate and maintain chronic pain and *ii)* to establish biochemical markers that permit an objective assessment of chronic pain and its therapeutic interventions.

1.2 SCIENTIFIC PREMISES

The main scientific premises guiding this thesis are:

i) The molecular mechanisms involved in chronic pain are not completely understood;
ii) Inflammation is part of the pathophysiological mechanisms of many chronic pain conditions;
iii) Inflammation induces the activation of the KYN and BH4 pathways;

iv) Preclinical evidence demonstrates that some metabolites from the KYN and BH4 pathways possess nociceptive activity;

v) No biomarker is currently available to assess chronic pain in biological fluids.

vi) Inflammation can be objectively assessed through the measurement of inflammatory markers in biological fluids;

vii) The activation of the KYN and BH4 pathways can be objectively assessed through the measurement of metabolites from the KYN and BH4 pathways in biological fluids.

1.3 HYPOTHESIS

Given the above-mentioned scientific premises, the overall hypothesis of this thesis is that:

The activation of KYN and BH4 pathways by inflammation is involved in the pathophysiological mechanisms promoting chronic pain.

1.4 AIMS

The main aim of this thesis is:

To explore the potential utility of KYN and BH4 pathways-related metabolites as biomarkers of chronic pain.

The specific aims of this study are:

i) To investigate the metabolic profile of KYN and BH4 pathways in multiple cohorts of patients affected by chronic pain conditions that possess an inflammatory component as part of the physiopathology.

Task 1- Recruitment of chronic pain cohorts with inflammatory pathophysiological component

Research partnerships were established with the University Hospital from Universidade Federal de Santa Catarina (Florianopolis, SC, Brazil) and Genesis Research Services (Broadmeadow, NSW, Australia) in order to recruit chronic pain participants. We had

access to three chronic pain cohorts: *i*) Diabetes Painful Neuropathy (DNP; a neuropathic pain condition), *ii*) Complex Regional Pain Syndrome type 1 (CRPS; a nociplastic pain condition) and *iii*) Colitis-related Abdominal Pain (an inflammatory pain condition). We identified from the literature that these three chronic pain conditions have a well-documented inflammatory pathophysiological component (Coates et al., 2013; Ge et al., 2016; Parkitny et al., 2013). Further, the presence of inflammation in the chronic pain cohorts recruited to our studies was assessed by the analysis of inflammatory markers in blood samples.

Task 2- Analysis of metabolites from the KYN and BH4 pathways in biological samples obtained from the participants

The plasma and urinary quantification of KYN and BH4 metabolites from the participants were performed by biochemical and analytical chemistry techniques.

ii) To examine if there is a correlation between the KYN and BH4 pathways with chronic pain features.

Task 1- Characterisation of clinical features from the participants' cohorts

To evaluate the clinical features from the participants' cohorts the use of validated self-reports tools, as well as specialised clinician evaluation were performed. Specifically, characterisation of disease features such as pain intensity, emotional functioning (stress, depression, and anxiety symptoms), pain-related psychosocial features (pain catastrophizing, pain self-efficacy, and kinesiophobia), as well as other disease-related features (years of disease, disease severity and distribution) were performed.

Task 2- Analysis of correlation between the inflammation-enhanced KYN and BH4 pathways metabolites with chronic pain features

The correlation between KYN and BH4 metabolites with the clinical features from the chronic pain cohorts studied was assessed by statistical analysis techniques.

1.5 OVERVIEW OF THE RESEARCH APPROACH

Despite robust preclinical efficacy, most attempts to develop new analgesics based on rational knowledge of pain pathways have failed mainly due to a lack of efficacy in clinical

trials (Arrowsmith & Miller, 2013; Woolf, 2010). There are many possible explanations, including the fact that chronic pain is caused by multiple processes, many of which remain poorly understood (Woolf et al., 2004). In addition, many analgesic targets have been exclusively uncovered in animal models, which may deter translations to humans (van der Worp et al., 2010). Additionally, chronic pain and its underlying causes are notoriously difficult to be accurately accessed across patients, as chronic pain assessment is based on subjective self-reports (Dworkin et al., 2005; Treede et al., 2019). This research project was designed to address some of these challenges and proposed the investigation of relevant biochemical pathways for mechanisms of chronic pain induction as biomarkers that can be objectively measured and, perhaps, targeted in the future to personalise chronic pain treatment.

Chronic pain arises from an interaction of many transmissions, amplifications, and suppressions systems, involving hundreds of molecules (Kissin, 2015). Humans may have a slightly different mix of these mechanisms than other species (Latremoliere & Costigan, 2017). These factors are likely to have contributed to the fact that some therapies designed exclusively in animal models have subsequently shown no efficacy in patients (Hill, 2000; Huggins, Smart, Langman, Taylor, & Young, 2012). One strategy to improve translational success in developing a therapy for chronic pain is to screen for potential molecular targets with pre-existing clinical relevance (Latremoliere & Costigan, 2017). Accordingly, one of the biochemical pathways of interest for this research (the BH4 pathway) has a genetic association with a pain protective phenotype in patients (Campbell et al., 2009; Lotsch, Klepstad, Doehring, & Dale, 2010; Tegeder et al., 2008; Tegeder et al., 2006). Specifically, a modification in the gene *GCH1* (that codifies for the rate-limiting enzyme of BH4 biosynthesis) in humans is associated with low levels of BH4 and chronic pain after injuries (Tegeder et al., 2006). We further observed from the literature that a molecule from another biochemical pathway (*i.e.*, the KYN pathway) was able to modulate the production of BH4 *in vitro* (Haruki, Hovius, Pedersen, & Johnsson, 2016). Therefore, we proposed that the overlapping roles of these two biochemical pathways (*i.e.*, BH4 and KYN), as well as, the crosstalk between them, could take place in a complex organism like humans and ultimately regulate synaptic plasticity and, therefore, pain sensibility (Staats Pires, Tan, Heng, Guillemin, & Latini, 2020). This proposal is detailed described in the literature review presented as part of this thesis - *Kynurenine and tetrahydrobiopterin pathways crosstalk in pain hypersensitivity* (Staats Pires et al., 2020).

Once the relevance of the BH4 and KYN pathways for chronic pain could be rationally justified, it was identified from the literature robust preclinical evidence that the levels of BH4 and KYN metabolites have been shown to spike in animals models of inflammatory and

neuropathic chronic pain, conversely, the pharmacologic or genetic inhibition of both biochemical pathways efficiently reduced chronic hypersensitivity in these animals (see review Staats Pires et al., 2020). The preclinical evidence from animal models is essential to understand the participation of these two biochemical pathways in chronic hypersensitivity and formally evidence causality. Given the lack of clinical evidence and bearing in mind that effective research translation is better achieved by regularly cycling preclinical work with patient analysis, the investigation of BH4 and KYN-related metabolites pathways in clinical studies was proposed. Three cross-sectional clinical studies developed as part of this thesis' research sought to explore the profile of KYN and BH4 pathways metabolites and its correlation with chronic pain features in three cohorts of participants with chronic pain. The candidate, with colleagues, combined the use of liquid and gas chromatography, as well as immunoassays techniques, to quantify molecules from the KYN and BH4 pathways in the blood and urine samples from the cohorts of participants. In addition, we combined the use of statistical analysis and machine learning algorithms to establish a relationship between the metabolites from the two biochemical pathways with chronic pain features.

The following two chapters detail the state-of-the-art, core findings, and publications arising or to result from the work done as part of this research. The research presented has been allocated to two categories, 1) one manuscript of literature review that evidence the involvement of the KYN and BH4 pathways on chronic pain hypersensitivity; 2) three cross-sectional clinical studies investigating the concentration of inflammatory markers and metabolites from KYN and BH4 pathways in samples from 3 different chronic pain cohorts. Articles will be noted if they have been published or are under revision for submission.

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CHAPTER 2

STATE-OF-THE-ART

This chapter provides a state-of-the-art on chronic pain, including essential taxonomy, the mechanisms proposed to underpin chronic pain and the effectiveness of current diagnostic tools and treatments. An article resulting from this thesis is added in chapter 2, and it brings evidence of metabolic pathways that can identify or be responsible for the development of chronic pain.

(P1) Frontiers in Neuroscience:
Kynurenine and tetrahydrobiopterin pathways crosstalk in pain hypersensitivity.
Staats Pires, A. C.; Tan, V. X.; Heng, B.; Guillemin, G.; Latini, A. (Accepted 24 Jun 2020).

CHAPTER 2: STATE-OF-THE-ART

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage (IASP, 2017). Acute pain is a direct outcome of a noxious event and is often classified as a symptom of the underlying tissue damage which precipitated it (Woolf, 2010b). When pain persists in the absence of noxious stimuli or long after the tissue damage has healed, pain is regarded as a disease in its own right (IASP, 2019).

Chronic pain is characterized by its long-term nature and abnormally increased sensitivity (Woolf, 2000). Chronic pain can lead to a series of physical and psychosocial changes such as disability, anxiety, depression and disturbed sleep (Davis, Kroenke, Monahan, Kean, & Stump, 2016). These secondary symptoms, in addition to the persistent pain itself, frequently have a devastating impact on the patient's quality of life (Andrew, Derry, Taylor, Straube, & Phillips, 2014).

Chronic pain is a major health problem worldwide responsible for prevalent human suffering and high expenditures to the nations (Dahlhamer et al., 2018; de Souza et al., 2017; Painaustralia, 2019). Although scientists have made great advances in the understanding of the molecular mechanisms through which persistent pain develops, this knowledge has not been translated into safe and effective therapies (Woolf, 2010a). Indeed, conventional pharmacotherapy is largely unsatisfactory in treating and eradicating chronic pain, and produces many side effects (Busse et al., 2018; Chu, Angst, & Clark, 2008). Additionally, the inherent subjectiveness of the pain experience further complicates the advance of chronic pain management (Woolf, 2010a). Overall, the lack of effective chronic pain treatment places an immense burden on patients, families, health care systems and society in general, therefore, better management of chronic pain is a major unmet need.

2.1 ACUTE PAIN – AN UNPLEASANT PROTECTIVE WARMING DEVICE

The nervous system detects and interprets a wide range of thermal and mechanical stimuli as well as environmental and endogenous chemical irritants. When these stimuli are intense and/or potentially damaging, they activate a class of high-threshold receptors (*i.e.,* the nociceptors) localised at the primary sensory neurons (Julius & Basbaum, 2001). This neural process of encoding noxious stimuli is defined as nociception (IASP, 2017), and is the initial step in triggering the experience of pain. The sensory inflow generated by the activation of nociceptors will be processed in the central nervous system (CNS) and will generate the experience of pain (IASP, 2017). By definition, according to the International Association for the Study of Pain (IASP), pain is "An unpleasant sensory and emotional experience associated with, or resembling that associated

with, actual or potential tissue damage."(IASP, 2017; see Box 1). Therefore, pain is associated with protective reactions for the maintenance of homeostasis and survival (Dib-Hajj, Yang, Black, & Waxman, 2013).

Box 1: The definition of pain by the International Association for the Study of Pain (IASP)

Pain: An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage.
Six key notes and etymology: 1. Pain is always a personal experience that is influenced to varying degrees by biological, psychological, and social factors. 2. Pain and nociception are different phenomena. Pain cannot be inferred solely from activity in sensory neurons. 3. Through their life experiences, individuals learn the concept of pain. 4. A person's report of an experience as pain should be respected. 5. Although pain usually serves an adaptive role, it may have adverse effects on function and social and psychological well-being. 6. Verbal description is only one of several behaviours to express pain; inability to communicate does not negate the possibility that a human or a nonhuman animal experiences pain. *Etymology:* Middle English, from Anglo-French ***peine*** (pain, suffering), from Latin ***poena*** (penalty, punishment), in turn from Greek ***poine*** (payment, penalty, recompense).

Extracted from the IASP website (https://www.iasp-pain.org/resources/terminology/) accessed on 18 Aug 2021.

Pain is one of the sensory modalities resulting from the integration of several neural levels, comprising the detection of the stimulus by the peripheral nervous system and subsequent processing in the CNS (Julius & Basbaum, 2001). The neurophysiologic underpinnings of pain can be divided into four stages: transduction, transmission, pain modulation, and perception (Woolf & Ma, 2007). Initially, harmful stimuli, or stimuli recognized as harmful, are detected by nociceptors that encoded them into electrical signals (Basbaum, Bautista, Scherrer, & Julius, 2009). This process of transduction is executed by small-diameter unmyelinated C fibers and medium-diameter myelinated (Aδ) somatosensory afferent terminals through specialised receptors or ion channels that can convert external noxious stimuli into electrical activity (Schmidt et al., 1995). These specialised receptors/ion channels include, for example, transient receptor potential cation channel subfamily V member 1 (TRPV1) to detect noxious heat (Caterina et al., 1997); transient receptor potential subfamily A member 1 (TRPA1) to detect chemical irritants (Bautista et al., 2006); transient receptor potential cation channel subfamily M member 8 (TRPM8) activated by cold temperatures (Bautista et al., 2007); the potassium channel TREK1 activated by mechanical stimulation and intracellular acidosis (Honoré, 2007); the sodium channel NaV1.7 (Dib-Hajj et al., 2013), and others.

The electrical signals originating from the activation of peripheral nerve endings are transmitted from primary sensory neurons to the CNS (Craig, 2003). Specifically, primary sensory

neurons are pseudounipolar neurons with a peripheral nerve ending at a target tissue to detect the noxious stimuli (Figure 1A), a cell body located in the dorsal root ganglia (DRG; or the in the trigeminal ganglia; Figure 1B), and a central axon projection to the dorsal horn of the spinal cord to convey the pain message to the CNS (Figure 1C) (for a review see Woolf & Ma, 2007). Projection neurons within the dorsal horn give rise to ascending pathways, including the spinoreticulothalamic and spinothalamic tracts, which carry pain messages to the brainstem and thalamus, respectively (Figure 1D) (Craig, 2003). From these brainstem and thalamic loci, information reaches cortical structures involved in the perception of pain (Figure 1E) (Apkarian, Bushnell, Treede, & Zubieta, 2005).

There is no single brain area essential for pain. Rather, pain results from integrated functions across brain networks (Craig, 2003). Some of the brain structures are more associated with the sensory-discriminative properties of pain (*e.g.*, the somatosensory cortex), and others with the emotional aspects of pain (*e.g.*, the anterior cingulate gyrus, prefrontal and insular cortices) (Apkarian et al., 2005). Throughout this pain signalling pathway, pain information is processed by complex circuits involving excitatory and inhibitory interneurons (Ossipov, Dussor, & Porreca, 2010). In addition, the neuronal activity at various points in these circuits can be modulated by ascending and descending neuronal pathways (Sivilotti & Woolf, 1994). This complex neuronal interaction along the pain signalling pathway will ultimately give rise to the perception of pain (Woolf, 2010b).

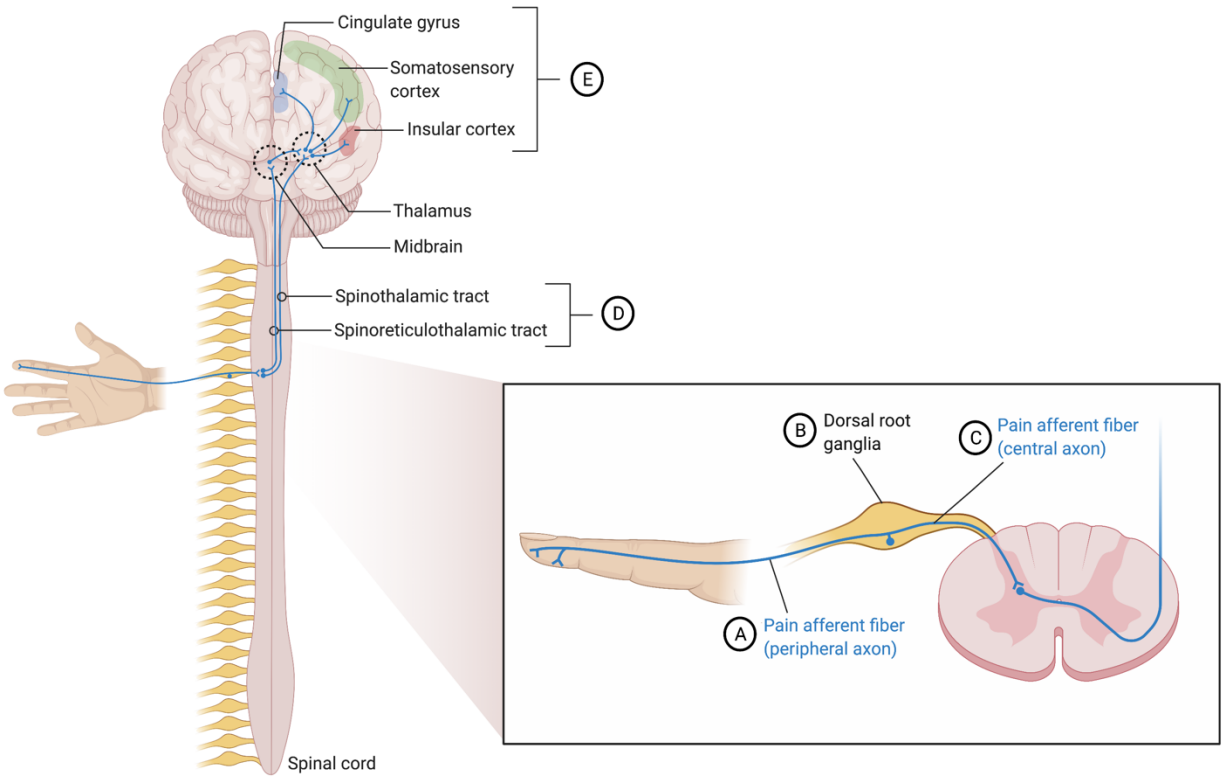

Figure 1. The pain signalling pathway convey noxious stimuli information from periphery to central circuits. An overview of the basic pain signalling pathway from the periphery to the brain. The pain usually starts with the activation of nociceptors at the primary sensory neurons. (A) Primary sensory neurons have a peripheral nerve ending at a target tissue to detect extremes of heat, cold, mechanical, and chemical signals to alert the body of potential

dangers. (B)The cell bodies of primary sensory neurons are located in the dorsal root ganglia or the trigeminal ganglia. (C) From the cell body, the primary sensory neurons project a central axon to the dorsal horn of the spinal cord to convey pain messages to the central nervous system. (D) Ascending pathways, including the spinoreticulothalamic and spinothalamic tracts, carry pain messages to higher brain centers (*e.g.,* brainstem and thalamus, respectively). (E) The sensory inflow generated by nociceptors activates cortical structures involved in the sensory-discriminative (*e.g.*, the somatosensory cortex) and emotional (*e.g.*, the anterior cingulate gyrus and insular cortex) aspects of pain, eliciting pain.

Pain cannot be assumed as simply a result of activity in sensory neurons (IASP, 2017; see Box 1, Note 2). Instead, pain is the interpretation of nociception signals into an unpleasant perception, which is personal and influenced by a myriad of factors (Baliki & Apkarian, 2015). Indeed, the interpretation of nociceptive signals is shaped by factors that include past experiences of pain, beliefs, cultural expectations, affect, psychological state, the context and meaning of the pain, among others (Paksaichol, Lawsirirat, & Janwantanakul, 2015). This scenario captures pain as a complex sensory and emotional experience that is influenced to varying degrees by biological, psychological, and social factors, representing the biopsychosocial model of pain (Figure 2; see Box 1, Notes 1 and 3) (Engel, 1977). Pain thereby reflects a multidimensional composite created by complex interactions between afferent sensory inputs and their processing throughout the nervous system from the periphery to the brain circuitry, with an additional layer of complexity given by memory, expectations, attention, affection and mood (Baliki & Apkarian, 2015).

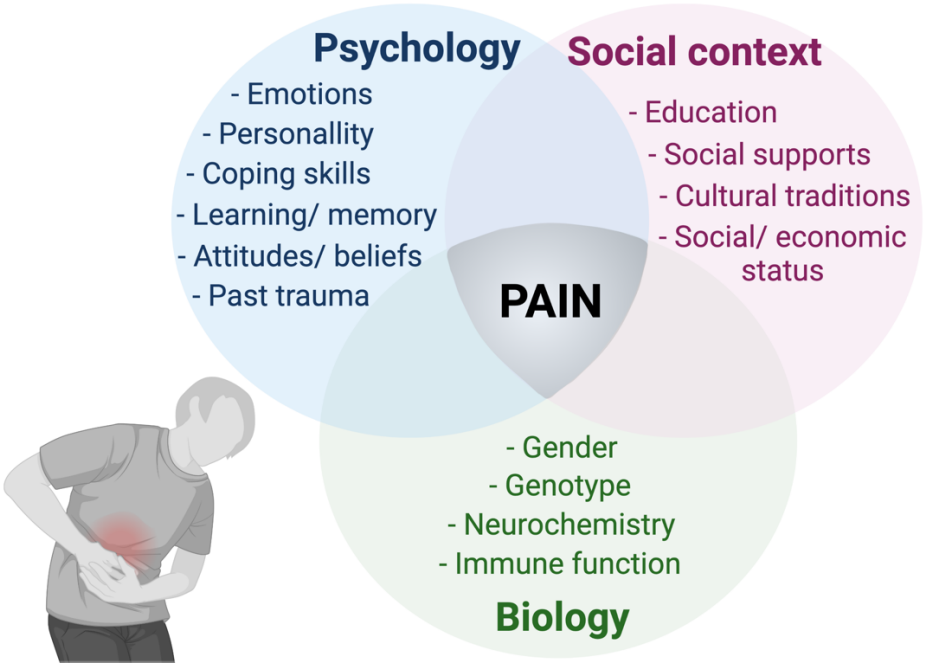

Figure 2. The biopsychosocial model of pain. The experience of pain is a multidimensional process that may include to varying degrees biological and psychosocial aspects. In this context, many factors influence the way individuals perceive and cope with pain. Past experiences, genetic factors, comorbidities, cultural background, emotional state, economic, and environmental factors all play a role. The interaction between the biological and psychosocial aspects of pain is complex as they can overlap and reciprocally influence each other to ultimately shape the experience of pain.

The ability to detect noxious stimuli is essential to an organism's survival and wellbeing. Hence, pain is part of an early-warning protection mechanism (Barik, Thompson, Seltzer, Ghitani, & Chesler, 2018). The intrinsic unpleasantness of pain allows the individual to promptly react to noxious stimuli, minimizing their effects and, therefore, having a protective role (Axelrod & Hilz, 2003). These protective reactions include motor withdrawal reflexes and complex nocifensive behaviours that involve learning and memory components to ultimately avoid damaging situations (Barik et al., 2018). The benefit of this unpleasant experience (*i.e.,* pain) is illustrated by the examination of individuals who suffer from congenital abnormalities that result in lacking the ability to perceive pain (Dib-Hajj et al., 2013). For example, patients carrying rare Mendelian recessive loss-of-function mutations for the sodium channel NaV1.7 are completely indifferent to pain (Dib-Hajj et al., 2013). These people cannot feel pain from a sharp drilling object, heat of an open flame, or even discomfort associated with internal injuries and infections. As a result, they do not engage in appropriate protective behaviours against these conditions, many of which can be life threatening.

2.2 THE PLASTICITY OF THE PAIN SIGNALLING PATHWAY – A DOUBLE-EDGED SWORD

The experience of pain is dependent upon many molecular events at multiple levels of the nervous system, from the peripheral nerve to the brain (Latremoliere & Woolf, 2009; Ma & Woolf, 1996). As part of this dynamic process, both peripheral and CNS components of the pain transmission pathway exhibit tremendous plasticity, enhancing pain signals and producing changes in the pain sensation (Woolf, 2000). The facilitation of protective reactions can be beneficial, but when the changes persist for long periods of months to years, chronic pain may result. Therefore, the duration of this increased synaptic activity will be an important determinant of the protective role (when transient) or detrimental effects (when persistent) of pain sensitisation (Woolf & Salter, 2000; Woolf & Walters, 1991). The exact mechanism for the establishment of a persistent sensitisation after an acute event of injury or disease, or even without any apparent cause, is not completely elucidated (Woolf, 2000).

An important property of all sensory neurons is that they sensitize (that is, their excitability can be increased) (Abraira & Ginty, 2013). Synaptic transmission can be facilitated in response to changes in transmitter release from presynaptic terminals or in transmitter responsiveness on the postsynaptic membrane (Ma & Woolf, 1996; Mannion et al., 1999; Neumann, Doubell, Leslie, & Woolf, 1996). In the specific case of pain sensation, this facilitation of the synaptic transmission leads to a reduction in pain threshold, amplification of pain responses and spread of pain sensitivity to non-injured areas (see Box 2; IASP, 2017). As consequence, pain may appear to arise

spontaneously, from stimuli that would not normally produce pain (allodynia) and noxious stimuli evoke a greater and more prolonged pain sensation (hyperalgesia) (see Box 2; IASP, 2017).

Box 2: The definition of pain threshold, sensitization, hyperalgesia and allodynia by the International Association for the Study of Pain (IASP)

Pain threshold - The minimum intensity of a stimulus that is perceived as painful.
Sensitization - Increased responsiveness of nociceptive neurons to their normal input, and/or recruitment of a response to normally subthreshold inputs. *Note:* Sensitization can include a drop in threshold and an increase in suprathreshold response. Clinically, sensitization may only be inferred indirectly from phenomena such as hyperalgesia or allodynia.
Hyperalgesia - Increased pain from a stimulus that normally provokes pain. *Note:* Hyperalgesia reflects increased pain on suprathreshold stimulation. This is a clinical term that does not imply a mechanism.
Allodynia - Pain due to a stimulus that does not normally provoke pain. *Note:* The stimulus leads to an unexpectedly painful response. This is a clinical term that does not imply a mechanism. It is important to recognize that allodynia involves a change in the quality of a sensation, whether tactile, thermal, or of any other sort. The original modality is normally nonpainful, but the response is painful. There is thus a loss of specificity of a sensory modality.

Extracted from the IASP website (https://www.iasp-pain.org/resources/terminology/) accessed on 18 Aug 2021.

Mechanistically, some of the modifications in sensory neurons responsible for pain sensitisation include phosphorylation of receptors/ion channels or associated regulatory proteins, changes in the expression and trafficking of proteins, increased expression of constitutively expressed genes and/or induction of expression of novel genes, among others (Ma & Woolf, 1996; Mannion et al., 1999; Neumann et al., 1996). These changes can take place in the primary sensory neuron (*i.e.,* nociceptor) or other neurons along the CNS (*i.e.,* spinal cord, brainstem, thalamus and other brain regions) — a phenomenon known as peripheral and central sensitization, respectively (Latremoliere & Woolf, 2009; Woolf & Salter, 2000).

Pain sensitisation is a phenomenon that further enhances the protective function of pain after repeated or particularly intense noxious stimuli, so that the threshold for its activation falls and responses to subsequent inputs are amplified (Woolf & Salter, 2000; Woolf & Walters, 1991). The capacity to increase its sensitivity following exposure to an injurious stimulus is part of the neurobiological protective process that makes the pain signalling system hyperalert in conditions in which a risk of further damage is high. This sensitization enables protective responses to be evoked readily and with a reduced threshold, protecting an injured organism from further injury (Walters, 1994). For example, after tissue damage, injury-related and inflammatory mediators such as prostaglandins, bradykinin, serotonin, adenosine and nerve growth factors are locally released

(Gao & Ji, 2010; Mannion et al., 1999). These chemicals act on receptors expressed on nociceptor peripheral terminals, activating intracellular signalling pathways that by phosphorylating receptors and ion channels change their threshold in the nociceptor terminal (Neumann et al., 1996). The increased excitability of the nociceptor peripheral terminal membrane will reduce the amount of depolarization required to initiate an action potential discharge and produce a temporary sensitization of the affected area. As a result, normally innocuous stimuli, such as touch or warmth, are perceived as painful (*i.e.*, allodynia), or normally painful stimuli elicit pain of greater intensity (*i.e.*, hyperalgesia) (Neumann et al., 1996).

In the absence of ongoing tissue injury, this state of heightened sensitivity returns over time to the normal baseline, where high-intensity stimuli are again required to initiate pain (Ma & Woolf, 1996). When plasticity and the associated changes in pain sensitivity persist long after the initial cause for pain has disappeared, then the pain has no longer a protective function (von Hehn, Baron, & Woolf, 2012). Changes in signal processing in the nervous system may contribute to or may become the sole cause for prolonged hyperalgesia and allodynia (Costigan, Scholz, & Woolf, 2009). Indeed, changes in the function of the pain signalling pathway have been implicated in the development and maintenance of chronic pain, although all the pathological changes at the molecular level are not fully appreciated (Woolf & Salter, 2000).

2.3 CHRONIC PAIN – A MALADAPTIVE CONDITION

When pain persists after the underlying cause is resolved or even in the absence of any damage or disease, it indicates that long-lasting alterations in the pain signalling pathway along the nervous system have occurred (Costigan et al., 2009). Maladaptation can result in chronic hypersensitivity, such that persistent pain outlives its usefulness as an acute warning system and instead becomes a chronic and debilitating disease (Woolf, 2010b). The pain that persists for more than three months is defined as chronic pain (See Box 3; IASP, 2019). The persistence of pain creates a complex biopsychosocial phenomenon that may negatively interfere with many aspects of a person's life, including the ability to work, social activities, and both physical and mental health. Therefore, pain when chronic is no longer considered a "symptom", but instead is promoted to the disease category and presents a maladaptive character (Treede et al., 2019).

In the 11th edition of the International Classification of Diseases (ICD-11), chronic pain diagnoses are represented systematically and classified as chronic primary and chronic secondary pain syndromes. In this lens, chronic pain conditions that are not related to any underlying disease, such as fibromyalgia, complex regional syndrome type 1 and non-specific back pain are considered as primary chronic pain syndromes. On the other hand, chronic pain may be secondary to osteoarthritis, diabetic polyneuropathy, or other diseases, where it is at least initially considered

as a symptom, but it is promoted to disease category when persists for more than three months (See Box 3; Treede et al., 2019). Regardless, chronic pain is a long-term condition that requires special investigation of the underlying cause and demands a target management strategy under the penalty to incapacitate the individuals.

Box 3: The definition of chronic pain by the International Association for the Study of Pain (IASP)

Chronic pain - Pain that lasts or recurs for longer than 3 months. *Note:* A systematic classification of chronic pain was developed by a task force of the IASP and distinguishes chronic primary and chronic secondary pain syndromes. These pain diagnoses have been implemented in the 11th version of the International Classification of Diseases that was released by the World Health Organization in June 2018.
Chronic primary pain – It is defined as pain in one or more anatomical regions that persists or recurs for longer than 3 months and is associated with significant emotional distress or functional disability (interference with activities of daily life and participation in social roles) and that cannot be better accounted for by another chronic condition.
Chronic secondary pain – It is defined as a syndrome linked to other disease as the underlying cause, for which pain may initially be regarded as a symptom. However, when this pain persists for more than 3 months and requires specific care for the patient it becomes a problem in its own right. In many cases, the chronic pain may continue beyond successful treatment of the initial cause; in such cases, the pain diagnosis will remain, even after the diagnosis of the underlying disease is no longer relevant.

Extracted from Treede et al., (2019); a publication from a task force of the IASP; in Pain, 160; 19–27.

Chronic pain takes place with adverse effects on the function, psychological and social well-being of the affected individuals (Walankar, Panhale, & Patil, 2020). In fact, chronic pain has been linked to restrictions in mobility and daily activities (Tsang et al., 2008) and individuals with chronic pain are almost 5 times more likely to report that their daily activities were 'limited a lot' (33 %) than those without chronic pain (according to the Australian Bureau of Statistics; ABS, 2017). To illustrate, the Global Burden of Disease Study 2013 evaluated "years lived with disability" (YLDs) for a broad range of diseases and injuries in 188 countries and the single greatest cause of YLDs around the world was a chronic pain condition - chronic low back pain (Rice, Smith, & Blyth, 2016). Other frequent causes of YLDs in this study included other chronic pain and related conditions, such as chronic neck pain, migraine, osteoarthritis, other musculoskeletal disorders, and medication overuse headache (Rice et al., 2016). In addition, a survey conducted by the World Health Organization (WHO) revealed that patients with chronic pain were more likely to have impaired work role functioning and to have missed three or more days from their usual activities in the prior month (Gureje, Von Korff, Simon, & Gater, 1998). The impact of chronic pain on life is substantial and performance of social responsibilities at work and family life can be significantly impaired, with approximately 20 % of people with chronic pain

unable to work due to pain (Andrew et al., 2014). This debilitating aspect of chronic pain can in turn cause worry and emotional distress (Walankar et al., 2020).

Decrease in quality of life, changes in mood, adverse effects in social relationships, sleep disturbances, and altered appetite are further debilitating consequences promoted by chronic pain (Tsang et al., 2008; Walankar et al., 2020). Additionally, high rates of emotional distress, depression and anxiety disorders are also reported in people affected by chronic pain (Lerman, Rudich, Brill, Shalev, & Shahar, 2015), which are 2.5 times more likely to have mental health problems than people without chronic pain (ABS, 2017). Furthermore, a recent systematic review indicates that people with chronic pain present increased indices of self-reported negative affect (*i.e.,* a cluster of negative emotions, thoughts, and behaviours) relative to pain-free subjects (Burke, Mathias, & Denson, 2015). These overwhelming challenges of living with chronic pain contribute to a suicide rate that is higher than that of the general population (Fishbain, Lewis, & Gao, 2014). Chronic pain reduces the quality of life, provokes high rates of emotional distress, depression and anxiety disorders; thus, it is associated with poor health (Figure 3).

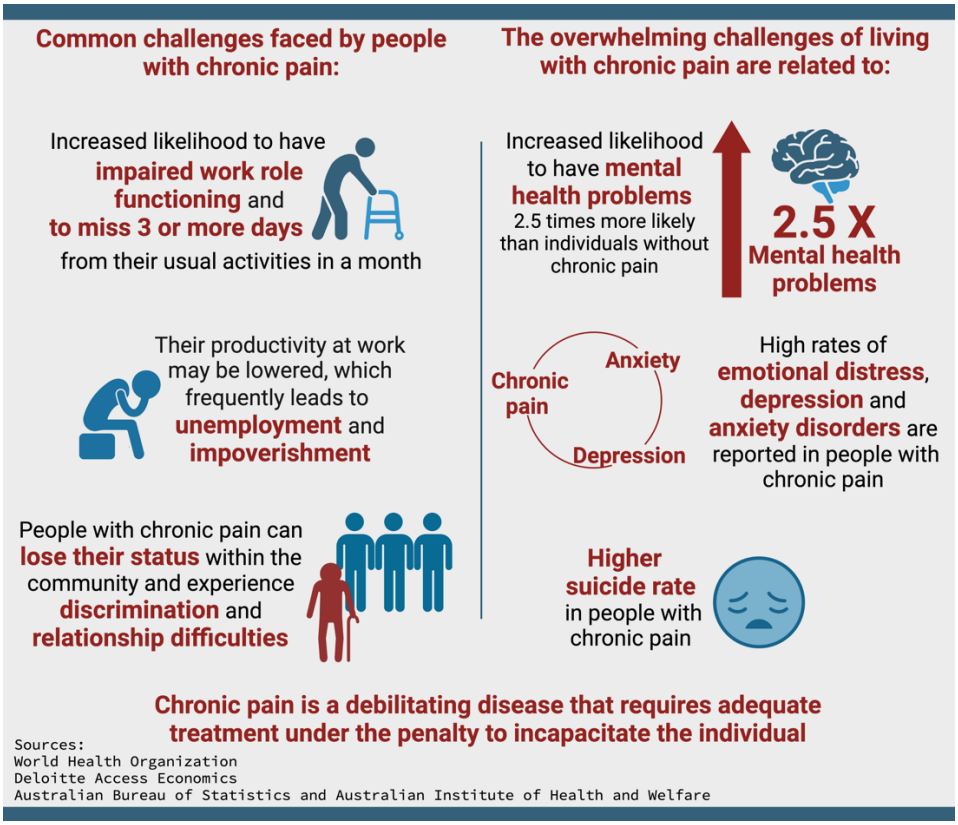

Figure 3*:* The effect and burden of chronic pain for individuals. Chronic pain affects every aspect of a individuals' life, contributing to a loss of both physical and emotional function.

Secondary psychosocial problems can in turn worsen chronic pain, posing escalating threats to health and well-being (Davis et al., 2016). For example, emotional distress negatively promotes catastrophizing (see details in Table 2), reduces the likelihood of practicing exercise and other health promoting behaviours, which may contribute to functional disability (Bousema,

Verbunt, Seelen, Vlaeyen, & Knottnerus, 2007). Indeed, a great body of prospective studies has shown that these conditions will engage the affected individual in extended periods of bed rest, least likely to exercise, and most likely to become physically deconditioned over time (Bousema et al., 2007; Verbunt, Sieben, Vlaeyen, Portegijs, & Andre Knottnerus, 2008). In turn, disuse and physical inactivity can result in more pain and disability (Geneen et al., 2017). Evidence shows that fear avoidance behaviours and physical inactivity besides being associated with more pain and functional disability, resulted in poorer treatment outcomes overall, and reduced probabilities of return to work in individual with chronic pain (Wertli et al., 2014a; Wertli, Rasmussen-Barr, Weiser, Bachmann, & Brunner, 2014b). The emotional burden is also associated with low levels of pain self-efficacy (*i.e.,* confidence in performing activities while in pain; see Table 2 for details), and produces attentional and information-processing biases that lead individuals to focus more on the pain and magnify pain-related stimuli (Crombez, Van Ryckeghem, Eccleston, & Van Damme, 2013; Van Damme, Crombez, & Eccleston, 2004).

The exact mechanisms by which psychosocial problems worsened chronic pain is complex and not completely understood; however, psychophysical studies have also shown that catastrophizing, anxiety, social isolation and other negative affective processes are related to reduced effectiveness in descending pain-inhibitory systems and impaired activity in regions of the prefrontal cortices (Loggia et al., 2015; van Wijk & Veldhuijzen, 2010).

Overall, chronic pain is a multidimensional and dynamic interaction among biological and psychosocial factors that reciprocally influence each other, resulting in a complex chronic disease (Figure 4) (Bandura, 1978). In this lens, negative biological and psychosocial processes can be outcomes from persistent pain but also risk factors that confer vulnerability for the development of chronic pain (Bandura, 1978; Jacobson, 2001). The various patterns of chronic pain-related biological and psychosocial processes interact with an array of neurobiological pathways to shape chronic pain and its long-term outcomes such as disability (Loggia et al., 2015; van Wijk & Veldhuijzen, 2010).

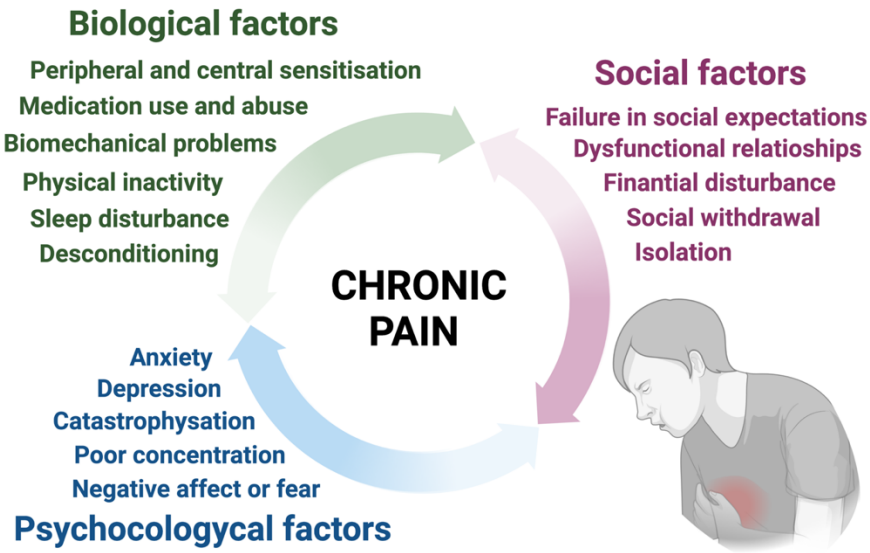

Figure 4. The biopsychosocial aspects of chronic pain and its related outcomes. Chronic pain is characterised by persistent physical pain, disability, emotional disturbance, and social withdrawal symptoms, existing together and influencing one another in reciprocal determinism. People with chronic pain will undergo several biological and psychosocial upheavals throughout their illness, including reduced mobility, which can lead to loss of strength, disturbed sleep, immune impairment, loss of independence, depression or anxiety, and withdrawal from social interaction. It is important to acknowledge that psychosocial factors are not solely secondary reactions to chronic pain; rather a number of these variables act as risk or resilience factors, influencing the probability of developing a chronic pain condition, the severity of pain-related consequences such as disability, and the success or failure of various pain treatments.

2.4 CHRONIC PAIN – A PREVALENT BURDEN FOR SOCIETIES

Chronic pain is one of the most common reasons by which adults seek medical care (Schappert & Burt, 2006). It is estimated that 20 % of the world population (1 in 5 people) suffers from chronic pain, meaning around 1.6 billion individuals worldwide with some degree of chronic pain (Gureje et al., 1998). A survey conducted in 10 developed and 7 developing countries suggested that the prevalence of chronic pain is 41 % and 37 %, respectively (Tsang et al., 2008). A survey conducted by the Center for Disease Control and Prevention (CDC, United States) estimated that 20.4 % (50 million) of adults had chronic pain and 8.0 % of adults (19.6 million) had high-impact chronic pain (*i.e.,* chronic pain that limited life or work activities on most days or every day) (Dahlhamer et al., 2018). The same CDC study revealed that both, chronic pain and high-impact chronic pain, were more frequent in women, older adults, previously but not currently employed adults, adults living in poverty, adults with public health insurance, and rural residents (Dahlhamer et al., 2018). Similar patterns of prevalence between 12 % and 30 %, has been reported in 16 European countries (Breivik, Collett, Ventafridda, Cohen, & Gallacher, 2006).

The Brazilian prevalence of chronic pain at a wide-country level is not available. However, some studies have revealed that some states from the Southern and Southeastern regions showed a prevalence for chronic pain of 40 %, that the most affected population were females with a mean age of 41 years (Carvalho, 2018; de Souza et al., 2017). Slightly lower prevalence have been shown in Australia; 1 in 5 (19 % or 1.6 million) individuals aged 45 and over have reported chronic pain, according to a survey conducted by the Australian Bureau of Statistics (ABS, 2017). Noteworthy, 68 % of people living with chronic pain are of working age in Australia, where 40 % of early retirement is due to chronic pain issues (Painaustralia, 2019).

Chronic pain is a public health epidemic placing burdens on those experiencing pain as well as a financial burden on nations' public funds (Figure 5). Chronic pain is costly to the nations because it requires medical treatment and complicates treatment for other ailments, but also because pain lowers worker productivity and quality of life. Accordingly, the presence of chronic pain has both direct health care and associated indirect costs (*e.g.,* disability payments, lost

productivity, reduction of quality of life) (Painaustralia, 2019). The total financial costs of chronic pain in 2010, including both direct and indirect costs, was USD $635 billion in the United States, which was greater than the annual costs for heart diseases ($309 billion), cancer ($243 billion), and diabetes ($188 billion) (Gaskin & Richard, 2012). The total annual financial costs associated with chronic pain, including healthy system, loss of productivity and well-being costs, were estimated to be AUD $139.33 billion in 2018 in Australia. These costs are estimated to reach AUD $215.6 billion by 2050 in the absence of changes to health system treatments and prevalence rates (Painaustralia, 2019).

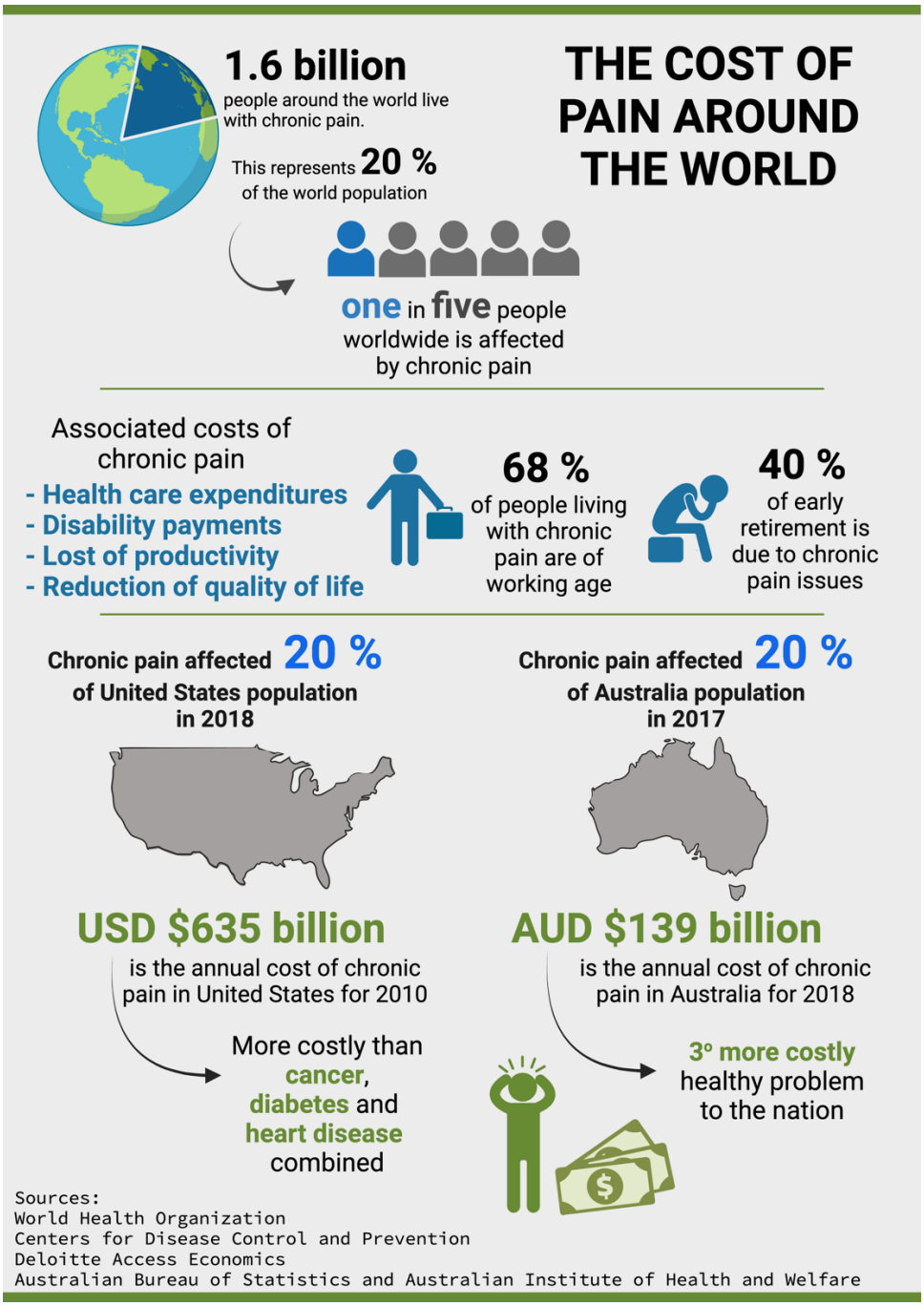

Figure 5. The prevalence and costs of chronic pain. Chronic pain of all sorts is not only responsible for considerable personal suffering worldwide; it also contributes to substantial costs to the nations. Chronic pain is related to high expenditures due to direct health care costs and indirect costs (*e.g.,* disability payments, lost productivity, and reduction of quality of life).

An evaluation of the health system and loss of productivity costs associated with chronic non-cancer pain in primary care patients in Canada revealed mean annual total costs of CAD $16,636 per patient with chronic pain (Lalonde et al., 2014). A study that evaluated the burden of chronic neuropathic pain in Europe, covering health system and other financial costs, indicated that the total cost of chronic neuropathic pain per patient were €10,313 in France, €14,446 in Germany, €9,305 in Italy, €10,597 in Spain and €9,685 in the United Kingdom (Liedgens, Obradovic, De Courcy, Holbrook, & Jakubanis, 2016). In Australia, the total annual financial costs associated with chronic pain were estimated to be AUD $42,979 per person (Painaustralia, 2019). Worldwide, governments are becoming aware of the huge impact that chronic pain is having on their communities, which translates into national strategies to deal with this chronic condition (IOM, 2011; IPRCC, 2016; Painaustralia, 2019). As a consensus, these initiatives call for more research in the field to elucidate the exact pathophysiology mechanisms of chronic pain and, ultimately, improve the management of this disease.

2.5 PATHOPHYSIOLOGY MECHANISMS OF CHRONIC PAIN

Chronic pain remains a global health problem and a challenge to basic and clinical sciences. Chronic pain involves diverse maladaptive plasticity processes, which have not been completely decoded in terms of the involvement of specific circuits and biochemical pathways. Diverse pathological situations at different anatomical sites can contribute to chronic pain. Causes of pain include cancer, long-term inflammation, chronic metabolic dysfunctions, pathogenic infections, tissue damage, as well as injury or lesions of the nervous system (Costigan et al., 2010; Honore et al., 2000; Ji, Xu, & Gao, 2014; von Hehn et al., 2012). Diverse chronic widespread chronic pain syndromes may also occur due to abnormal amplification states within the CNS (Basbaum et al., 2009; Latremoliere & Woolf, 2009). The heterogeneity of clinical pain conditions and the complexity and multiplicity of underlying pathophysiological mechanisms are majors obstacles to preventing and treating chronic pain (Vardeh, Mannion, & Woolf, 2016).

Chronic pain is a multifactorial condition with complex biology and proposed pathophysiology that includes multiple body systems and biochemical pathways affected (Woolf, American College of, & American Physiological, 2004). Even if a solitary event precipitates the initiation of chronic pain (*e.g.*, injury, surgery or disease), a combination of factors will determine the duration, intensity, as well as physical and emotional associated effects (Diatchenko, Fillingim, Smith, & Maixner, 2013). All these factors can to some extend influence the pain signalling pathway through maladaptive changes in the nervous system (Woolf, 2010b). Most acute insults *(e.g.,* sunburn, sprained ankle or a surgical incision) resolve without persisting pain, which

emphasizes that the processes of pain sensitization are typically reversible (Woolf & Costigan, 1999). Some chronic pain states are, however, associated with long-term activation of nociceptors by noxious stimulus (*e.g*., due to anatomical deformities or biomechanical problems) (IASP, 2019). Additionally, a variety of chronic pain conditions are closely associated with tissue pathology (*e.g.,* after nerve transection or amputation) and apparent irreversibility sensitization (Costigan et al., 2009; IASP, 2015). Also, many chronic pain conditions are related to permanent pain sensitisation by inflammatory factors (*e.g*., during chronic inflammatory and autoimmune diseases) (IASP, 2019). Furthermore, a plethora of chronic pain states have no obvious tissue inflammation or pathology associated (*e.g*., fibromyalgia, irritable bowel syndrome, painful bladder syndrome or migraine) (ICSI, 2009), although central sensitisation seems to be present (Staud, Craggs, Perlstein, Robinson, & Price, 2008). The exact molecular biochemical and neuroanatomical basis for the variety of chronic pain conditions are not completely elucidated and are also the focus of this and several worldwide current studies (Latremoliere & Costigan, 2017).

Despite the complexity of this multifactorial condition, there have been attempts to systematically define the pathophysiological mechanisms for chronic pain induction. Based on the current knowledge, the IASP definition of mechanistic terminology of pain are *i*) nociceptive pain as a "pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors"; *ii*) neuropathic pain as a "pain caused by a lesion or disease of the somatosensory nervous system"; and *iii*) nociplastic pain as a "pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain" (IASP, 2017; see Table 1). All these mechanistic pain types are thought to be common and to play a role in many common chronic pain conditions such as nociceptive pain in osteoarthritis, neuropathic pain in painful diabetic neuropathy, and nociplastic pain in fibromyalgia (IASP, 2019). Another frequently used terminology is "mixed pain", which is widely used, yet it is not defined in the IASP taxonomy. The proposed definition for mixed pain is "a complex overlap of the different known pain types (nociceptive, neuropathic and nociplastic) in any combination, acting simultaneously and/or concurrently to cause pain in the same body area" (Freynhagen et al., 2019).

Table 1: The definition of mechanistic pain terminology by the International Association for the Study of Pain (IASP) and Freynhagen et al. (2019)

Types of pain	Definition	Examples of chronic pain conditions
Nociceptive pain	Pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors.	Osteoarthritis; Myofascial pain; Bursitis; Tendonitis.
Neuropathic pain	Pain caused by a lesion or disease of the somatosensory nervous system.	Painful diabetic neuropathy; Postherpetic neuralgia; Complex regional pain syndrome type 2. nal
Nociplastic pain	Pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain.	Fibromyalgia; Irritable bowel syndrome; Temporomandibular disorder; Painful bladder syndrome; Trigeminal neuralgia; Complex regional pain syndrome type 1; Migraine;
Mixed pain	It is a complex overlap of the different known pain types (nociceptive, neuropathic, nociplastic) in any combination, acting simultaneously and/or concurrently to cause pain in the same body area. Either mechanism may be more clinically predominant at any point of time. Mixed pain can be acute or chronic.	Low back pain; Neck pain; Chronic postsurgical pain; Lumbar spinal stenosis; Cancer pain.

Extracted from Current Medical Research and Opinion (2019) and the IASP website (https://www.iasp-pain.org/resources/terminology/) accessed on 18 Aug 2021.

Literature also supports inflammation as an important factor that drives self-perpetuation and pathologic plasticity changes in sensory neurons that sustain the chronicity of pain (Gao & Ji, 2010; Kawasaki, Zhang, Cheng, & Ji, 2008). Some authors even consider pain driven by the inflammatory response as a distinct type of pain mechanism – inflammatory pain as a "pain caused by activation of the immune system by tissue injury or infection" (Woolf, 2010b). Growing evidence suggests that the activation of pro-inflammatory pathways is a key process driving neuronal changes in many types of chronic pain, including nociceptive, neuropathic and nociplastic chronic pain (Austin & Moalem-Taylor, 2010; Chua et al., 2019; Sweitzer, Hickey, Rutkowski, Pahl, & DeLeo, 2002). Although the current knowledge supports the crosstalk between pain-signalling and immune systems to the sustained sensitization of sensory neurons (for a review

see McMahon, La Russa, & Bennett, 2015), the exact mechanisms by which inflammation would promote a shift from acute to chronic pain is not well understood and deserves further investigation.

2.6 THE CHALLENGE OF MEASURING CHRONIC PAIN

Pain is an individual human experience that is entirely subjective and can only be truly appreciated by the individual experiencing the pain (IASP, 2017). The personal and subjective nature of pain turns the objective measurement of pain into a difficult and biased task. Accordingly, the current gold standard assessment of pain is subjective and based on individual reports (Dworkin et al., 2005; Treede et al., 2019).

Different self-report tools are used to measure pain intensity and to determine its impact on physical, social and emotional functioning. These assessment tools are valuable to address chronic pain, where a myriad of factors reciprocally influence each other to determine the features of chronic pain (Bandura, 1978; Jacobson, 2001). Accordingly, the IASP and the Initiative on Methods, Measurement, and Pain Assessment in Clinical Trials (IMMPACT) endorse the use of self-reported biopsychosocial aspects of chronic pain to support its assessment in the clinical and research context (Dworkin et al., 2005; Treede et al., 2019). Although a variety of self-report tools for chronic pain and its biopsychosocial aspects are available, the lack of objective assessment for chronic pain represents a barrier to advance the management of chronic pain in both clinical and research fields. Alternatively, the use of biomarkers for chronic pain has been proposed as a valuable tool to support the diagnosis and management of chronic pain (Tracey, Woolf, & Andrews, 2019). However, no quantifiable biomarker is available to identify or characterize chronic pain or its degree. Thus, the identification and validation of biomarkers for chronic pain represent a desired focus of research.

Self-reports have been widely used for decades and are still considered the golden standards measurement of pain (Dworkin et al., 2005; Treede et al., 2019). Some of the pain self-report tools validated and widely used include unidimensional scales such as the Numeric Rating Scale (von Baeyer et al., 2009) and Visual Analogue Scale (VAS; Price, Mcgrath, Rafii, & Buckingham, 1983), which scores pain in a scale of 11 points ranging from 0 to 10 (or 0 to 100), with one far extremity described as “no pain” and the other one described as “worst pain imaginable/ unbearable pain”. Multidimensional questionaries are also available to measure more than one dimension of pain, including intensity, quality, affect, interference with functioning and quality of life. The short-form McGill Pain Questionnaire (SF-MPQ) is an example of a well-validated multidimensional pain questionary with extensive clinical and research use (Melzack,

1987). The SF-MPQ consists of three major classes of word descriptors summarizing sensory, affective and evaluative qualities of pain (Melzack, 1987).

Across chronic pain conditions, a diverse array of psychological and social factors need to be considered as outcomes or potential risk/ resilience factors within the dynamic system of aspects that constitutes chronic pain (Dworkin et al., 2005). Instruments used to assess symptoms of depression, anxiety, and distress have been recommended for use as outcomes or risk factors measures in chronic pain trials (Dworkin et al., 2005). For example, the Short-form Depression, Anxiety and Stress Scale – 21 Items is a validated set of three self-report scales designed to measure the emotional states of depression, anxiety and stress (Lovibond & Lovibond, 1995). Other factors associated with negative outcomes and vulnerability are pain catastrophizing and fear, which can be measured through self-report questionaries (Roelofs et al., 2011; Sullivan, Bishop, & Pivik, 1995). The Pain Catastrophizing Scale is a widely used measure of catastrophic thinking (see Table 2) and encompasses different perspectives from this phenomenon (*i.e.,* rumination, magnification and helplessness) (Sullivan et al., 1995). One tool to address pain-related fear is the Tampa Scale for Kinesiophobia (Roelofs et al., 2011), which assesses fear of movement and (re)-injury (see Table 2). Finally, factors associated with resiliency and positive outcomes on functionality, such as pain self-efficacy (See Table 2) can be assessed by self-report tools. The Pain Self-Efficacy Questionnaire (PSEQ), for example, is a validated 7-item tool assessing self-efficacy for functioning normally while in pain (Nicholas, 2007).

Table 2: The pain-related psychosocial features

Pain-specific features	Definition
Pain catastrophizing	Pain catastrophizing is a pain-specific psychosocial construct comprised of negative cognitive and emotional processes such as helplessness, pessimism, rumination about pain-related symptoms, and magnification of pain reports (Sullivan et al., 1995).
Pain self-efficacy	Pain-related self-efficacy is defined as the beliefs held by people with pain that certain activities can be carried out despite the pain (Bandura, 1977).
Kinesiophobia	A situation where a patient has an excessive, irrational, and debilitating fear of physical movement and activity resulting from a feeling of vulnerability to painful injury or reinjury (Miller, Kori, & Todd, 1991).

Extracted from Psychological Assessment (1995), Psychological Review (1977) and The Clinical Journal of Pain (1991).

The systematic diagnosis of chronic pain developed by a task force of the IASP for ICD-11 describe characteristic features of chronic pain diagnoses that can be accessed by self-report tools, including the severity of pain, its temporal course, and evidence for psychological and social

factors (See Box 4: Treede et al., 2019). Furthermore, the IMMPACT recommendations for chronic pain clinical trials include assessment of six core outcome domains: 1) pain; 2) physical functioning; 3) emotional functioning; 4) participant ratings of improvement and satisfaction with treatment; 5) symptoms and adverse events; and 6) participant disposition. Of note, the IMMPACT group endorse the assessment of the outcome domains 1 to 5 with the use of self-reports (Dworkin et al., 2005).

Box 4: Specifiers that characterise chronic pain conditions in the 11th edition of the International Classification of Diseases (ICD-11)

Severity of the pain

Mild pain VAS: 1- 3 (< 31);
Moderate pain VAS: 4 - 6 (31-54);
Severe pain VAS: 7-10 (55-100).

Temporal characteristics of the pain

"Continuous" (the pain is always present);
"Episodic recurrent" (there are recurrent pain attacks with pain-free intervals);
"Continuous with pain attacks" (there are recurrent pain attacks as exacerbations of underlying continuous pain).

Presence of psychosocial factors

This extension code permits coding problematic cognitive (*e.g.*, catastrophizing, excessive worry), emotional (*e.g.*, fear, anger), behavioural (*e.g.*, avoidance) and/or social factors (*e.g.*, work, relationships) that accompany the chronic pain.

The extension code is appropriate if there is positive evidence that psychosocial factors contribute to the cause, the maintenance and/or the exacerbation of the pain and/or associated disability and/or when the chronic pain results in negative psychobehavioral consequences (*e.g.*, demoralisation, hopelessness, avoidance, withdrawal).

Extracted from Treede et al., (2019); a publication from a task force of the IASP; in Pain, 160; 19–27.

Although subjective self-reports have been considered golden standards for the measurement of chronic pain features, their accuracy and utility are limited under certain circumstances. For instance, the reliability of self-report scales for pain intensity can be affected by psychological and environmental factors. To illustrate, patients with chronic pain can hold negative attitudes and magnify their pain severity, a common phenomenon in pain catastrophizing (Campos et al., 2019; Sullivan et al., 2001). Also, self-reporting relies on effective communications and thus is not suitable for patients under general anesthesia, with cognitive disorders or infants/ young children (Painaustralia, 2021). The subjectivity of chronic pain assessment can, therefore, represent an obstacle for a proper patient's treatment, as well as, the advance of the knowledge in the field.

Considering the limitations of self-reports for measuring chronic pain, an objective assessment is desired, and the use of biomarkers could represent a valuable tool in clinical practice and research. The FDA-NIH Biomarker Working Group glossary, BEST (Biomarkers, EndpointS, and other Tools Resource) (2016) defines biomarkers as a characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to a therapeutic intervention (Box 5). Given the inherently subjective nature of pain, it is unlikely that a single biomarker could translate perfectly the experience of pain on its complexity. However, the identification of markers that inform about the presence or pathophysiological mechanism responsible for chronic pain would improve the ability to accurately diagnoses and treat chronic pain disorders. Finally, the biomarkers could also support the development of disease-modifying drugs for chronic pain, which may increase the likelihood of success, reduce off-target effects, and result in durable benefits, all high-desired outcomes for chronic pain management (Tracey et al., 2019).

Box 5: The definition of biomarker by FDA-NIH Biomarker Working Group glossary

Biomarker: A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions. Biomarkers may include molecular, histologic, radiographic, or physiologic characteristics. A biomarker is not a measure of how an individual feels, functions, or survives.

Extracted from FDA-NIH Biomarker Working Group glossary, BEST (Biomarkers, EndpointS, and other Tools Resource) (2016).

2.7 THE CHALLENGE OF TREATING CHRONIC PAIN

Pharmacological interventions have been the mainstay of treatment for chronic pain during past centuries, and the use of drugs to treat chronic pain has expanded exponentially in recent years, with increases in expenditures of 188% between 1996 and 2005 (Martin et al., 2008). Despite the widespread use of pharmacological treatments for chronic pain, none are universally endorsed and specific pharmacotherapy for chronic pain is not available (Woolf et al., 2004). Currently, all common palliative treatments prescribed for acute pain and chronic cancer pain are also chronically used for the treatment of chronic pain conditions (ICSI, 2009). However, these pharmacological treatments do not target the specific cause of chronic pain and, therefore, have limited evidence of efficacy for the treatment of chronic pain conditions (Busse et al., 2018; Moore, Chi, Wiffen, Derry, & Rice, 2015). Moreover, the long-term use of these drugs is associated with undesirable and counterproductive side effects (Chu et al., 2008; McGettigan & Henry, 2006). As a result, patients report great dissatisfaction with the management of their condition and chronic pain persists as a debilitant and untreated condition (Breivik et al., 2006). This scenario is not surprising given the complexity of chronic pain and lack of understanding of

its physiopathology. A better understanding of the cellular and molecular mechanisms of chronic pain is necessary to take an ideal approach towards effective and safe chronic pain treatment.

Currently, all pharmacological treatments for chronic pain are palliative approaches focusing on relieving pain without dealing with the cause of the condition (Woolf et al., 2004). Drugs for this purpose include opioids and nonopioid analgesics such as nonsteroidal anti-inflammatory drugs (NSAIDs), acetaminophen and topic anesthetic, as well as adjuvant drugs such as antidepressants and anticonvulsants (ICSI, 2009). Acetaminophen (paracetamol) and the NSAIDs act to reduce the production of inflammatory chemicals by inhibiting the cyclooxygenase enzymes that decrease the production of pro-inflammatory prostaglandins. Systematic reviews indicate low evidence of efficacy for acetaminophen and NSAIDs in the treatment of chronic pain conditions (Enthoven, Roelofs, Deyo, van Tulder, & Koes, 2016; Moore et al., 2015; Radner et al., 2012). One review focusing on the treatment of chronic low back pain concluded that there is low-quality evidence that NSAIDs are more effective than placebo on reduction of pain and disability (Enthoven et al., 2016). Furthermore, there was no indication of any significant pain reduction with NSAIDs administration for management of neuropathic pain (Moore et al., 2015) and paucity of evidence regarding the safety and efficacy of NSAIDs treatment in inflammatory arthritis (Radner et al., 2012). Meanwhile, the long-term use of acetaminophen and NSAIDs is associated with the development of adverse reactions that greatly limited their use. Specifically, prolonged use of paracetamol can cause impairment in liver function and induce liver toxicity (Bunchorntavakul & Reddy, 2013). While long-term use of NSAIDs is associated with gastrointestinal, and cardiovascular side effects (Griffin & Scheiman, 2001). In addition, meta-analyses of long-term use of high-dose NSAIDs have found that all increase the risk of coronary heart disease and thrombotic cardiovascular events (Coxib et al., 2013; McGettigan & Henry, 2006). Frequently, the use of topic anesthetic such as lidocaine (an amide anesthetic that inhibits nerve depolarisation by blocking sodium channels) has been recommended for localised pain relief of peripheral neuropathic pain (Dworkin et al., 2007). Despite individual studies reporting efficacy in the relief of neuropathic pain, a recent systematic review found no good quality evidence to support its use (Derry, Wiffen, Moore, & Quinlan, 2014).

Opioids drugs are commonly used for pain relief (IASP, 2018). Essentially, opioid analgesia results from the binding and signalling through mu-opioid receptors present along the pain signalling pathway (Matthes et al., 1996). Opioids are the most efficient class of drug against acute pain, but their efficacy for the treatment of chronic pain is controversial regarding the analgesic and adverse effects (Chou, Ballantyne, Fanciullo, Fine, & Miaskowski, 2009). A recent meta-analysis published by Busse et al. (2018) concluded that opioids provided only minor improvements for people dealing with chronic pain caused by conditions other than cancer.

Furthermore, the effectiveness of opioids can diminish over time because of tolerance upon their chronic use (Vorobeychik, Gordin, Mao, & Chen, 2011), which means that dosage needs to be increased over time to achieve pain relief. As a result, some adverse effects are commonly observed with the chronic use of opioids, including nausea, constipation, cognitive impairment, sedation, and various hormonal changes (Kalso, Edwards, Moore, & McQuay, 2004). Paradoxically, opioid-induced hyperalgesia is a common counterproductive effect resulted from the chronic use of opioids (Chu et al., 2008). Of special concern is the fact that long-term opioid use is associated with a risk of fatal and non-fatal overdose, as well as problematic patterns of use (*i.e.,* abuse and dependence) (CDC, 2020). In the United States, opioids were involved in 70.6 % of all drug overdose deaths (which represents a total of 49,860 deaths) in 2019 (CDC, 2020). The increased opioid prescription has been accompanied by a sharp rise in the incidence of addiction and opioid-related mortality, a phenomenon termed the Opioid Epidemic (NIDA, 2020). These concerns, as well as the restricted efficacy, have resulted in some re-assessment and debate regarding practices surrounding chronic use of opioids (IASP, 2018).

Overall, chronic pain patients are treated as though they are suffering from an extended period of acute pain, which means that palliative analgesics are tried and tested, to reduce the pain to an acceptable level (ICSI, 2009). Not surprisingly, evidence suggests that none of the most commonly prescribed treatment regimens are, by themselves, sufficient to eliminate chronic pain and to have a major effect on physical and emotional function in most patients with chronic pain (Turk, Wilson, & Cahana, 2011). According to a report from the Institute of Medicine (IOM), most Americans who live with chronic pain do not receive appropriate care (IOM, 2011). Indeed, a survey of patients with chronic pain conducted in 15 European countries and Israel observed that 40 % of patients had inadequate management of their pain (Breivik et al., 2006). Similarly, a dissatisfaction with chronic pain management was reported by 49 % of the Brazilians that participated in a population-level survey (de Souza et al., 2017). Clearly, chronic pain represents a major cause of human suffering worldwide, especially because none of the currently available treatments has proven to be capable of eliminating pain and restoring functioning to a high proportion of patients (Breivik et al., 2006). Therefore, the development of more efficient and safe treatment for chronic pain is an unmet need, and the elucidation of the molecular mechanism underlying chronic pain is key for the discovery of new drug targets.

In the light of these challenges, current efforts have focused on identifying new therapeutic targets that selectively and specifically modulate chronic pain (Latremoliere & Costigan, 2017). Understanding the participation of some biochemical pathways that underlie chronic pain and the development and maintenance of plasticity in sensory neurons will reveal novel targets for the discovery of more specific chronic pain therapies. In this context, the first article originated from this thesis (Staats Pires, Tan, Heng, Guillemin, & Latini, 2020) reviewed the literature and

provided evidence that the KYN and BH4 pathways are exacerbated during pain, having a possible pathological role in pain maintenance.

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ORIGINAL RESEARCH ARTICLE: KYNURENINE AND TETRAHYDROBIOPTERIN PATHWAYS CROSSTALK IN PAIN HYPERSENSITIVITY

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REVIEW
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Kynurenine and Tetrahydrobiopterin Pathways Crosstalk in Pain Hypersensitivity

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Despite the identification of molecular mechanisms associated with pain persistence, no significant therapeutic improvements have been made. Advances in the understanding of the molecular mechanisms that induce pain hypersensitivity will allow the development of novel, effective, and safe therapies for chronic pain. Various pro-inflammatory cytokines are known to be increased during chronic pain, leading to sustained inflammation in the peripheral and central nervous systems. The pro-inflammatory environment activates additional metabolic routes, including the kynurenine (KYN) and tetrahydrobiopterin (BH4) pathways, which generate bioactive soluble metabolites with the potential to modulate neuropathic and inflammatory pain sensitivity. Inflammation-induced upregulation of indoleamine 2,3-dioxygenase 1 (IDO1) and guanosine triphosphate cyclohydrolase I (GTPCH), both rate-limiting enzymes of KYN and BH4 biosynthesis, respectively, have been identified in experimental chronic pain models as well in biological samples from patients affected by chronic pain. Inflammatory inducible KYN and BH4 pathways upregulation is characterized by increase in pronociceptive compounds, such as quinolinic acid (QUIN) and BH4, in addition to inflammatory mediators such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). As expected, the pharmacologic and genetic experimental manipulation of both pathways confers analgesia. Many metabolic intermediates of these two pathways such as BH4, are known to sustain pain, while others, like xanthurenic acid (XA; a KYN pathway metabolite) have been recently shown to be an inhibitor of BH4 synthesis, opening a new avenue to treat chronic pain. This review will focus on the KYN/BH4 crosstalk in chronic pain and the potential modulation of these metabolic pathways that could induce analgesia without dependence or abuse liability.

Keywords: chronic pain, neuropathic pain, inflammatory pain, neuroinflammation, kynurenine, tetrahydrobiopterin, xanthurenic acid, central sensitization

INTRODUCTION

The immune and pain-signaling systems are evolutionarily designed to protect the organism by acutely responding to danger (Woolf and Ma, 2007; Woolf, 2010; O'neill et al., 2016). Typically, stimuli that activate both systems elicit inflammation and pain that are adaptive response to overcome the threat, increasing the life-time reproductive success (Beringer and Miossec, 2019). However, many injuries and diseases may perpetuate maladaptive inflammatory reactions, in which pro-inflammatory mediators persistently activate and sensitize neurons at different levels of the nociceptive pathway (Woolf and Costigan, 1999; Costigan et al., 2009a, 2010; Oikawa et al., 2019). These long-lasting sensory changes known as chronic pain, represents a major unmet clinical need (for a review see Woolf and Salter, 2000). This is a significant problem due to the high incidence worldwide (Rice et al., 2016) and lack of effective, specific and safe therapies (Varrassi et al., 2010; Dart et al., 2015; Jones, 2017).

Neuroinflammation is characterized by the infiltration of peripheral immune cells, activation of glial cells and production of inflammatory mediators in the peripheral and central nervous systems (PNS; CNS). This contributes to generate a peripheral and central sensitization that causes long-term pain (Kawasaki et al., 2008; Gao and Ji, 2010). Therefore, targeting these neuroinflammatory processes and molecules may result in an effective analgesic treatment for chronic pain.

CHRONIC PAIN

Chronic pain is a dysfunctional process defined by longstanding pain sensations of more than three months (IASP, 2019; Treede et al., 2019). Chronic pain is a major health problem worldwide that negatively impacts on the quality of life of the affected individuals, and represents a huge health public costs in both developed and emerging countries (Goren et al., 2014). Approximately 20% of the adult population in the United States (Dahlhamer et al., 2018), Canada (Shupler et al., 2019), and Europe (Breivik et al., 2006) are affected by chronic pain. Similar prevalence is observed in Australia, about 15% (Miller et al., 2017), and higher in countries like Brazil and Japan, with a prevalence of around 40% (Inoue et al., 2015; De Souza et al., 2017). Chronic pain is a major health burden to the society, with annual costs over $635 billion per year in the United States alone (Gereau et al., 2014). This exceeds the combined costs of common chronic conditions including, cancer, heart disease, and diabetes (Gereau et al., 2014). The global high incidence of chronic pain is aggravated by the lack of effective and safe treatments; in particular, the development of side effects such as addiction and the risk overdose leading to death (NIDA, 2020).

There are numerous classes of drugs used to treat pain, including serotonin reuptake inhibitors, non-steroidal anti-inflammatory drugs (NSAIDs), and opioids (Nalamachu, 2013). Each of these drugs are associated with different adverse events impacting the gastrointestinal, cardiovascular, and renal systems, and their efficacy against chronic pain is controversial (Macario and Lipman, 2001; Brueggemann et al., 2010; Skljarevski et al., 2012; Chou et al., 2014). A recent meta-analysis showed that opioids, the most efficient class of drug against acute pain, provided only minor improvements for people dealing with chronic pain caused by conditions other than cancer (Busse et al., 2018). Moreover, the repeated use of opioids is strongly associated with addiction and risk of death (NIDA, 2020). Therefore, more research is urgently needed to develop pain medications with higher efficacy and safety.

The exact mechanism driving pain persistence is poorly understood. Chronic pain conditions are likely to have distinctive underlying mechanisms that ultimately alter the long-term nociceptive signaling in patients. Indeed, a key and common feature for all chronic pain conditions is a long-term neuronal plasticity in pain-signaling circuits that result in increased neuronal responsiveness to their normal input and/or recruitment of a response to subthreshold inputs (for a review see Woolf and Salter, 2000). The pain-induced neuronal plasticity involves the sensitization of sensory neurons in different anatomical locations along of the PNS and CNS (Woolf, 1983; Latremoliere and Woolf, 2009).

NOCICEPTIVE SIGNALING PATHWAY AND PAIN HYPERSENSITIVITY

The peripheral primary sensory neurons from the pain-signaling pathway are activated by different noxious stimuli, including thermal, mechanical, or chemical stimuli that have potential or are currently damaging tissue. These specialized primary sensory cells are pseudo-unipolar neurons with the soma anatomically located in the dorsal root ganglia (DRG) and in the trigeminal ganglia (Kandel, 2013). The peripheral terminals of nociceptors are equipped with a range of receptors that transduce noxious stimuli into action potentials, which are then transmitted through the nervous systems. All primary sensory nociceptors, through

Abbreviations: 1-MT, 1-methyl-tryptophan, an IDO1 inhibitor; 3-HAA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; AA, anthranilic acid; AKR, aldoketo reductase; BH4, tetrahydrobiopterin; CAIA, collagen antibody-induced arthritis; CCI, chronic constriction injury; CFA, complete Freund's adjuvant; CNS, central nervous system; CR, carbonyl reductase; CTLA4, cytotoxic T lymphocyte antigen-4; DHFR, dihydrofolate reductase; DRG, dorsal root ganglia; FDA, Food and Drug Administration; *GCH1*, gene that codes for the human guanosine triphosphate cyclohydrolase I enzyme; *Gch1*, gene that codes for the murine guanosine triphosphate cyclohydrolase I; Glu, glutamate, GTP, guanosine triphosphate; GTPCH, guanosine triphosphate cyclohydrolase I; HIV, human immunodeficiency virus; IASP, International Association for the Study of Pain; IC_{50}, concentration at half-maximal inhibition; IDO1, indoleamine 2,3-dioxygenase 1; IFN-γ, interferon gamma; IL-1β, interleukin-1beta; IL-6, interleukin-6; JM6, a KMO inhibitor; KATs, kynurenine aminotransferases; KMO, kynurenine 3-monooxygenase; KYN, kynurenine; KYNA, kynurenic acid; KYNU, kynureninase; LPS, lipopolysaccharides; NAD^+, nicotinamide adenine dinucleotide; NMDA, N−methyl−d−aspartate; NMDAR, N-methyl-d-aspartate receptor; NSAIDs, non-steroidal anti-inflammatory drugs; PCD, pterin 4a-carbinolamine dehydratase; PNS, peripheral nervous system; PTPS, 6-pyruvoyl tetrahydropterin synthase; Q-1195, a SPR inhibitor; QM385, a SPR inhibitor; QUIN, quinolinic acid; Ro61-6048, a KMO inhibitor; SNI, spared nerve injury; SPR, sepiapterin reductase; *Spr*, gene that codes for the murine sepiapterin reductase; SPRi, sepiapterin reductase inhibitors; SPRi3, a SPR inhibitor; SSZ, sulfasalazine; TDO, tryptophan 2,3-dioxygenase; TNF-α, tumor necrosis factor alpha; Trp, tryptophan; XA, xanthurenic acid.

their central terminals, make synaptic connections with second-order neurons in the spinal cord (for a review see Woolf and Ma, 2007). Some subsets of spinal dorsal horn neurons project axons and transmit pain messages to higher brain centers, including the reticular formation, thalamus, amygdala, and finally the cerebral cortex. These brain regions are associated with autonomic, hormonal, emotional and cognitive aspects of pain, including the perception and consciousness of pain (Mobbs et al., 2009). The neural activity along the pain transmission pathway is inhibited or amplified by ascending and descending neural circuits (for a revision see Woolf, 2018). This modulation allows a wide range of factors to modulate pain sensation and perception, including psychosocial and environmental factors (for a review see Chayadi and Mcconnell, 2019).

NEUROINFLAMMATION IN CHRONIC PAIN

Growing evidence suggests that persistent inflammation within the PNS and CNS is a factor that drives self-perpetuation and pathologic plasticity changes in sensory neurons, sustaining the chronicity of pain (Kawasaki et al., 2008; Gao and Ji, 2010). Injuries and diseases that directly or indirectly affect the PNS and CNS can elicit neuroinflammatory responses that include activation of resident immune cells, changes in capillary permeability and infiltration of peripheral blood cells (for a review see Zhuang et al., 2005). It has been demonstrated that during inflammation peripheral leukocytes (including neutrophils, monocytes/macrophages and T cells) are able to infiltrate the PNS and CNS, leading to overproduction of a variety of pro-inflammatory cytokines, chemokines, and other pain-related mediators (Costigan et al., 2009b; Kigerl et al., 2009). The neuroinflammatory response is further amplified by the activation of resident glial cells, including microglia and astrocytes. Once activated, these cells undergo hypertrophic changes and increase the release of glial mediators, including numerous trophic factors, chemokines and proinflammatory cytokines that can modulate pain sensitivity (Rojewska et al., 2014b; **Figure 1**).

While acute neuroinflammation can produce transient peripheral and central sensitization, permanent or repeated neuroinflammation is associated with a long-lasting and even permanent sensitization (Christianson et al., 2011). The literature supports the association between neuroinflammation and various chronic pain conditions, such as neuropathic pain triggered by diabetes, nerve and spinal cord injury, inflammatory pain caused by arthritis, inflammatory bowel disease, cancer related pain, complex regional pain syndrome, and pain caused by drug therapy (Sweitzer et al., 2002; Chua et al., 2019). As an example, a study on *post mortem* spinal cord samples from human immunodeficiency virus (HIV)-infected patients with neuropathic pain showed increased glial activation and increased inflammatory cytokine levels (Shi et al., 2012).

The exact mechanisms by which neuroinflammation favors the transition from acute pain to persistent pain is still poorly defined. This lack of understanding of the basic mechanisms of pain perpetuation is reflected in the limited efficacy of anti-inflammatory drugs, in addition to the significant side effects (Enthoven et al., 2016). Therefore, new avenues need to be explored in order to manage this unmet clinical condition. In this paradigm, emerging mediators related to inflammation-enhanced metabolic pathways, *i.e.*, tetrahydrobiopterin (BH4) and quinolinic acid (QUIN; a KYN pathway metabolite) have been proposed to favor pain hypersensitivity (Latremoliere et al., 2015; Laumet et al., 2017). The innate immune system once activated elicits the synthesis of pro-inflammatory mediators in order to coordinate the inflammatory response. Many of these mediators can transcriptionally upregulate the expression of inducible enzymes, activating the pathological production of BH4 and QUIN (for a review see Guillemin, 2012; Ghisoni et al., 2015a; **Figure 1**).

THE BIOSYNTHESIS OF BH4

BH4 is traditionally known as an essential cofactor for the catalytic activity of phenylalanine hydroxylase, tyrosine-3-hydroxylase, tryptophan-5-hydroxylase, alkylglycerol monooxygenase, and all nitric oxide synthase isoforms (Thöny et al., 2000). As a consequence, BH4 is crucial for hydroxylation of the aromatic amino acids, resulting in the catabolism of phenylalanine and the synthesis of the catecholaminergic neurotransmitters dopamine and serotonin. BH4 is also mandatory for the cleavage of ether lipids as well as the biosynthesis of nitric oxide (Thöny et al., 2000; Werner et al., 2011). Recently, our group has uncovered other fundamental physiological roles for basal BH4 levels, as having antioxidant and anti-inflammatory properties, and being a mitochondrial activator as well as a memory enhancer (Ghisoni et al., 2015a,b, 2016; De Paula Martins et al., 2018; Latini et al., 2018). We also described that exacerbated production of BH4 is pathogenic, causing pain, increasing the aggressiveness of the immune system and the progression of the symptoms of chronic diseases, including, chronic pain, asthma, multiple sclerosis, ulcerative colitis, rheumatoid arthritis, and cognitive impairment (Latremoliere et al., 2015; Cronin et al., 2018; Fujita et al., 2019).

Physiological basal levels of BH4 are at tightly controlled concentrations, requiring therefore, a tuned regulation of BH4 synthesis. Three metabolic pathways, namely *de novo* synthesis, recycling, and salvage pathways cooperate to maintain appropriate intracellular levels of BH4 (**Figure 2**). The *de novo* pathway generates BH4 from guanosine triphosphate (GTP) through a three-step enzymatic cascade starting with the rate-limiting enzyme guanosine triphosphate cyclohydrolase I (GTPCH), followed by 6-pyruvoyl tetrahydropterin synthase (PTPS) and sepiapterin reductase (SPR) (for a review see Ghisoni et al., 2015a). Alternative to *de novo* synthesis, intracellular BH4 levels can be produced via the salvage pathway using sepiapterin and 7,8-dihydrobiopterin as intermediates. Although this pathway is not fully understood, SPR and dihydrofolate reductase (DHFR) appear to be essential enzymes to maintain BH4 levels without consuming high-energy phosphate containing compounds (Werner et al., 2011). In addition, the catalytic activity of SPR can also be performed by non-specific enzymes, the aldoketo and carbonyl reductases

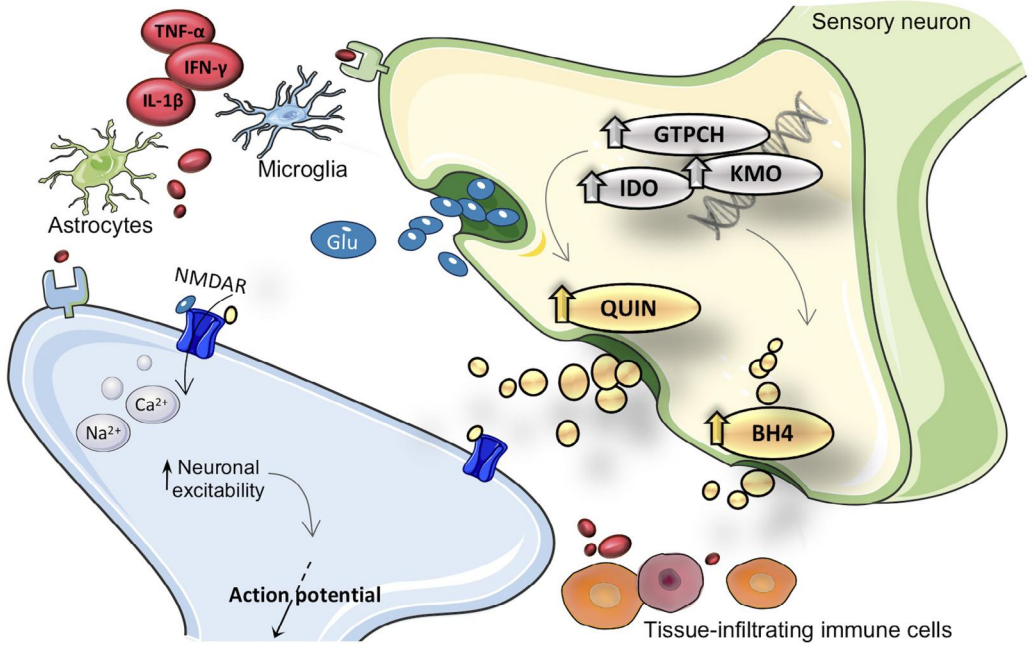

FIGURE 1 | Merger of kynurenine (KYN) and tetrahydrobiopterin (BH4) pathways into inflammation cascade involved in pain hypersensitivity. After inflammatory activation, expression and activity from GTPCH (guanosine triphosphate cyclohydrolase I), IDO1 (indoleamine 2,3-dioxygenase), and KMO (kynurenine 3-monooxygenase) enzymes are enhanced in brain and immune cells. Inflammatory inducible KYN/BH4 pathways upregulation is characterized by increased nociceptive compounds, such as QUIN (quinolinic acid) and BH4. As a consequence, neurons undergo profound changes in activity, which eventually result in exacerbated pain sensations. The neuroactive compound QUIN is NMDAR (N-methyl-d-aspartate receptor) agonist it is proposed to exacerbate hypersensitivity throughout modulation of this receptor. The exact molecular mechanism by which BH4 induces pronociceptive effects is not fully elucidated. TNF-α, tumor necrosis factor alpha; IL-1β, interleukin-1beta; IFN-γ, interferon gamma; Glu, glutamate.

(Hirakawa et al., 2009; Werner et al., 2011). Finally, the recycling pathway represents a mechanism that preserves energy and generates large quantities of pterin in high-BH4 demanding organs (e.g., hepatic metabolism of aromatic amino acids). After BH4 participates as a mandatory enzymatic cofactor, the unstable intermediate 4a-hydroxy-tetrahydrobiopterin is formed and undergoes a dehydration leading to the formation of quinonoid dihydrobiopterin, which is reduced back to BH4 by dihydropteridine reductase (Thöny et al., 2000; Longo, 2009).

Increased levels of BH4 are expected under cellular stress and requires the production of new blocks of BH4. GTPCH, the rate-limiting enzyme of the *de novo* BH4 pathway, is an inducible enzyme, having its expression controlled by pro-inflammatory mediators, such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-1beta (IL-1β), and lipopolysaccharides (LPS) (Werner et al., 1990). During the pro-inflammatory response, expression of *GCH1* (which codes for GTPCH), and GTPCH activity are markedly increased, while the downstream enzymes, PTPS and SPR, are only slightly augmented, resulting in PTPS as the rate-limiting enzyme of the BH4 *de novo* pathway during inflammation. Consequently, this pseudometabolic blockage favors the accumulation of the PTPS substrate, which non-enzymatically will be transformed in neopterin, a well-established and sensitive biomarker for immune system activation, and for the activation of the *de novo* BH4 pathway (for a review see Ghisoni et al., 2015a; **Figure 2**).

BH4 AND CHRONIC PAIN

The first human validation linking chronic pain to the BH4 metabolism comes from the identification of single nucleotide polymorphisms in the *GCH1* loci, which correlated with reduced experimental and clinical persistent pain sensitivity (Tegeder et al., 2006, 2008). Human homozygous haplotypes were associated with decreased upregulation of *GCH1* upon inflammatory stimulation, but without full loss of function of GTPCH, which would preserve baseline BH4 concentrations (Campbell et al., 2009; Doehring et al., 2009; Dabo et al., 2010; Kim et al., 2010, 2013). Indeed, it was demonstrated that this human protective-pain haplotype only influences nociceptive thresholds after pain sensitization (Tegeder et al., 2008). In addition, individuals carrying the homozygous from of the haplotype are less sensitive to persistent leg pain after discectomy or persistent pain in fibromyalgia (Tegeder et al., 2008; Kim et al., 2010, 2013). However, other studies demonstrated that carrying this human homozygous polymorphism does not protect from pancreatic pain, post dental surgery or post mastectomy pains, suggesting that the association between *GCH1* and pain may be disease- or tissue-specific (Kim and Dionne, 2007; Lazarev et al., 2008; Holliday et al., 2009; Hickey et al., 2011).

In rodent models, the genetic ablation of *Gch1* in DRGs generated evidence supporting the contribution of excessive

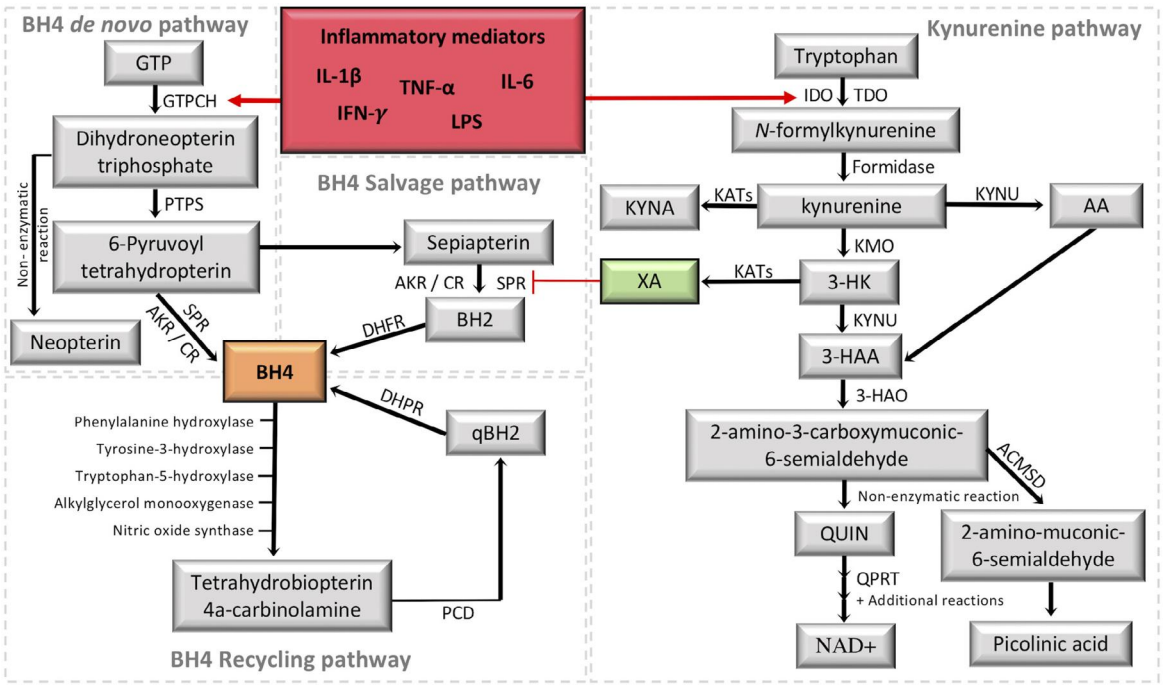

FIGURE 2 | Crosstalk between the tetrahydrobiopterin (BH4) and kynurenine (KYN) pathways. It is highlighted in red the activation of the two metabolic pathways under inflammation. It is highlighted in green the KYN intermediate xanthurenic acid (XA), which recently was demonstrated to be an inhibitor of sepiapterina reductase (SPR) (Haruki et al., 2016). The formation of BH4 (highlighted in orange) by the *de novo* pathway involves the catalytic activity of GTPCH (guanosine triphosphate cyclohydrolase I), PTPS (followed by 6-pyruvoyl tetrahydropterin synthase) and SPR (sepiapterin reductase). SPR deficiencies may be overcome in target tissues by unspecific reductases of the salvage pathway, including aldoketo and carbonyl reductases (AKR; CR) (Hirakawa et al., 2009; Werner et al., 2011), which transform 6-pyruvoyl-tetrahydropterin into sepiapterin and BH2 (7,8-dihydrobiopterin), then the final reduction back to BH4 is performed by DHFR (dihydrofolate reductase). The recycling pathway maintains high levels of BH4 in the liver, where it is mainly used to metabolize phenylalanine. After BH4 oxidation, PCD (Pterin 4a-carbinolamine dehydratase) forms qBH2 (quinonoid dihydrobiopterin) to be reduced back to BH4 by DHPR (dihydropteridine reductase). The KYN pathway transforms tryptophan into a series of intermediates with different properties by combining the activity of several enzymes in different cells/tissues. The central intermediate KYN allows the production of XA, which inhibits SPR activity in the BH4 pathway. GTP, guanosine triphosphate; IDO1, indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; KYNU, kynureninase; KYNA, kynurenic acid; KATs, kynurenine aminotransferases; KMO, kynurenine 3-monooxygenase; 3-HK, 3-hydroxykynurenine; AA, anthranilic acid; 3-HAA, 3-hydroxyanthranilic acid; 3-HAO, 3-hydroxyanthranilic acid 3,4-dioxygenase; QUIN, quinolinic acid; QPRT, quinolinate phosphoribosyltransferase; NAD^+, nicotinamide adenine dinucleotide; ACMSD, 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase; IFN-γ, interferon gamma, TNF-α, tumor necrosis factor alpha; IL-1β, interleukin 1beta; IL-6, interleukin-6; LPS, lipopolysaccharides.

levels of BH4 in chronic pain hypersensitivity (Kim et al., 2009; Latremoliere et al., 2015). DRG *Gch1* null rats showed decreased mechanical pain hypersensitivity and microglial activation in the dorsal horn 14 days following spared nerve injury (SNI) (Kim et al., 2009). Similarly, the absence of *Gch1* in the DRG of mice prevented excessive BH4 production in sensory neurons and mechanical pain hypersensitivity induced by nerve injury following 21 days of SNI and chronic constriction injury (CCI) as measured by the normalization of the mechanical threshold for pain (Latremoliere et al., 2015).

Expression and functional profiling in rodents has shown that enhanced BH4 biosynthetic enzymes transcription and activity in sensory neurons and immune cells lead to increased BH4 levels, which results in greater chronic pain hypersensitivity (Tegeder et al., 2006; Kim et al., 2009; Latremoliere et al., 2015; Cronin et al., 2018). For example, *Gch1* and *Spr* were upregulated up to sixfold 21 days after SNI, indicating that elevated transcription for BH4 biosynthesis persists for some time after injury (Tegeder et al., 2006; Kim et al., 2009; Latremoliere et al., 2015). In parallel with persistent mechanical hypersensitivity, BH4 levels are increased in rat sensory neurons in response to both axonal injury and peripheral inflammation induced by SNI and intraplantar complete Freund's adjuvant (CFA) injection, respectively (Tegeder et al., 2006). Furthermore, enhanced *GCH1* transcription and increased BH4 levels were identified not only in injured sensory neurons, but also in leukocytes that infiltrated the tissue after SNI in mice, which underline the contribution of the immune system in the persistence of hypersensitivity induced by BH4 overproduction (Latremoliere et al., 2015). Collectively, the enhanced expression of the transcripts for the BH4 biosynthetic enzymes and increased BH4 levels correlate with the persistence of mechanical and cold hypersensitivity in rat and mice models for inflammatory and neuropathic pain, reinforcing the contribution of excessive levels of BH4 with chronic pain hypersensitivity (Tegeder et al., 2006; Latremoliere et al., 2015; Fujita et al., 2019).

TABLE 1 | Half maximal inhibitory concentration (IC_{50}) for the known sepiapterin reductase inhibitors (SPRi).

Compounds	IC_{50} value (μM)		
	In vitro		*In vivo*
	Protein-based assay	**Cellular system**	
Natural SPRi			
N-acetyl-serotonin	3.8[h] 35[m] 1.2[r] (Haruki et al., 2016) 11.61[h] (Moore et al., 2019)	Ø	Ø
Xanthurenic acid	0.15[h] 0.053[m] 0.045[r] (Haruki et al., 2016)	Ø	Ø
Synthetic SPRi			
SPRi3	0.053[h] (Haruki et al., 2016)	0.45[a] Latremoliere et al., 2015	Ø
QM385	Ø	0.036[b] 0.074[c] Cronin et al., 2018	Ø
Q-1195	0.008[h] 0.004[r] Meyer et al., 2019	Ø	2.2[d] (Meyer et al., 2019)
FDA-approved			
Sulfasalazine	0.0070[h] 0.0078[m] 0.043[r] (Haruki et al., 2016) 0.023[h] (Haruki et al., 2013)	Ø	Ø
Tranilast	5.889[h] (Moore et al., 2019)	Ø	Ø

Values are mean of at least 2 independent experiments. Ø, not determined. h, human SPR; m, mouse SPR; r, rat SPR. a, mouse primary dorsal root ganglia cell culture. b, mouse splenocytes cell culture. c, human peripheral blood mononuclear cell culture. d, adult male Sprague-Dawley rats after spinal nerve injury. FDA, Food and Drug Administration.

Inhibition of Pathological BH4 Levels as a Novel Pathway to Induce Analgesia

One of the most effective current pharmacological therapies for controlling certain types of pain is the use of opioids. However, chronic opioid use lacks safety, effectiveness and has a substantial liability for abuse and high risk of death from overdose (NIDA, 2020). Therefore, the inhibition of inflammation-triggered BH4 production may represent an innovative and non-addictive strategy for managing persistent pain.

Analgesia induced by the pharmacological inhibition of GTPCH activity was demonstrated in rodents subjected to SNI and CCI. The use of 2,4-diamino-6-hydroxypyrimidine (DAHP; a GTPCH inhibitor) reversed the mechanical and cold hypersensitivity induced by the nerve injury (Tegeder et al., 2006). Similarly, DAHP treatment reduced tumor-evoked microglial activation in the spinal cord and reduced cancer-induced systemic hyperalgesia in mice (Pickert et al., 2012). These initial data indicated that targeting the flux of this metabolic pathway could represent new horizons in the clinical management of chronic pain. However, since GTPCH activity is essential for BH4 production, any pharmacological approaches should aim to reduce exacerbated BH4 levels back to basal levels, without compromising its physiological roles on endothelial function and metabolisms of neurotransmitters, lipids and nitric oxide.

A yeast three hybrid screen revealed that a Food and Drug Administration (FDA)-approved anti-inflammatory compound, sulfasalazine (SSZ), is an inhibitor of the BH4 synthesizing enzyme SPR (Haruki et al., 2013). The use of SSZ in mice subjected to SNI showed reduced mechanical pain hypersensitivity, without compromising essential BH4-related functions (Latremoliere et al., 2015). However, the analgesia induced was mild, probably due to the limited bioavailability, low potency, and complex metabolism of this drug in the gut (Pieniaszek and Bates, 1979). Thus, using a structure-based design, our group has developed new more potent SPR inhibitors (SPRi), SPRi3 and QM385. These SPRi have been shown to induce potent analgesic effects in neuropathic and inflammatory pain models, without inducing tolerance or adverse effects (Latremoliere et al., 2015; Fujita et al., 2019). Either a single or repeated SPR inhibitor intraperitoneal injection alleviated intraplantar CFA injection-, SNI-, and CCI-induced pain hypersensitivity in mice, with a maximal activity 1 h after the administration (Latremoliere et al., 2015). The analgesic effect of these SPRi were also demonstrated in the chronic phase of the collagen antibody-induced arthritis (CAIA) model of inflammatory joint pain, in which a rapid onset of clinical signs of arthritis is followed by a persistent pain-related hypersensitivity syndrome lasting at least 55 days (Fujita et al., 2019). We also demonstrated that SPRi reduced pain scores in mice submitted to a colitis experimental model, and that this effect was in part due to the absolute requirement of T cells for BH4 in order to expand and infiltrate tissues (Cronin et al., 2018). Another FDA-approved drug, Tranilast – an anti-allergic agent, was also demonstrated to inhibit SPR in protein- and cell-based assays (Moore et al., 2019) and to reduce pelvic pain caused by endometriosis in a clinical study (Honda et al., 2013).

Table 1 summarizes the half maximal inhibitory concentration (IC_{50}) values of key SPRi, including the natural SPR inhibitors (e.g., N-acetyl-serotonin), the synthetic and FDA-approved compounds.

Sepiapterin as a Biological Marker for the Analgesic Effects of SPRi

Sepiapterin is a metabolic intermediate of the BH4 salvage pathway. It does not accumulate intracellularly, but is found

at increased concentrations in the biological fluids of patients affected by mutations in the SPR gene (Carducci et al., 2015). The chemical stability of sepiapterin and its accumulation upon genetic and/or pharmacological manipulation of SPR make this metabolite a sensitive and specific biological marker of SPR inhibition (Fujita et al., 2019). Indeed, a dose-dependent increase of sepiapterin levels in plasma and urine from rodents, and humans were observed after the administration of SPRi (SSZ, SPRi3, QM385, and Q1195 in rodents and SSZ in humans (Latremoliere et al., 2015; Fujita et al., 2019; Meyer et al., 2019). In a recent publication by our group, the urinary sepiapterin was established as a reliable biomarker of SPR inhibition with high sensitivity (70–85%) and specificity (82–88%) in both mice and human health volunteers after the administration of SPRi3 and SSZ, respectively (Fujita et al., 2019).

In an effort to associate changes of sepiapterin and BH4 with analgesic effects induced by SPRi, **Table 2** shows the changes on sepiapterin and BH4 levels identified in biological samples after treatment with different SPRi and the correspondent analgesic effects in different experimental pain models.

THE KYN PATHWAY BIOSYNTHESIS

The KYN pathway is active in a variety of different tissues, but more notably in the liver through the enzyme tryptophan 2,3-dioxygenase (TDO); and in cells of the immune and nervous systems (including neurons, microglia and astrocytes) by indoleamine 2,3-dioxygenase 1 (IDO1) (Moroni et al., 1988a). IDO1 is the rate-limiting enzyme of the pathway in immune cells, playing key roles in immune system activation and regulation (Guillemin et al., 2005). The most potent activator of IDO1 is IFN-γ (Werner-Felmayer et al., 1989), but this enzyme is also activated by other mediators such as LPS, amyloid peptides, cytotoxic T lymphocyte antigen-4 (CTLA4) and HIV proteins (Guillemin et al., 2003; Jones et al., 2015). Around 95% of the dietary tryptophan (Trp) is metabolized into the KYN pathway, which can follow three different metabolic routes, synthesizing the essential cofactor nicotinamide adenine dinucleotide (NAD^+), kynurenic acid (KYNA), or xanthurenic acid (XA) (Beadle et al., 1947; Wolf, 1974; **Figure 2**).

Initially, Trp can be oxidized into the instable metabolite N-formyl-kynurenine by TDO or IDO1, to be further transformed into KYN, the central intermediate of the pathway, by formamidase. KYN can be metabolized into anthranilic acid or KYNA by kynureninase and kynurenine aminotransferases (KATs I, II, and III), respectively. Additionally, kynurenine 3-monooxygenase (KMO) can transform KYN into 3-hydroxykynurenine (3-HK) to produce 3-hydroxyanthranilic acid (3-HAA) by kynureninase. 3-HAA in turn, forms picolinic acid, QUIN and NAD^+ through the action of additional enzymes. 3-HK can be also metabolized by KATs into XA (**Figure 2**).

KYN PATHWAY AND CHRONIC PAIN

There is extensive evidence in literature that proinflammatory stimuli, mitochondrial dysfunction, oxidative stress, and the formation of neuroactive metabolites that can modulate glutamatergic receptors and neurotransmitter production are relevant to pain sensation (Moroni et al., 1988b; Turski et al., 1988). Several of the KYN pathway metabolites are neuroactive compounds able to regulate, for example, the activity of glutamatergic N−methyl−d−aspartate (NMDA) receptors inducing toxicity, favoring the excessive generation of reactive species (Stone and Perkins, 1981; Kessler et al., 1989), or compromising the activity of the energy metabolism by deficiencies in the synthesis of Trp-linked NAD^+ (Massudi et al., 2012; Gomes et al., 2013; Zhu et al., 2015). For example, QUIN is a potent NMDA receptor agonist, which at nM levels induces excitotoxicity, mitochondrial damage, oxidative stress, destabilization of the cellular cytoskeleton, and disruption of autophagy, among other negative effects (Schwarcz et al., 1984; Guillemin, 2012). Thus, perturbations on the KYN pathway may favor the transition from acute to persistent pain by inducing these deleterious reactions. In this scenario, it is well established that glutamatergic neurotransmission is essential for peripheral (Pardutz et al., 2012) and central sensitization (Latremoliere and Woolf, 2009). Thus, the pharmacological antagonism of NMDA receptors has been explored as a key therapeutic target in pain disorders (Nasstrom et al., 1992; Chapman and Dickenson, 1995; Bannister et al., 2017).

It has been also demonstrated that increased IDO1 activity is inversely related to serotonin concentrations in human plasma (Lood et al., 2015), which has a relevant role in the pain inhibitory descendant modulation (Mico et al., 1997; Sawynok et al., 2001). Sustained Trp catabolism throught KYN pathway during chronic inflammation can compromise the availability of this aromatic amino acid to form serotonin, and thus decrease the serotonin inhibitory descendant pain modulation (Capuron and Dantzer, 2003; Kim et al., 2012). In line with this, a persistent mechanical and thermal hyperalgesia has been shown to be associate with a decreased serotonin/Trp ratio, and an increased KYN/Trp ratio in the hippocampus of rats under chronic arthritis inflammatory pain model induced by a joint CFA injection (Kim et al., 2012).

Clinical and preclinical studies have also demonstrated that the excessive IDO1 activation contributes to inflammation-induced pain (Kim et al., 2012; Huang et al., 2016). IDO1 expression is increased in several inflammatory and pain conditions, for example in the lungs and lymphoid tissue of mice with mechanical pain hypersensitivity induced by an acute and a chronic viral infection, respectively (Huang et al., 2016). In contrast, virus-induced mechanical pain hypersensitivity was not evident in mice lacking IDO1 genes (Huang et al., 2016). In a separate study of chronic arthritis inflammatory pain model, the IDO1 gene expression, protein content and activity were elevated in the hippocampus of rats, resulting in persistent pain hypersensitivity after 21 days of the CFA injection in the tibiotarsal joint (Kim et al., 2012). Blockage of IDO1 with the inhibitor 1-methyl-tryptophan (1-MT) attenuated persistent mechanical and thermal hyperalgesia in rats with chronic arthritis inflammatory pain (Kim et al., 2012). In a clinical observational study, patients affected with chronic back pain showed elevated plasma IDO1 and increased KYN/Trp ratio as compared with healthy controls (Kim et al., 2012).

TABLE 2 | Analgesic effects, tetrahydrobiopterin (BH4) and sepiapterin changes for the main sepiapterin reductase inhibitors (SPRi).

SPRi	Analgesic effect	Experimental pain model	BH4 changes	Sepiapterin changes
N-acetyl-serotonin	⇓ Cold allodynia; ⇓ Mechanical allodynia (Tegeder et al., 2006)	Spared nerve injury neuropathic pain model	Ø	Ø
	⇓ Heat hyperalgesia (Tegeder et al., 2006)	Granulomatous skin inflammatory pain model (intra-plantar injection of CFA)	Ø	Ø
SPRi3	⇓ Heat hyperalgesia; ⇓ Mechanical allodynia (Fujita et al., 2019)	Collagen antibody-induced arthritis model	⇓ in urine	⇑ in urine
	⇓ Mechanical allodynia (Latremoliere et al., 2015)	Chronic constriction injury and spared nerve injury neuropathic pain models	⇓ in DRG, sciatic nerve and plasma	⇑ in DRG, sciatic nerve and plasma
	⇓ Heat hyperalgesia; No changes in mechanical allodynia (Latremoliere et al., 2015)	Granulomatous skin inflammatory pain model (intraplantar injection of CFA)	⇓ in plantar skin	Ø
QM385	⇓ Heat hyperalgesia (Fujita et al., 2019)	Collagen antibody-induced arthritis model	Ø	⇑ in plasma
Q-1195	No changes in mechanical allodynia (Meyer et al., 2019)	Spinal nerve ligation neuropathic pain model	⇓ in DRG	⇑ in plasma and DRG
Sulfasalazine	⇓ Mechanical allodynia (Latremoliere et al., 2015)	Spared nerve injury neuropathic pain model	Ø	⇑ in plasma

Ø not evaluated. CFA, complete Freund's adjuvant; DRG, dorsal root ganglia.

In murine pre-clinical models, alterations in the KYN pathway and immune system contributing to pain hypersensitivity were demonstrated. For example, in sensory neurons from the DRG and spinal cord, sustained IDO1 and KMO activation due to nerve injury were associated with mechanical and thermal hypersensitivity in rats after 21 the CCI in the sciatic nerve (Rojewska et al., 2016, 2018). Furthermore, in cell culture models we demonstrated that the overexpression of KMO, and subsequent increase in QUIN production is mainly enhanced in monocytic cells, including macrophages and microglia during inflammation (Guillemin et al., 2003). Indeed, the administration of the microglial inhibitor minocycline was able to reduce mechanical hypersensitivity in parallel with a reduction of KMO expression in sensory neurons from rat submitted to the chronic neuropathic pain model induced by the CCI (Rojewska et al., 2014a, 2016). Similarly, treatment with inhibitors for IDO1 (1-MT) or KMO (Ro61-6048 and JM6) attenuated the persistent mechanical and thermal hypersensitivity, along with reduced markers of peripheral inflammation in rat the model of chronic neuropathic pain induced by the sciatic nerve CCI (Rojewska et al., 2016, 2018). These evidence strengthens the correlation between upregulation of KYN pathway, dysregulation of immune system and the development of persistent pain hypersensitivity.

XA, the Link Between the BH4 Metabolism and the KYN Pathway in Pain

XA was identified as a potent inhibitor of SPR during a screening of a collection of natural compounds (Haruki et al., 2016). This implies that this KYN pathway metabolite has potential to limit the pathological overproduction of BH4 observed in experimental pain models, and therefore induce similar analgesic effects as those elicited by synthetic SPRi, SPRi3 and QM385.

A role for XA in neurotransmission and neuromodulation has been suggested based on (i) the capacity to cross the BBB and spread heterogeneously within different mouse brain regions (Gobaille et al., 2008); (ii) the inhibitory effect on the rat brain vesicular release of glutamate, reducing the glutamatergic transmission (Neale et al., 2013); and (iii) the activity as an agonist of metabotropic glutamate receptors type II, which have been implicated in the negative modulation of nociceptive transmission (Fazio et al., 2016). Thus, the better understanding of the metabolic interaction between these two pathways during inflammation may open new avenues to pharmacologically modulate chronic pain. Indeed, XA has been proposed as an antinociceptive compound, as intraperitoneal administration of XA increased the threshold for nociception in rats (Heyliger et al., 1998) and lower levels of plasma XA were observed in patients affected by episodic and chronic cluster headache in a clinical observational study (Curto et al., 2016). However, to the best of our knowledge, XA-induced decreased SPR activity in a cellular system or a correlation between analgesic effects and SPR inhibition have not been explored yet.

CONCLUSION

Advances in the understanding of the mechanisms behind the development of chronic pain have identified a critical interaction between the immune system and the nervous system. These two systems synergistically promote local and systemic responses that restore homeostasis after tissue injury and/or infection. However, this bidirectional communication is also involved in maladaptive feedforward inflammatory loops at multiple levels of the neuroaxis contributing to the development of chronic pain. Neuro-immune interactions are able to control the metabolic flux of various metabolic pathways, including the BH4 and KYN pathways. Both are rapidly activated by inflammation, resulting in the production of several biologically active metabolites, which have been involved in pain states. Recently, XA, a KYN pathway intermediate, was identified as an endogenous inhibitor of the BH4 metabolism. This raises the possibility that XA can potentially modulate the pathological

overproduction of BH4 reported in chronic pain hypersensitivity experimental models. The modulation of the KYN pathway can be directed toward the production of XA (through the kynureninase inhibition e.g.), resulting in alterations of BH4. Therefore, understanding the interaction between these two pathways during inflammation is likely to open new avenues to pharmacologically modulate chronic pain.

Altogether, strategies aiming to manipulate the production of bioactive metabolites from the BH4 and KYN pathways, especially in sensory neurons, immune and glia cells might represent promising new analgesic approaches to reduce the hypersensitivity triggered by chronic inflammation. However, given the major central functions of the metabolites produced through the BH4 and KYN pathways in physiological conditions, potential undesirable side effects could be triggered by therapeutic approaches involving the manipulation of both pathways. The relationship between BH4 and KYN pathways, and especially its possible relevance for inflammation-induced pain hypersensitivity, should be critically assessed, and pre-clinical experiments exploring the complex interconnection between both pathways and the production of clinical evidence are encouraged.

AUTHOR CONTRIBUTIONS

AS designed the concept and drafted the manuscript. AL, GG, VT, and BH reviewed and edited the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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CHAPTER 3

FINDINGS - INVESTIGATION OF KYNURENINE AND TETRAHYDROBIOPTERIN PATHWAYS IN COHORTS OF PATIENTS WITH CHRONIC PAIN

Three manuscripts are part of this chapter

(P1) Frontiers in Neuroscience:
Kynurenine, tetrahydrobiopterin and cytokine inflammatory biomarkers in individuals affected by diabetic neuropathic pain.
Staats Pires, A. C.; Heng, B.; Tan, V. X.; Latini, A.; Russo, M. A.; Santarelli, D. M.; Bailey, D.; Wynne, K.; O'brien, J. A.; Guillemin, G.; Austin, P. (Accepted 21 Aug 2020).

(P2) Journal of Neuroimmunology:
Novel immune biomarkers in Complex Regional Pain Syndrome.
Russo, M.; Georgius, P.; **Staats Pires A.;** Heng, B. R.; Allwright, M.; Guennewig, B.; Santarelli, D.; Bailey, D.; Fiore, N.; Tan, V.; Latini, A.; Guillemin, G.; Austin, P (Accepted 16 Jul 2020).

(P3) Gastroenterology:
Tetrahydrobiopterin as a key factor promoting colitis abdominal pain.
Staats Pires, A. C.; Turnes, B. L.; Scheffer, D. L.; Menegassi, V. S.; Rizzatti, S. M.; Niero, L.; Guillemin, G.; Latini, A. (Final revisions).

3 FINDINGS - INVESTIGATION OF KYNURENINE AND TETRAHYDROBIOPTERIN PATHWAYS IN COHORTS OF PATIENTS WITH CHRONIC PAIN

Chronic pain is a prevalent condition that negatively affects the economy and quality of life of the affected individuals. There is no objective assessment available for diagnosis, prognosis, or monitoring of the disease, and no effective or safe treatments. Although several mechanisms are proposed as part of chronic pain pathophysiology, few treatment options have been translated through to the clinic. The following studies will examine the potential of metabolites from the KYN and BH4 pathways as biomarkers of chronic pain.

The candidate, with colleagues, used biochemical and analytical chemistry techniques for analysis of inflammatory markers and KYN and BH4 pathways metabolites profile in blood and urine samples from patients with chronic pain. Whilst clinical parameters such as pain intensity, emotional functioning, and pain-related psychosocial features were assessed in the same cohorts. The results arising from these analyses reveal an abnormal profile of KYN and BH4 pathways metabolites and inflammatory markers in chronic pain patients, suggesting an exacerbation of these metabolic pathways in this disease. Furthermore, inflammatory markers and metabolites of the KYN and BH4 were shown to be associated with clinical features of chronic pain, including pain intensity, emotional functioning and pain-related psychosocial features. This has important ramifications for clarification of molecular mechanisms underlying chronic pain conditions, which can be objectively assessed to support diagnosis and treatment. Future studies are needed to explore in detail the specific participation of the KYN and BH4 pathways metabolites in neuronal abnormalities that underlying pain hypersensitivity in chronic pain.

This section details the core findings and publications arising or resulting from the work done as part of this research. Before each manuscript, a brief background identifying the research gap is addressed, followed by the presentation of key results and implications of these findings. Articles will be noted if they have been published or are under revision for submission.

ORIGINAL RESEARCH ARTICLE: KYNURENINE, TETRAHYDROBIOPTERIN AND CYTOKINE INFLAMMATORY BIOMARKERS IN INDIVIDUALS AFFECTED BY DIABETIC NEUROPATHIC PAIN

(P1) Frontiers in Neuroscience:
Kynurenine, tetrahydrobiopterin and cytokine inflammatory biomarkers in individuals affected by diabetic neuropathic pain.
Staats Pires, A. C.; Heng, B.; Tan, V. X.; Latini, A.; Russo, M. A.; Santarelli, D. M.; Bailey, D.; Wynne, K.; O'brien, J. A.; Guillemin, G.; Austin, P. (Accepted 21 Aug 2020).

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ASP contributed to the acquisition of data, data curation, writing the original draft and revising the article. BH contributed to the acquisition of data, data curation, and revising the article. VT contributed to revising the article. AL contributed to revising the article and provided intellectual and technical support for tetrahydrobiopterin pathway analysis. MR contributed to the conception of the study, participant recruitment, and funding acquisition. DS contributed to participant recruitment, clinical database management, and revising the article. DB contributed to participant recruitment, sample collection, and clinical database management. KW contributed to participant recruitment and revising the article. JO'B contributed to sample preparation and revising the article. GG contributed to funding acquisition and revising the article. PA contributed to the conception of the study, project supervisor, funding acquisition, data curation, writing the original draft, and revising the article.

Research Gap

Diabetes is a prevalent chronic problem worldwide (Saeedi et al., 2019) and one of the leading causes of neuropathy (Boulton et al., 2005). Painful neuropathy is one of the main clinical consequences of diabetic neuropathy - a symptom that affects 25 % to 60 % of this population (Abbott, Malik, van Ross, Kulkarni, & Boulton, 2011; Tavakoli & Malik, 2008). Diabetic neuropathic pain (DNP) is a debilitating condition that can substantially affect the quality of life of patients, impacting the ability to perform daily activities, participate in labour and social activities, and is associated with high rates of depression (Gore et al., 2005).

Management of DNP can be challenging, as such pain is often unresponsive, or only partially responsive, to existing pharmacological approaches (Jose, Bhansali, Hota, & Pandhi, 2007). Thus, a demand exists to develop a greater understanding of the underlying molecular mechanisms responsible for this debilitating condition to advance the management of DNP.

Advances in the elucidation of the mechanisms eliciting neuropathic pain have identified a critical role for an abnormal activation of the tetrahydrobiopterin (BH4) and the kynurenine (KYN) pathways in preclinical models of neuropathic pain (Latremoliere et al., 2015; Rojewska, Ciapala, Piotrowska, Makuch, & Mika, 2018). Therefore, the measurement of BH4- and KYN-related metabolites in biological fluids from patients with DNP may offer relevant information about the molecular mechanisms that might underlie this chronic pain condition.

Key Results & Implications

We present the first comprehensive analysis of cytokines and KYN/ BH4 metabolites levels in serum from individuals affected by DNP. 1) We demonstrate increased levels of the pro-inflammatory cytokines, GM-CSF and IL-8, in serum from DNP patients. 2) The serum levels of pro-inflammatory cytokines positively correlated with metabolites of the KYN and BH4 pathways, suggesting that immune mediators drive activation of these pathways. 3) The KYN/Trp ratio and neopterin levels were increased in DNP, with the former correlating with pain intensity. These findings suggest that inflammatory activation and the upregulation of KYN and BH4 pathways may participate in the pathophysiology of neuropathic pain emergence as a complication of diabetes. These findings are crucial as *i*) we demonstrate for the first time, that the activation of the KYN and BH4 pathways may be involved in the pathophysiology that leads to the appearance of neuropathic pain in type 1 diabetes mellitus; *ii*) the measurement of the metabolites from the KYN and BH4 pathways in the serum may be used as indicators of the participation of the mentioned pathways as pathophysiological mechanisms of this chronic pain condition. These results are important to advance the elucidation of the mechanisms responsible for the

development of neuropathic pain in type 1 diabetes mellitus, as well as to provide possible biomarkers that can support the diagnosis and guide the treatment of DNP.

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ORIGINAL RESEARCH
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Kynurenine, Tetrahydrobiopterin, and Cytokine Inflammatory Biomarkers in Individuals Affected by Diabetic Neuropathic Pain

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Neuropathic pain is a common complication of diabetes with high morbidity and poor treatment outcomes. Accumulating evidence suggests the immune system is involved in the development of diabetic neuropathy, whilst neuro-immune interactions involving the kynurenine (KYN) and tetrahydrobiopterin (BH4) pathways have been linked to neuropathic pain pre-clinically and in several chronic pain conditions. Here, using a multiplex assay, we quantified serum levels of 14 cytokines in 21 participants with type 1 diabetes mellitus, 13 of which were classified as having neuropathic pain. In addition, using high performance liquid chromatography and gas chromatography-mass spectrometry, all major KYN and BH4 pathway metabolites were quantified in serum from the same cohort. Our results show increases in GM-CSF and IL-8, suggesting immune cell involvement. We demonstrated increases in two inflammatory biomarkers: neopterin and the KYN/TRP ratio, a marker of indoleamine 2,3-dioxygenase activity. Moreover, the KYN/TRP ratio positively correlated with pain intensity. Total kynurenine aminotransferase activity was also higher in the diabetic neuropathic pain group, indicating there may be increased production of the KYN metabolite, xanthurenic acid. Overall, this study supports the idea that inflammatory activation of the KYN and BH4 pathways occurs due to elevated inflammatory cytokines, which might be involved in the pathogenesis of neuropathic pain in type 1 diabetes mellitus. Further studies should be carried out to investigate the role of KYN and BH4 pathways, which could strengthen the case for therapeutically targeting them in neuropathic pain conditions.

Keywords: neuropathic pain, kynurenine, type 1 diabetes, tetrahydrobiopterin, pro-inflammatory cytokines

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INTRODUCTION

Peripheral diabetic neuropathy is a disorder of the peripheral nervous system that preferentially targets sensory axons, autonomic axons and later, to a lesser extent, motor axons (for a review see Feldman et al., 2019). Diabetic neuropathy is responsible for the greatest morbidity in terms of depression, anxiety, loss of sleep and non-compliance with treatment in diabetic patients (Holzer, 1998; Jensen et al., 2007). A sizable proportion of patients with peripheral diabetic neuropathy (25–60%) also develop neuropathic pain (diabetic neuropathic pain; DNP) (Tavakoli and Malik, 2008; Abbott et al., 2011; van Hecke et al., 2014), which is defined as "pain caused by a lesion or disease affecting the somatosensory system" (IASP, 2017). Management of DNP can be challenging for both the clinician and the patient, with pain being unresponsive, or only partially responsive, to existing pharmacological approaches (Jose et al., 2007). Thus, a pressing need exists to develop a greater understanding of the underlying molecular mechanisms responsible for the characteristic intractable chronic pain associated with diabetic neuropathy in order to develop more effective therapies.

Over the last two decades, advances in the understanding of the mechanisms eliciting chronic neuropathic pain have identified a critical interaction between the immune system and the nervous system (Austin and Moalem-Taylor, 2010). The two systems are tightly integrated, cooperating in local and systemic reflexes that restore homeostasis in response to tissue injury and infection. They further share a broad common language of cytokines, growth factors, and neuropeptides that enables bidirectional communication. However, this reciprocal crosstalk permits amplification of maladaptive feedforward inflammatory loops at multiple levels of the neuraxis, contributing to the development of both sensory and emotional aspects of chronic pain (for detailed reviews see Grace et al., 2014; Talbot et al., 2016; Austin and Fiore, 2019). A number of recent studies have identified elevated immune markers (C-reactive protein, tumor necrosis factor alpha (TNF-α), interleukin (IL) 6 (IL-6), toll-like receptor (TLR) 4, transforming growth factor beta 1 (TGF β 1) and the presence of antinuclear and anti-ganglioside auto-antibodies in diabetic neuropathy (Hussain et al., 2013, 2016; Janahi et al., 2015; Zhu et al., 2015; Ge et al., 2016), however these studies do not distinguish between painless and painful neuropathy.

The kynurenine (KYN) and tetrahydrobiopterin (BH4) pathways are critical regulators of neuro-immune crosstalk (Staats Pires et al., 2020). Inflammation rapidly activates both pathways, producing several neuroactive metabolites (**Figure 1**), and there is emerging evidence that KYN pathway (KP) activation contributes to the pathogenesis of chronic pain and co-morbid depression through resultant neuroinflammation and neurotoxicity (Kim et al., 2012; Walker et al., 2014; Zhou et al., 2015; Rojewska et al., 2016, 2018; Laumet et al., 2017). Inflammatory cytokines, such as interferon gamma (IFN-γ) and TNF-α, are activators of indoleamine 2,3-dioxygenase (IDO1), a major biosynthetic enzyme in the conversion of tryptophan (TRP) to KYN. KYN is metabolized by kynurenine 3-monooxygenase (KMO) into 3-hydroxy-kynurenine (3-HK). 3-HK is converted to the neurotoxic metabolite quinolinic acid (QUIN) in both macrophages and microglia by the enzymes kynureninase (KYNU) and 3-hydroxyanthralinic acid dioxygenase (HAO) (Guillemin, 2012). A recent study in over 17000 chronic pain patients identified QUIN as the most commonly elevated biomarker, with the authors suggesting a role in enhanced nociception through peripheral NMDA-receptor activation (Gunn et al., 2020). Moreover, the inhibition of IDO and KMO reduces allodynia and depressive-like behavior in rodent models of neuropathic pain (Rojewska et al., 2016, 2018; Laumet et al., 2017).

GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme of BH4 biosynthesis, is also activated by IFN-γ and TNF-α (Werner et al., 1990, 1993; Connor et al., 2008; Dias et al., 2016). BH4 has recently been identified as a key mediator of chronic pain. Elevated levels have been found in axotomized sensory neurons and macrophages infiltrating injured nerves, whilst inhibiting its production reduces pain sensitivity (Latremoliere et al., 2015; Fujita et al., 2019). BH4 acts as a mandatory cofactor for the production of catecholamines and nitric oxide (for a review see Ghisoni et al., 2015) and it is essential for the effective activation and proliferation of mature T cells (Cronin et al., 2018). Neopterin (NEO) is also downstream of GTPCH, however whilst production of BH4 relies on further enzymatic conversion from 6-pyruvoyl-tetrahydropterin synthase (PTPS) and others, NEO is produced by a non-enzymatic reaction. Consequently NEO is considered a more sensitive biomarker of immune activation than BH4 (Werner et al., 1990, 1993; Ghisoni et al., 2015).

Therefore, measurement of BH4, NEO and KP metabolites in biological fluids may offer relevant information about the progression and pathogenesis of DNP. Here, we present a comprehensive analysis of KP metabolites, as well as BH4 and NEO, in serum from diabetic individuals with chronic neuropathic pain (DNP) compared to diabetic controls with no pain (DC). Since pro-inflammatory cytokines activate KYN and BH4 pathways, we also analyzed a panel of 14 cytokines in the serum of these individuals.

MATERIALS AND METHODS

Participants

Participants were recruited by Genesis Research Services (Broadmeadow, NSW, Australia) between April 2018 and Sept 2019. The study included both male and female participants aged 40–71 years old, with definite clinical diagnosis of type 1 diabetes mellitus (n = 21). Thirteen of these participants were in the diabetic neuropathic pain group (DNP), as they had a definitive clinical diagnosis of painful diabetic peripheral neuropathy, whilst the remaining eight participants were classified in the diabetes control group (DC). Classification of the diabetes patients in to the DNP (n = 13) or DC (n = 8) groups was further confirmed using the Douleur Neuropathique 4 (DN4) questionnaire (Bouhassira et al., 2005), based on the absence (score $<$ 4) or presence (score $\geq$ 4) of neuropathic pain. Exclusion criteria included age less than 18 years; chronic neuropathic pain lasting less than 3 months;

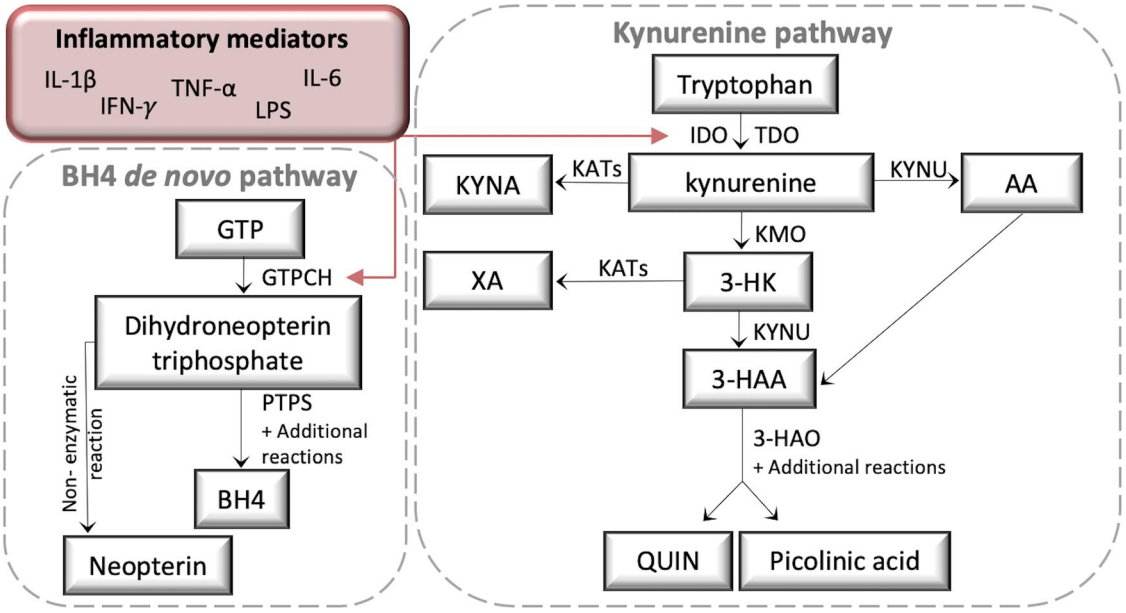

FIGURE 1 | A schematic diagram of the kynurenine (KYN) and tetrahydrobiopterin (BH4) pathways. Both KYN and BH4 pathways are activated by inflammatory cytokines such as IL-1β, Il-6, IFN-γ, TNF-α, and the immune activator LPS. LPS, lipopolysaccharide; GTP, Guanosine-5'-triphosphate; GTPCH, GTP cyclohydrolase I; PTPS, 6-pyruvoyl-tetrahydropterin synthase; IDO, Indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; KATs, Kynurenine aminotransferases; KYNA, Kynurenine acid; XA, Xanthurenic acid; KYNU, Kynureninase; AA, Anthranilic acid; KMO, Kynurenine 3-monooxygenase; 3-HK, 3-Hydroxykynurenine; 3-HAA, 3-Hydroxyanthranilic acid; 3-HAO, 3-hydroxyanthranilate 3,4-dioxygenase; QUIN, Quinolinic acid.

presence of acute pain, non-neuropathic pain, or mixed pain; any neurological, psychiatric or pain condition that could confound the study endpoints; and pregnancy. For the DC group, further exclusion criteria included any evidence of peripheral neuropathy (i.e., numbness, non-painful paresthesias or tingling, non-painful sensory distortions or misinterpretations), or any other microvascular complications of diabetes (i.e., retinopathy, nephropathy). All participants were taking medication to manage their diabetes. On the day of blood collection, participants had ceased taking immune-modulating medications (e.g., NSAIDs, steroids or opioids) for at least 7 days, which was the longest wash-out clinically practicable.

Ethics Approval and Consent to Participate

This study was approved by the Human Ethics Committee from University of Sydney (HREC #2017/019) and Macquarie University (HREC #5201600401). Authorization for access to participants who fit the inclusion criteria was also granted by the Hunter New England Local Health District. Participation in the study was on a completely voluntary basis, and all participants signed informed consent. All demographic information and blood samples were de-identified from the study team.

Pain and Psychological Profiling

All participants rated pain intensity from 0 to 100 on a visual analog scale (VAS) and completed 5 questionnaires to assess pain and psychological variables: Short-form McGill Pain Questionnaire (SF-MPQ-2) (Dworkin et al., 2009), Short-form Depression, Anxiety and Stress Scale (DASS21) (Lovibond and Lovibond, 1995), Pain Self-Efficacy Questionnaire (PSEQ) (Nicholas, 2007), Tampa Scale for Kinesiophobia (TSK) (Roelofs et al., 2011) and Pain Catastrophizing Scale (PCS) (Sullivan et al., 1995).

Blood Collection Protocol

A non-fasted blood sample was taken by venepuncture from the antecubital fossa by a trained phlebotomist into a 5 mL tube. Samples were then incubated at room temperature for 15 min to allow the blot to clot, then centrifuged at 2,000 × *g* for 10 min. The supernatant was removed and stored in polypropylene aliquot tubes at −80°C until analysis.

Sample Preparation

Serum samples were deproteinised by adding 1 vol. of 10% trichloroacetic acid containing 6.5 mM dithioerythritol. Afterward, samples were centrifuged at 12,000 × *g* at 4°C for 15 min and filtered through a 0.20 μm PTFE syringe filter (Merck-Millipore, CA, United States). Supernatants were transferred to high performance liquid chromatography (HPLC) vials for analysis.

Metabolites Quantification by HPLC

Serum levels of xanthurenic acid (XA), TRP, KYN, 3-HK, 3-hydroxyanthranilic acid (3-HAA) and anthranilic acid (AA) were determined by HPLC and quantified using a sequential diode-array UV and fluorescence detection as previously described (Guillemin et al., 2007). The HPLC analysis was carried out in

an uHPLC system (Agilent 1290 Infinity, CA, United States) by using an Agilent ZORBAX Rapid Resolution High Definition C18, reversed phase column (2.1 × 150 mm, 1.8 μm, Agilent Technologies, CA, United States). The temperature of the column compartment was set at 38°C. Injection volume was 20 μL and the autosampler tray temperature was set at 4°C to prevent sample degradation. The flow rate was set at 0.75 mL/min with an isocratic elution of 100% of 100 mM sodium acetate, pH 4.65. The identification and quantification of XA, KYN and 3-HK were performed by a UV detector (G4212A, Agilent, CA, United States) with absorbance at 250 nm and a reference signal at 350 nm for XA; and with absorbance at 365 nm and reference signal 'off' for KYN and 3-HK. The identification and quantification of TRP, 3-HAA and AA were performed by a fluorescence detector (G1321B xenon flash lamp, Agilent, CA, United States) with an emission wavelength of 438 nm and an excitation wavelength of 280 nm for TRP and 320 nm for 3-HAA and AA. The results were calculated by interpolation using a 6-point calibration curve and expressed as μmol/L or nmol/L.

NEO concentrations in serum samples were determined as previously described with some modifications (de Paula Martins et al., 2018). The same HPLC system and column described previously were used with a mobile phase of 100% of 15 mM potassium phosphate buffer, pH 6.4. The flow rate was set at 0.7 mL/min with an isocratic elution. The identification of NEO was performed by a fluorescence detector (G1321B xenon flash lamp, Agilent, CA, United States) with an emission wavelength of 438 nm and an excitation wavelength of 355 nm. The results were calculated by interpolation using 6-point calibration curve. Levels of NEO were calculated and expressed as nmol/L.

Kynurenic acid (KYNA) concentrations in serum samples were determined by HPLC (Agilent 1260 Infinity, Agilent, CA, United States) and an Agilent ZORBAX Rapid Resolution High Definition C18, reversed phase (4.6 × 100 mm, 3.5 μm, Agilent Technologies, CA, United States). Mobile phase consisted of 95% of 50 mM sodium acetate and 50 mM zinc acetate, pH 5.2 and 5% HPLC grade acetonitrile. The flow rate was set at 1.00 mL/min with an isocratic elution. The identification of KYNA was performed by a fluorescence detector (G1321B xenon flash lamp, Agilent, CA, United States) with emission wavelength of 388 nm and an excitation wavelength of 344 nm. The results were calculated by interpolation using a 6-point calibration curve. Levels of KYNA were calculated and expressed as nmol/L.

Metabolites Quantification by Gas Chromatography/Mass Spectrometry

QUIN and picolinic acid (PIC) concentrations in serum samples were determined using an Agilent 7890 gas chromatograph coupled with an Agilent 5975 mass spectrometer following a protocol previously described (Guillemin et al., 2007). Briefly, deproteinised-serum samples and deuterated internal standards were dried under vacuum and derivatized with trifluoroacetic anhydride and 1,1,1,3,3,3-hexafluoroisopropanol for an hour at 60 °C. Fluorinated esters were then extracted into toluene and washed with 5% sodium bicarbonate. The upper organic layer was collected and washed with 1 mL MilliQ water, and dried using sodium sulfate packed pipette tips. Samples were then injected under a splitless mode onto a HP-5MS GC capillary column (Agilent, CA, United States) and the analysis was carried out with the MS operating in negative chemical ionization mode. Selected ions (m/z 273 for PIC, m/z 277 for 4-PIC, m/z 467 for QUIN and m/z 470 for d3-QUIN) were simultaneously monitored. GC oven settings were as follows: oven temperature was held at 75°C for 3 min and then ramped to 290°C at a rate of 25°C/min and held at 290°C for 4 min for a total run time of 15.6 min. Quantification was achieved through normalization with respect to the internal standards and interpolation using 6-point calibration curves for each metabolite. Levels were calculated and expressed as nmol/L.

BH4 Quantification by ELISA

BH4 concentrations in serum samples were assessed by ELISA, using a commercial kit (Novus Biologicals, Colorado, United States), and following the manufacturer's instructions. The levels of BH4 were estimated by interpolation from a standard curve by colorimetric measurements at 450 nm on a plate reader (PHERAstar® *FSX*, Offenburg, Germany). Results were calculated as nmol of BH4 per liter (nmol/L).

Cytokine Analysis

Aliquoted serum samples were thawed and filtered 0.22μm before being analyzed in duplicate by Eve Technologies (Calgary, AB, Canada) using a Milliplex human high sensitivity T-cell discovery array 14-plex assay kit (Millipore). Sample concentrations of the 14 analytes were determined using a 7-point standard curve using the manufacturer's software.

Statistical Analysis

Fisher's exact test was used to compare the proportion of male and female participants between the groups (Prism 6, GraphPad Software Inc.). All other data were first analyzed for the presence of multiple outliers using ROUT test and all the outliers were excluded. Subsequently, the data were analyzed for normality using the D'Agostino-Pearson normality test. Unpaired Student's *T*-test (for normally distributed data), or a Mann–Whitney *U*-test (for non-normally distributed data) were used to test for statistically significant differences between the groups. Unpaired and two-tailed tests were used, and $P < 0.05$ was considered significant for these group comparisons. The relationship of pain and psychological variables, and cytokine levels to KYN- and BH4- metabolites was assessed using linear regression analyses, with the family-wise error rate corrected using the Benjamini-Hochberg procedure, at a false discovery rate of $q < 0.1$. This is the most appropriate and stringent methodology to correct for multiple comparisons and has been used by similar studies (Luchting et al., 2015; Russo et al., 2019). Only relationships that were significant following this correction are reported.

RESULTS

The demographics, clinical and psychological measures in DNP and DC participants are shown in **Table 1**. Among patients who had type 1 diabetes mellitus, 61.9% ($n = 13$) had DN4 scores ≥ 4

TABLE 1 | Demographics, clinical and psychological measures in type 1 diabetic neuropathic pain and type 1 diabetic control groups.

		DC (*n* = 8)	DNP (*n* = 13)
Sex (F/M)		4/4	5/8
Age (years)		54.1 (43 – 70)	60.4 (40 – 71)
Painful peripheral neuropathy onset (years ago)		n/a	9.08 ± 9.6
Lower limbs affected (L/R)		n/a	13/13
Upper limbs affected (L/R)		n/a	3/2
DN4 Score (0–10)		0	5.69 ± 1.44
BMI (kg m^{-2})		30 (29 – 37)	34.9 (25.5 – 50.1)
Pain score (VAS 0–100)		3.8 ± 3.7	55.8 ± 24.4***
SF-MPQ-2 (0–220)		1.5 ± 2	95.9 ± 45.8**
DASS 21 (0–42)	Depression	2.5 ± 3.3	13.2 ± 10.6**
	Anxiety	2.2 ± 3.7	12 ± 8.7**
	Stress	7.2 ± 9.2	14.6 ± 10.2
TSK (0–68)		29.6 ± 3.8	44.3 ± 6.3***
PCS (0–52)		1.8 ± 2.8	29.4 ± 15.1***
PSEQ (60–0)		57.2 ± 4.4	39.5 ± 13.7***

*DN4, Douleur Neuropathique 4 questionnaire; VAS, Visual analog scale; SF-MPQ-2, Short-form McGill Pain Questionnaire; DASS21, Short-form Depression, Anxiety and Stress Scale; TSK, Tampa Scale for Kinesiophobia; PCS, Pain Catastrophizing Scale; PSEQ, Pain Self-Efficacy Questionnaire. Data are presented as mean ± standard deviation (X ± SD). **P < 0.01, ***P < 0.001, unpaired two-tailed Mann–Whitney U-test.*

TABLE 2 | Cytokine protein levels in the serum of diabetic neuropathic pain and diabetic control groups.

Cytokine (pg/mL)	DC (X ± SD; *n* = 8)	DNP (X ± SD; *n* = 13)
GM-CSF	21.1 ± 11.6	72.6 ± 60.9*
IFN-γ	10.6 ± 6.8	14.6 ± 10
TNF-α	7.4 ± 3.4	8.1 ± 4.8
IL-1b	1.6 ± 0.6	2.5 ± 1.5
IL-2	3.8 ± 1.9	7.1 ± 4.4
IL-4	7.3 ± 7.7	6.6 ± 4.3
IL-5	1.5 ± 0.9	1.7 ± 0.7
IL-6	1.6 ± 0.9	1.8 ± 0.7
IL-8	6.1 ± 1.6	9.1 ± 1.4**
IL-10	5.4 ± 3.0	4.9 ± 3.3
IL-12	3.4 ± 1.8	4.1 ± 2.4
IL-13	4.9 ± 2.9	3.8 ± 1.9
IL-17A	13.5 ± 8.1	18.3 ± 11.6
IL-23	323.4 ± 230.9	474.2 ± 358.0

**P < 0.05, **P < 0.01, unpaired two-tailed Mann–Whitney U-test.*

and presented with persistent pain for at least 3 months, meeting the criteria for chronic DNP (Bouhassira et al., 2005). This matched the clinical diagnosis of diabetic painful peripheral neuropathy in this group. The average time since pain onset in the DNP group was 9.08 years. All 13 DNP patients had pain in both lower limbs, 3 had pain in the left upper limb, and 2 had pain in the right upper limb. All of the DC group reported no neuropathic pain symptoms, scoring 0 on DN4, nor did they have any sensory deficits in the limbs or have any microvascular complications of diabetes. Therefore, we confirmed that there was no evidence of peripheral neuropathy (painful or otherwise) in the diabetic control group, but clear evidence of painful peripheral neuropathy in the DNP group.

The proportion of females in the DNP group was greater than in the DC group, but this was not statistically significant ($P = 0.67$). Severe pain was confirmed in the DNP participants using the VAS pain scale ($U = 0$, $P < 0.001$) and the SF-MPQ-2 questionnaire ($U = 0$, $P < 0.001$). Psychological profiles were examined using the DASS21 scale, which showed moderate depression ($U = 14$, $P < 0.01$), moderate anxiety ($U = 15$, $P < 0.01$), and mild stress ($U = 26$, $P = 0.06$) in the DNP group. Kinesiophobia, pain catastrophizing and reduced pain self-efficacy were also found in the DNP group, which had significantly higher scores on the TSK ($U = 3$, $P < 0.001$), and PCS ($U = 1.5$, $P < 0.001$) scales, and lower scores in the PSEQ scale ($U = 6$, $P < 0.001$).

The serum protein levels of a panel of 14 cytokines were quantified in the DNP and DC groups (**Table 2**). Of particular interest are the significant increases in the levels of inflammatory cytokines GM-CSF ($U = 16$, $P < 0.05$, **Figure 2A**) and IL-8 ($U = 6$, $P < 0.01$, **Figure 2B**) in the DNP group compared to controls. There was no obvious pattern of differential cytokine levels between male and female participants. None of the other 12 cytokines showed significant differences between the two groups, although it is important to highlight that major pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-1b, IL-2, IL-17A, and IL-23 were highest in the DNP, whereas anti-inflammatory cytokines, IL-4 and IL-10, were lowest in the DNP group. The significant increases in IL-8 and GM-CSF suggest the presence of an inflammatory state in the DNP group.

This is further supported by a significant increase in the KYN/TRP ratio in the DNP group [$t_{(18)} = 2.348$, $P < 0.05$] (**Figure 3A**). The KYN/TRP ratio is an indirect marker of the enzymatic activity of IDO1, which is activated by pro-inflammatory cytokines. Furthermore, the KYN/TRP ratio positively correlated with pain intensity in the DNP group ($P < 0.05$, $R^2 = 0.39$), but not in diabetic controls (**Figure 3C**). There was no clear pattern of differential KYN/TRP ratio between male and female participants. Overall, these findings provide support that there is increased inflammatory activation in individuals with diabetic neuropathic pain.

Metabolites of the KP and BH4 pathway have been quantified in the serum of the DNP and diabetic control participants (**Table 3**). No significant alterations were observed in the different metabolic intermediates or end products of the KP. Lower levels of TRP and higher levels of KYN in the DNP group support the aforementioned significant increase in the KYN/TRP ratio (**Figure 3A**). The neurotoxic metabolite QUIN was 248.8 ± 191.2 nmol/L in the DNP group and 187.6 ± 110.6 nmol/L in the DC group, however this difference failed to reach significance due to high levels of individual variation [$t_{(19)} = 1.303$, $P = 0.21$]. While BH4 levels were unaltered, the levels of the macrophage produced inflammatory biomarker NEO were significantly increased in the serum of DNP compared to DC participants [$t_{(17)} = 2.717$, $P < 0.05$] (**Figure 3B** and **Table 3**).

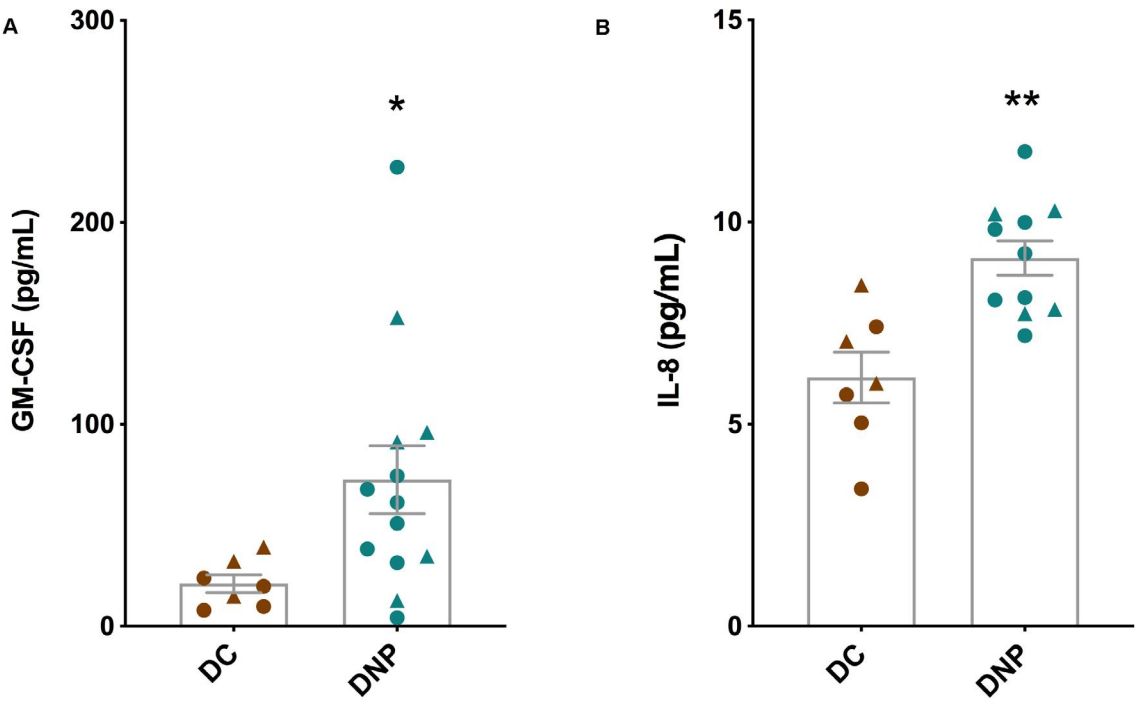

FIGURE 2 | Cytokine levels in the serum of diabetic neuropathic pain and diabetic control groups. **(A)** GM-CSF and **(B)** IL-8 in serum of DNP participants and diabetic controls. *$P < 0.05$, **$P < 0.01$, unpaired two-tailed Mann–Whitney *U*-test. Circles represent male participants; triangles represent female participants.

In addition to the elevated activity of IDO1 predicted by the ratio of TRP/KYN, the activity of the other KP enzymes were estimated by the product/substrate ratios (**Table 4**). The kynurenine aminotransferase (KAT) activity responsible for the production of XA from the substrate 3-HK (identified here as KAT B) was significantly higher ($U = 9$, $P < 0.01$) in the DNP group. This is in line with lower levels of 3-HK and higher levels of XA (**Table 3**). Furthermore, the total KAT activity (calculated as the sum of KAT A and KAT B activities) was also significantly higher ($U = 15$, $P < 0.05$) in DNP participants, however given KAT A activity is not significantly different this is most likely the result of a significant change in KAT B. No other alterations were observed in the activity of the different enzymes of the KP.

Given the known relationship between inflammatory cytokines and the KP and BH4 metabolites (**Figure 1**), linear regression was performed separately within the DNP and DC groups to uncover any relationships. Significant relationships were identified between inflammatory cytokines TNF-α and IL-1β and metabolites KYN, PIC and BH4 in the DNP group, but not the DC group (**Figure 4**). There were significant positive correlations between KYN ($P < 0.01$, $R^2 = 0.54$, **Figure 4A**) and PIC ($P < 0.001$, $R^2 = 0.70$, **Figure 4B**) with TNF-α within the DNP group. There was a significant positive correlation between IL-1β and BH4 in the DNP group ($P < 0.01$, $R^2 = 0.63$, **Figure 4C**). These relationships exist in the DNP group despite the fact that none of these markers were significantly elevated compared to DC (**Tables 2**, **3**). There were no obvious patterns between males and females. Overall, these findings suggest some degree of individual variation across the DNP group and that increased pro-inflammatory cytokine levels could be responsible for activation of the KP and BH4 pathway in some DNP participants.

DISCUSSION

In this study, we have demonstrated that the pro-inflammatory cytokines GM-CSF and IL-8, and the inflammatory biomarkers NEO and the KYN/TRP ratio, were elevated in participants with type 1 diabetes mellitus and neuropathic pain. The serum levels of a number of other pro-inflammatory cytokines positively correlated with metabolites of the KYN- and BH4-pathways, suggesting that immune mediators drive activation of these pathways.

It has been well documented that the appearance of peripheral neuropathy in type 1 and type 2 diabetes mellitus is strongly associated with increases in inflammatory biomarkers, including C-reactive protein, TNF-α, and IL-6 (Hussain et al., 2013; Zhu et al., 2015; Ge et al., 2016). Here, we specifically examined the serum levels of cytokines in participants with type 1 diabetes mellitus complicated by neuropathic pain (DNP group). We found that GM-CSF and IL-8 were elevated in the DNP group. GM-CSF promotes the survival and activation of macrophages, neutrophils and eosinophils, and has been shown to polarize macrophages toward an M1 pro-inflammatory phenotype (Hamilton, 2008). It has also been shown to sensitize peripheral nociceptors indirectly through the activation of macrophages, and cause sprouting of sensory nerve endings

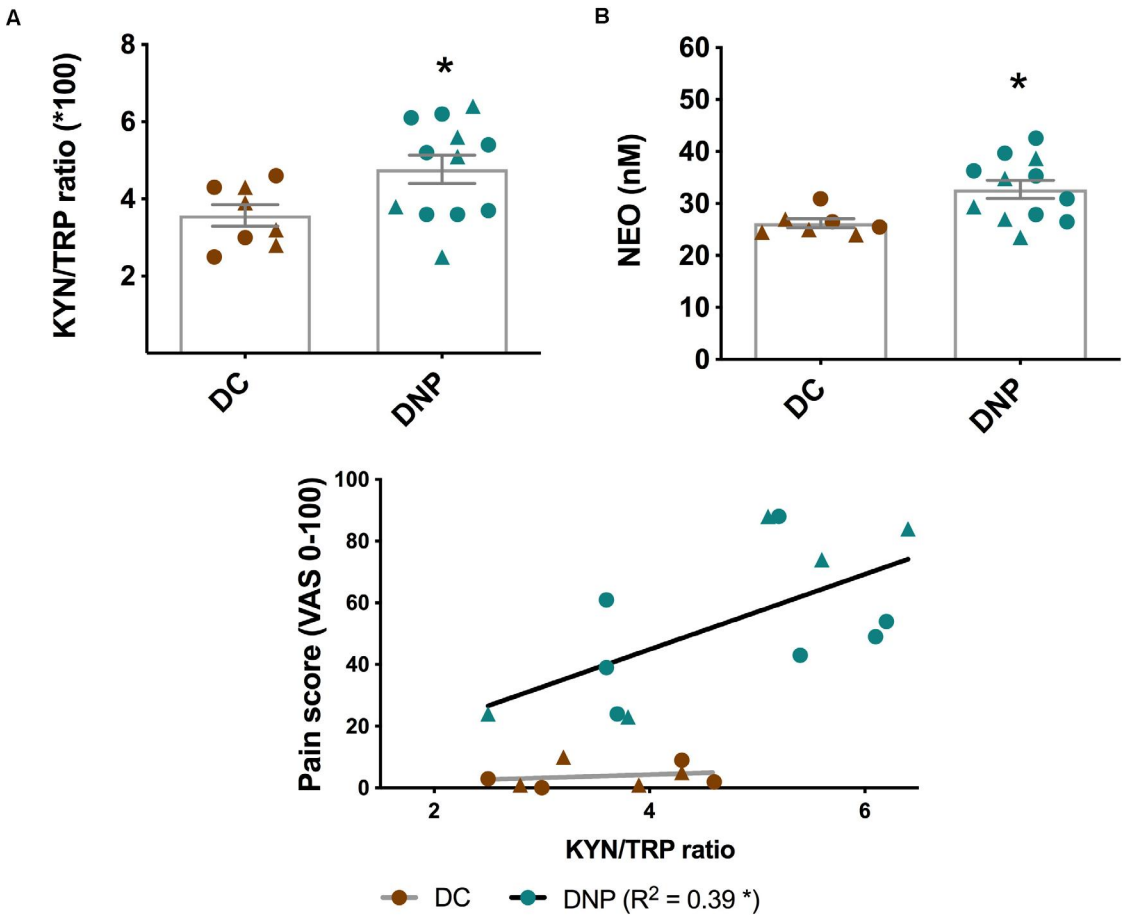

FIGURE 3 | Levels of two major inflammatory biomarkers in the serum of diabetic neuropathic pain and diabetes control groups. **(A)** KYN/TRP ratio and **(B)** NEO in serum of DNP participants and diabetic controls. *$P < 0.05$, unpaired two-tailed Student's *T*-test. **(C)** The relationship between pain intensity score and the KYN/TRP ratio in DNP and DC groups. *$P < 0.05$ & R^2 linear regression analysis. Circles represent male participants; triangles represent female participants. TRP, Tryptophan; KYN, Kynurenine; NEO, neopterin; VAS, Visual analog scale.

in the skin (Schweizerhof et al., 2009; Tewari et al., 2020). A recent study has confirmed a role for GM-CSF in neuropathic pain, where it can potentiate the release of pro-inflammatory mediators from spinal glial cells (Nicol et al., 2018). Moreover, GM-CSF was identified as an important biomarker in our recent study in complex regional pain syndrome (Russo et al., 2020). The chemokine IL-8 is predominantly released from macrophages, recruits both neutrophils and T lymphocytes, and has recently been associated with neuropathic pain in burning mouth syndrome and peripheral neuropathies (Barry et al., 2018; Langjahr et al., 2018). An imbalance of pro- and anti-inflammatory cytokines has previously been reported in other painful peripheral neuropathies (Uceyler et al., 2007). The mean serum level of IFN-γ was ∼40% higher in the DNP group compared to the control group (see **Table 2**), and although not a significant increase, it could be biological important as it is a major activator of IDO1. The cytokine profiles of DNP participants suggest an inflammatory state, involving GM-CSF and IL-8, may play a role in the development of neuropathic pain in type 1 diabetes mellitus. Given these cytokines may act indirectly through the activation of immune cells, particularly macrophages, an immunophenotyping study of blood from diabetics with neuropathic pain would shed further light on these findings.

Under inflammatory conditions, the rate-limiting enzyme of BH4 biosynthesis, GTP cyclohydrolase I (GTPCH), is upregulated up to 100-fold (Shi et al., 2004). This is in keeping with our observation of a strong positive relationship between BH4 serum levels and the pro-inflammatory cytokine IL-1β in the DNP group. This suggests that BH4 may be increased in individuals with higher levels of IL-1β despite not being significantly elevated in all DNP participants (Werner et al., 1990; Shi et al., 2004). Several pre-clinical studies have identified BH4 as a key mediator of chronic pain (Latremoliere et al., 2015; Fujita et al., 2019). The activity of PTPS and sepiapterin reductase, enzymes of the BH4 pathway downstream of GTPCH, are only slightly increased by inflammation, with the metabolic blockage resulting in the accumulation of NEO. NEO is therefore considered a far more sensitive biomarker for inflammatory activation, and proxy for GTPCH activity,

than BH4 (Werner et al., 1990, 1993; Ghisoni et al., 2015). NEO is a macrophage activation marker, produced by macrophages stimulated by the Th1 T lymphocyte derived cytokine, IFN-γ (Huber et al., 1983), and is elevated in the dorsal root ganglia (DRG) (Tegeder et al., 2006). A study in HIV-infected patients with peripheral neuropathy found an increase in blood and CSF NEO levels, compared to HIV patients without neuropathy, together with an increase in CD14 + CD16 + monocytes (Wang et al., 2014). Given the significant increases in macrophage-related cytokines, GM-CSF and IL-8, in the DNP group, and the link to nerve injury and neuropathy, it is perhaps unsurprising that we were also able to observe an increase in NEO levels in the DNP group. These findings make NEO a potentially useful biomarker in diabetic neuropathic pain.

Two major enzymes catabolize TRP via the KP: tryptophan (2,3)-dioxygenase (TDO) in the liver, and IDO1 elsewhere (Schrocksnadel et al., 2006). The extrahepatic KP enzyme, IDO1, contributes very little (< 2%) to TRP degradation under normal conditions, however it assumes a greater quantitative

TABLE 3 | Levels of KYN and BH4 pathway metabolites in diabetic neuropathic pain and diabetic control groups.

Metabolite	DC (X ± SD; *n* = 8)	DNP (X ± SD; *n* = 13)
TRP (μmol/L)	52.9 ± 6.1	46.8 ± 7.1
KYN (μmol/L)	1.8 ± 0.4	2.2 ± 0.5
3-HAA (nmol/L)	18.8 ± 3.6	19.4 ± 6
AA (nmol/L)	15.1 ± 3.5	15.05 ± 3.9
KYNA (nmol/L)	30.4 ± 10.4	27.3 ± 14.7
3-HK (nmol/L)	14.5 ± 4.5	12.1 ± 3.5
XA (nmol/L)	9.3 ± 1.7	11.1 ± 2.5
PIC (nmol/L)	29.01 ± 5.4	31.9 ± 12.6
QUIN (nmol/L)	187.6 ± 110.6	248.8 ± 191.2
BH4 (nmol/L)	2.575 ± 0.6076	2.458 ± 0.4672
NEO (nmol/L)	26.20 ± 0.8794	32.72 ± 1.741*

*TRP, Tryptophan; KYN, Kynurenine; 3-HAA, 3-Hydroxyanthranilic acid; AA, Anthranilic acid (AA); KYNA, Kynurenine acid; 3-HK, 3-Hydroxykynurenine; XA, Xanthurenic acid; QUIN, Quinolinic acid; PIC, Picolinic acid; BH4, Tetrahydrobiopterin; NEO, Neopterin. All data are presented as mean ± standard deviation (X ± SD). *P < 0.05, unpaired two-tailed Student's T-test.*

TABLE 4 | Activity of KP enzymes in the serum of DNP and diabetic control participants.

Enzymes	Expression	DC (X ± SD; *n* = 8)	DNP (X ± SD; *n* = 13)
KMO	100 × 3-HK/KYN	0.8 ± 0.3	0.58 ± 0.2
KAT A	100 × KYNA/KYN	1.6 ± 0.4	1.2 ± 0.4
KAT B	100 × XA/3-HK	56.2 ± 14	111 ± 50**
Total KAT	KAT A + KAT B	57.8 ± 14	112.2 ± 50**
KYNU A	100 × AA/KYN	0.81 ± 0.1	0.73 ± 0.2
KYNU B	100 × 3-HAA/3-HK	148.7 ± 73	166.2 ± 71
Total KYNU	KYNU A + KYNU B	149.5 ± 74	167.0 ± 71

*KMO, kynurenine 3-monooxygenase; KYNU, kynureninase; KAT, kynurenine aminotransferases; KYN, Kynurenine; 3-HAA, 3-Hydroxyanthranilic acid; AA, Anthranilic acid (AA); KYNA, Kynurenine acid; 3-HK, 3-Hydroxykynurenine; XA, Xanthurenic acid. All data are presented as mean ± standard deviation (X ± SD). *P < 0.05, **P < 0.01, unpaired two-tailed Mann–Whitney U-test.*

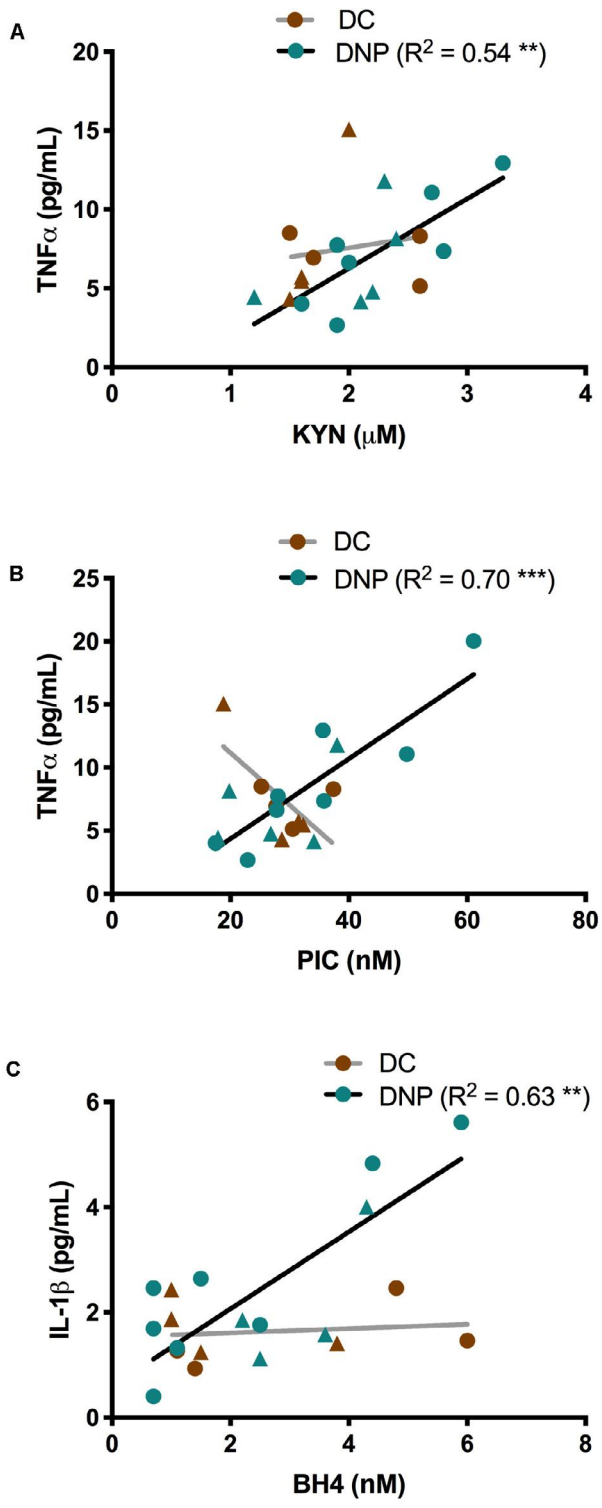

FIGURE 4 | The relationships between TNF-α and IL-1β, and metabolites of the KYN and BH4 pathways in serum of diabetic neuropathic pain and diabetic control groups. **(A)** TNF-α and KYN expression correlate in the DNP group. **(B)** TNF-α and PIC expression correlate in the DNP group. **(C)** IL-1β and BH4 levels correlate in the DNP group. **$P < 0.01$, $P < 0.001$ & R^2 from linear regression analysis. Circles represent male participants; triangles represent female participants.

significance after activation by cytokines, such as IFN-γ and TNF-α, and results in a decrease of TRP and an increase of KYN concentration in blood (Schrocksnadel et al., 2006). As a result, the KYN/TRP ratio is a well-established marker for IDO1 and inflammatory activation, with an elevated ratio observed in both chronic lower back pain and complex regional pain syndrome (Kim et al., 2012; Alexander et al., 2013). Here, we observed an increase in the KYN/TRP ratio in the DNP group compared to pain-free diabetic controls. Given the positive relationship between the KYN/TRP ratio and the pain intensity score, the KP is a candidate for direct involvement in pathological processes leading to diabetic neuropathic pain. However, we caution that an important follow up study should be undertaken to directly confirm an increase in IDO1 activity and mRNA levels in peripheral blood mononuclear cells from diabetics with neuropathic pain. Interestingly, KYN and PIC showed a significant positive correlation with the serum level of TNF-α in DNP, suggesting that TNF-α levels may be responsible for individual differences in KYN and PIC levels in the DNP group. A previous study showed that TNF-α works synergistically with IFN-γ to induce IDO1 expression, which increases production of KYN and other downstream KP metabolites (Pemberton et al., 1997; Robinson et al., 2003). Little is known about the physiological role of PIC, however a neuroprotective role has been suggested (Cockhill et al., 1992; Beninger et al., 1994). In summary, levels of GM-CSF and IL-8 were higher in the DNP group, and TNF-α and IL-1β positively correlated with KYN, PIC and BH4 levels, supporting the idea that immune activation leads to pathological activation of the KYN and BH4 pathways.

The pivotal metabolite KYN can be metabolized by different enzymes along distinct routes to produce several neuroactive metabolites that have been implicated in immune regulation and tolerance mechanisms (Munn et al., 1998). Evidence from pre-clinical studies has revealed the contribution of KP enzymes within the DRG, the spinal cord and the hippocampus in the development of neuropathic pain (Zhou et al., 2015; Rojewska et al., 2016, 2018; Laumet et al., 2017). An up-regulation of IDO1 and KMO in DRG and spinal cord neurons was associated with allodynia and hypersensitivity in a neuropathic pain model (Rojewska et al., 2016, 2018). Concurrently, the administration of KMO inhibitors (Ro61-6048 and JM6) or IDO1 inhibitor (1-methyl-D-tryptophan) decreased the pain hypersensitivity (Rojewska et al., 2016, 2018).

The present study suggests an activation of the KP with a possible conversion into QUIN and XA production in participants with type 1 diabetes mellitus and neuropathic pain. The QUIN serum levels were ∼30% higher in the DNP group than in the diabetic control group, and although this difference was not significant due to high variance amongst individuals, it provides the impetus for a larger follow up study. Similarly, our data demonstrated XA serum concentration was ∼20% higher in diabetes participants with neuropathic pain than in the diabetes pain-free control group, and this observation is supported by a significant increase in KAT activity, the enzyme responsible for the XA production. Furthermore, QUIN and XA were commonly elevated biomarkers in samples from a large cohort of patients with chronic pain (Gunn et al., 2020). Given these KP metabolites (i.e., QUIN and XA) are neuroactive compounds that act via glutamatergic receptors (Guillemin, 2012; Neale et al., 2013), further studies exploring their relevance for inflammation-induced pain hypersensitivity would shed further light on these findings.

Study Limitations

The limitations of this study are the small cohorts, and despite being non-significant, the different proportion of females and males in each group. In particular, sex differences in immune function and pain processing could represent a confounding factor in our findings (see review Mapplebeck et al., 2016), especially given sex differences in TRP and KYN metabolite serum levels (Badawy and Dougherty, 2016). The most significant limitation of our study is that subjects were not fasted prior to obtaining blood samples, which could lead to fluctuations in levels of some metabolites through food intake and dietary components (Badawy, 2010). Also, future studies should control for TRP and KYN modulators such as glucocorticoids and insulin, which play an important role in lowering serum TRP levels (Badawy, 2017). It is possible that the 7-day drug wash-out could leave residual effects on the immune system, since although myeloid cells have a rapid turnover, lymphocyte turnover is much slower, only 2% daily (De Boer et al., 2003).

CONCLUSION

In summary, our results suggest that inflammatory activation through elevated pro-inflammatory cytokines, neopterin and upregulation of the kynurenine pathway might be involved in the pathophysiology that leads to the appearance of neuropathic pain in type 1 diabetes mellitus. Our research encourages further studies exploring the role of neuro-immune interactions, particularly through the cytokines GM-CSF and IL-8 as well as the kynurenine and tetrahydrobiopterin pathways, in chronic pain conditions.

DATA AVAILABILITY STATEMENT

All raw datasets generated for this study are available on reasonable request from the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Ethics Committee from University of Sydney (HREC #2017/019) and Macquarie University (HREC #5201600401). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS: acquisition of data, data curation, and writing the original draft. BH: acquisition of data, data curation, and

revising the article. VT: investigator of the kynurenine and tetrahydrobiopterin analysis and revising the article. AL: intellectual and technical support for tetrahydrobiopterin pathway analysis and revising the article. MR: conception of the study, participant recruitment, and funding acquisition. DS: participant recruitment, clinical database management, and revising the article. DB: participant recruitment, sample collection, and clinical database management. KW: participant recruitment and revising the article. JO'B: sample preparation and revising the article. GG: principle investigator of the kynurenine and tetrahydrobiopterin analysis, funding acquisition, and revising the article. PA: conception of the study, project supervisor, funding acquisition, data curation, writing the original draft, and revising the article. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH ARTICLE: NOVEL IMMUNE BIOMARKERS IN COMPLEX REGIONAL PAIN SYNDROME

(P2) Journal of Neuroimmunology:
Novel immune biomarkers in Complex Regional Pain Syndrome.
Russo, M.; Georgius, P.; **Staats Pires A.;** Heng, B. R.; Allwright, M.; Guennewig, B.; Santarelli, D.; Bailey, D.; Fiore, N.; Tan, V.; Latini, A.; Guillemin, G.; Austin, P (Accepted 16 Jul 2020).

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Research Gap

Chronic pain in general is a diagnostic and therapeutic challenge, and the management of Complex Regional Pain Syndrome (CRPS) is particularly demanding. CRPS is a chronic pain condition that usually affects a single limb, often following an injury. The ongoing pain is disproportionate to the initiating event, with sensory, motor and trophic symptoms, as well as impairment of autonomic control of the limb (Birklein & Dimova, 2017). The clinical diagnosis of CRPS is based on the internationally agreed criteria, which considers the reported symptoms, presence of signs and exclusion of alternative causes (Harden et al., 2010). Research into the elucidation of CRPS pathophysiological mechanisms and related markers to support diagnosis and disease management are a scientific focus that demands attention.

The underlying pathophysiology of CRPS seems to be complex, however, it has become evident that the immune and nervous systems communicate closely and its interaction plays an important role in the mechanisms of CRPS (Parkitny et al., 2013). One of the likely mechanisms of neuro-immune interactions in chronic pain states is through the activation of the kynurenine (KYN) pathway, which is responsible for the metabolism of tryptophan (Trp) into several biologically active metabolites implicated in disease (Arnone et al., 2018; Savitz, 2019). In animal models of neuropathic and inflammatory pain, there is evidence of increases in KYN pathway enzymes and metabolites in both the blood and CNS, which are associated with increased pain measures and depressive-like behaviour (Kim et al., 2012; Laumet et al., 2017; Rojewska, Ciapala, Piotrowska, Makuch, & Mika, 2018; Rojewska, Piotrowska, Makuch, Przewlocka, & Mika, 2016; Zhou et al., 2015).

Another well documented neuro-immune crosstalk in chronic pain is the upregulation of the tetrahydrobiopterin (BH4) pathway (Costigan et al., 2002; Latremoliere et al., 2015; Tegeder et al., 2006). The activation of the BH4 pathway is associated with pain in preclinical models, and the use of inhibitors for the BH4 biosynthetic enzymes reduces chronic pain (Costigan, Latremoliere, & Woolf, 2012; Latremoliere et al., 2015; Tegeder et al., 2006). Therefore, inflammation-enhanced KYN and BH4 pathways can be involved in the pathophysiology of CRPS emergence and their metabolites are attractive candidates as biomarkers for CRPS and its comorbidity.

Key Results & Implications

We used a combined approach of targeted analysis related to the immune and metabolic status of CRPS subjects compared to healthy controls, which yielded several novel results. 1) We observed significant decreases in serum IL-37 and Trp in CRPS participants, and particularly high

levels of GM-CSF in a subset of CRPS participants. 2) Additionally, we identified several correlations between different KYN and BH4 pathways metabolites and CRPS symptoms and psychological metrics. Specifically, *i*) Neopterin correlates with depression scores; *ii*) Xanthurenic acid correlates with depression and stress scores; *iii*) The KYN/Trp ratio correlates with kinesiophobia; *iv*) TNF-α also correlates with kinesiophobia; *v*) BH4 correlates with pain score in CRPS participants. The association of these markers with pain intensity and psychological variables suggests a link between chronic pain and its related features with underlying immune and metabolic markers. 3) Finally, utilizing machine learning analysis, we identified a set of four binary variables that may represent a simple set of biomarkers for distinguishing chronic CRPS from healthy controls. This biomarker set combined IL-37 (low), GM-CSF (high), Treg cell number (high) and CD8+ T cell number (high) and gave an AUC of 0.79 (approaching clinical usefulness). Our findings can inform future studies and guide the validation of potential biomarker sets for CRPS that may assist in clinical diagnosis and guide prognosis and treatment.

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Novel immune biomarkers in complex regional pain syndrome

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ABSTRACT

We investigated serum levels of 29 cytokines and immune-activated kynurenine and tetrahydrobiopterin pathway metabolites in 15 complex regional pain syndrome (CRPS) subjects and 14 healthy controls. Significant reductions in interleukin-37 and tryptophan were found in CRPS subjects, along with positive correlations between kynurenine/tryptophan ratio and TNF-α levels with kinesiophobia, tetrahydrobiopterin levels with McGill pain score, sRAGE, and xanthurenic acid and neopterin levels with depression, anxiety and stress scores. Using machine learning, we identified a set of binary variables, including IL-37 and GM-CSF, capable of distinguishing controls from established CRPS subjects. These results suggest possible involvement of various inflammatory markers in CRPS pathogenesis.

1. Introduction

The highly debilitating chronic pain condition, Complex Regional Pain Syndrome (CRPS), typically develops after trauma or surgery. According to the Budapest criteria, the incidence rate of CRPS is approximately 8% after distal radius fracture (Roh et al., 2014), and less than 1% after foot and ankle fractures (Bullen et al., 2016). CRPS leads to ongoing pain in the affected limb that is disproportionate to the initiating event, with sensory, motor and trophic symptoms and impairment of autonomic control of the limb, with symptoms spreading beyond single nerve innervation territories (Birklein and Dimova, 2017). CRPS patients often have comorbid psychological disturbances, such as depression, kinesiophobia and pain catastrophizing. CRPS is classified into types I and II, with the latter being associated with documented nerve injury. CRPS-II has neuropathic pain like symptoms, but whether it is a true neuropathic pain condition is disputed (Oaklander et al., 2006).

Multiple individual findings of immune system involvement have been documented in CRPS and it is now considered an immunoneurological disorder (Birklein et al., 2014; Birklein et al., 2018; David Clark et al., 2018). There are many reports of increased expression of pro-inflammatory cytokines in the skin, blood and cerebrospinal fluid of CRPS patients at both acute and chronic stages (Alexander et al., 2005; Schinkel et al., 2006; Uceyler et al., 2007; Kramer et al., 2011; Parkitny et al., 2013; Bharwani et al., 2017; Konig et al., 2017). There is evidence for local neurogenic inflammation, with elevation of neuropeptides that mediate oedema and inflammation and may lead to an immune mediator cascade (Huygen et al., 2002; Kingery, 2010; Fan et al., 2018). There is also evidence for autoantibody involvement. Autoantibodies to the α-1a adrenoceptor, ß2 adrenergic receptor and/or the muscarinic-2 receptor have been found in the majority of chronic CRPS patients where they play a role in the maintenance and severity of painful hypersensitivity by sensitising nociceptors (Kohr et al., 2011; Dubuis et al., 2014; Cuhadar et al., 2019). It has recently been identified that chronic inflammation (e.g. rheumatoid arthritis) or prior infection increases the risk of CRPS (Goebel et al., 2005; Jo et al., 2019), suggesting some priming of the immune system may occur.

Our recent mass cytometry study has shown that chronic CRPS is associated with expanded populations of circulating central memory T lymphocytes (CD4+ and CD8+) (Russo et al., 2019). In particular,

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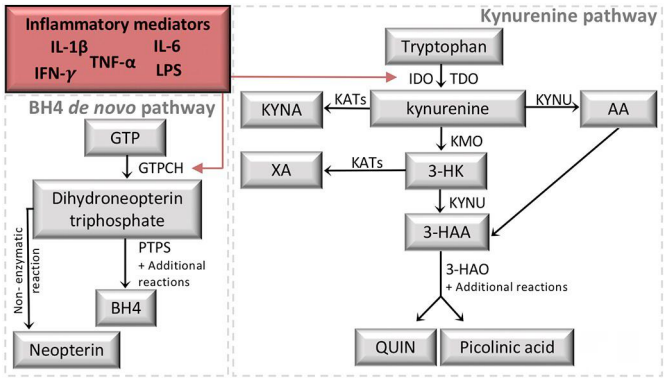

Fig. 1. A schematic diagram of the kynurenine (KYN) and tetrahydrobiopterin (BH4) pathways. Both KYN and BH4 pathways are activated by inflammatory cytokines such as IL-1β, Il-6, IFN-γ, TNF-α and the immune activator LPS. Abbreviations: LPS: lipopolysaccharide; GTP: Guanosine-5′-triphosphate; GTPCH: GTP cyclohydrolase I,; PTPS: 6-pyruvoyl-tetrahydropterin synthase; IDO: Indoleamine 2,3-dioxygenase; TDO: tryptophan 2,3-dioxygenase; KATs: Kynurenine aminotransferases; KYNA: Kynurenine acid; XA: Xanthurenic acid; KYNU: Kynureninase; AA: Anthranilic acid; KMO: Kynurenine 3-monooxygenase; 3-HK: 3-Hydroxykynurenine; 3-HAA: 3-Hydroxyanthranilic acid; 3-HAO: 3-hydroxyanthranilate 3,4-dioxygenase; QUIN: Quinolinic acid.

type 1 helper T lymphocyte (Th1) and regulatory T lymphocyte (Treg) CD4+ subsets showed significant increases in number and increased NFκB signalling. Interestingly, in animal models, Th1 and Tregs have been shown to be pro-nociceptive and antinociceptive respectively (Moalem et al., 2004; Austin et al., 2012), suggesting there may be an overall imbalance of these two populations in CRPS patients. Further, our study provided evidence of reduced numbers of activated myeloid dendritic cells in the peripheral circulation, indicative of tissue trafficking and involvement in T lymphocyte activation.

The kynurenine pathway (KP) (Fig. 1) is a major immune-activated signalling pathway that modulates neural function through neuroactive KP metabolites. It has been implicated in a variety of diseases and disorders, including Alzheimer's disease and depression (Chen and Guillemin, 2009). The full suite of KP enzymes is expressed in peripheral monocytes, macrophages and dendritic cells (Jones et al., 2015). The rate-limiting enzyme of the KP - indoleamine 2,3-dioxygenase (IDO), which converts tryptophan (TRP) to kynurenine (KYN) - is increased by pro-inflammatory cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (Guillemin et al., 2001). KYN is metabolised by kynurenine 3-monooxygenase (KMO) into 3-hydroxy-kynurenine (3−HK), which is subsequently converted into the neurotoxic quinolinic acid (QUIN) by enzymes kynureninase (KYNU) and 3-hydroxyanthralinic acid dioxygenase (HAAO) (Guillemin, 2012). QUIN is an *N*-methyl-D-aspartate (NMDA) receptor agonist, and given these receptors are found on peripheral nociceptor terminals, a role in chronic pain has been proposed (Gunn et al., 2020). QUIN produced in the central nervous system by microglia has been linked to glutamate excitotoxicity (Guillemin, 2012). Alternatively, within astrocytes, KYN is broken down by kynurenine aminotransferase (KAT) to the neuroprotective kynurenic acid (KYNA), which is an *N*-methyl-D-aspartate (NMDA) receptor antagonist that blocks the effects of QUIN. Thus, the KP has been suggested as a critical link between the immune and nervous systems in inflammatory diseases and is likely to be important in chronic pain states. To date, there has only been a single study to investigate the KP in CRPS (Alexander et al., 2013). Alexander et al. observed a decrease in TRP, as well as an increase in KYN/TRP ratio, a marker of IDO activity, with the latter being significantly correlated with pain intensity (Alexander et al., 2013). However, they did not investigate psychological symptoms, or whether changes in KP metabolites were related to changes in cytokine levels or immune cell populations.

The tetrahydrobiopterin (BH4) biosynthesis pathway (Fig. 1) is another metabolic pathway that is rapidly activated by inflammation and has also been implicated in chronic pain (Tegeder et al., 2006; Fujita et al., 2020). Neopterin (NEO) is one of the major metabolites of the BH4 pathway, and a well-known biomarker of immune activation (Murr et al., 2001). Alexander et al. observed a significant increase in NEO in CRPS (Alexander et al., 2013), but did not investigate BH4.

In this study, we took a comprehensive assessment of the immune status in chronic CRPS patients compared to healthy controls via metabolomic, mass cytometric and cytokine multiplex approaches. There were 3 aims to this study: 1) to analyse serum cytokine levels, as well as KP and BH4 pathway metabolite levels; 2) to interrogate the relationships of serum cytokine and metabolite levels to pain and psychological metrics; 3) to uncover the most predictive disease biomarkers amongst the 55 variables collected here, combined with the 12 immune cell variables previously collected from this cohort of CRPS participants using machine learning approaches (Russo et al., 2019).

2. Materials and methods

2.1. Participants

Male and female participants aged 19–70 years were recruited by Genesis Research Services (Broadmeadow, NSW, Australia) between September 2017 and April 2018. All 15 CRPS participants met the Budapest Criteria for clinical CRPS diagnosis (Harden and Bruehl, 2005) (see supplementary Table 1). The control group consisted of age-matched pain-free participants. Previous findings from this cohort have been reported in Russo et al. (Russo et al., 2019).

2.2. Ethics approval and consent to participate

This study was approved by the Human Ethics Committee from University of Sydney (HREC #2017/019) and Macquarie University (HREC #5201600401). Participation in the study was on a completely voluntary basis. All participants signed informed consent. All demographic information and blood samples were de-identified from the study team.

2.3. Pain and psychological profiling

All participants rated pain intensity from 0 to 100 on a visual analogue scale (VAS) and completed 5 questionnaires to assess pain and psychological variables: Short-form McGill Pain Questionnaire (SF-MPQ-2) (Dworkin et al., 2009), Short-form Depression, Anxiety and Stress Scales (DASS21) (Lovibond and Lovibond, 1995), Pain Self-Efficacy Questionnaire (PSEQ) (Nicholas, 2007), Tampa Scale for Kinesiophobia (TSK) (Roelofs et al., 2011) and Pain Catastrophizing Scale (PCS) (Sullivan et al., 1995).

2.4. Blood collection protocol

A non-fasted blood sample was taken by venepuncture from the antecubital fossa by a trained phlebotomist, into a 5 mL tube. Samples were then incubated at room temperature for 15 min to allow the blot to clot, then centrifuged at 2000 ×*g* for 10 min. The supernatant was removed and stored in polypropylene aliquot tubes at −80 °C until analysis.

2.5. Sample preparation

Serum samples were deproteinised by adding 1 vol. of 10% trichloroacetic acid containing 6.5 mM dithioerythritol. Afterwards, samples were centrifuged at 12,000 ×*g* at 4 °C for 15 min and filtered through a 0.20 μm PTFE syringe filter (Merck-Millipore, CA, USA).

Supernatants were transferred to high performance liquid chromatography (HPLC) vials for analysis.

2.6. Metabolite quantification by HPLC

Serum levels of KP metabolites xanthurenic acid (XA), TRP, KYN, 3-HK, 3-hydroxyanthranilic acid (3-HAA) and anthranilic acid (AA) were determined by uHPLC and quantified using a sequential diode-array UV and fluorescence detection as previously described (Guillemin et al., 2007). The HPLC analysis was carried out in an uHPLC system (Agilent 1290 Infinity, CA, USA) by using an Agilent ZORBAX Rapid Resolution High Definition C18, reversed phase column (2.1 × 150 mm, 1.8 µm, Agilent Technologies, CA, USA). The temperature of the column compartment was set at 38 °C. Injection volume was 20 µL and the autosampler tray temperatures was set at 4 °C to prevent sample degradation. The flow rate was set at 0.75 mL/min with an isocratic elution of 100% of 100 mM sodium acetate, pH 4.65. The identification and quantification of XA, KYN and 3-HK were performed by a UV detector (G4212A, Agilent, CA, USA) with absorbance at 250 nm and a reference signal at 350 nm for XA; and with absorbance at 365 nm and a reference signal off for KYN and 3-HK. The identification and quantification of TRP, 3-HAA and AA were performed by a fluorescence detector (G1321B xenon flash lamp, Agilent, CA, USA) with an emission wavelength of 438 nm and an excitation wavelength of 280 nm for TRP and 320 nm for 3-HAA and AA. The results were calculated by interpolation using a 6-point calibration curve and expressed as µmol/L or nmol/L (indicated in results tables).

NEO concentrations in serum samples were determined as previously described with some modifications (de Paula Martins et al., 2018). The same HPLC system and column were used with a mobile phase of 100% of 15 mM potassium phosphate buffer, pH 6.4. The flow rate was set at 0.7 mL/min with an isocratic elution. The identification of NEO was performed by a fluorescence detector (G1321B xenon flash lamp, Agilent, CA, USA) with emission wavelength of 438 nm and an excitation wavelength of 355 nm. The results were calculated by interpolation using 6-point calibration curve.

KYNA concentrations in serum samples were determined by HPLC (Agilent 1260 Infinity, Agilent, CA, USA) and an Agilent ZORBAX Rapid Resolution High Definition C18, reversed phase (4.6 × 100 mm, 3.5 µm, Agilent Technologies, CA, USA). Mobile phase consisted of 95% of 50 mM sodium acetate and 50 mM zinc acetate, pH 5.2 and 5% HPLC grade acetonitrile. The flow rate was set at 1.00 mL/min with an isocratic elution. The identification of KYNA was performed by a fluorescence detector (G1321B xenon flash lamp, Agilent, CA, USA) with an emission wavelength of 388 nm and an excitation wavelength of 344 nm. The results were calculated by interpolation using a 6-point calibration curve.

2.7. Metabolite quantification by gas chromatography/mass spectrometry (GC/MS)

QUIN and picolinic acid (PIC) concentrations in serum samples were determined using an Agilent 7890 gas chromatograph coupled with an Agilent 5975 mass spectrometer following a protocol previously described (Guillemin et al., 2007). Briefly, deproteinised-serum samples and deuterated internal standards were dried under vacuum and derivatized with trifluoroacetic anhydride and 1,1,1,3,3,3-hexafluoroisopropanol for an hour at 60 °C. Fluorinated esters were then extracted into toluene and washed with 5% sodium bicarbonate. The upper organic layer was collected and washed with 1 mL MilliQ water, and dried using sodium sulphate packed pipette tips. Samples were then injected under a splitless mode onto a HP-5MS GC capillary column (Agilent, CA, USA) and the analysis was carried out with the MS operating in negative chemical ionization mode. Selected ions (*m/z* 273 for PIC, m/z 277 for 4-PIC, m/z 467 for QUIN and m/z 470 for d3-QUIN) were simultaneously monitored. GC oven settings were as follows: oven temperature was held at 75 °C for 3 min and then ramped to 300 °C at a rate of 25 °C/min and held at 300 °C for 4 min for a total run time of 15.6 min. Quantification was achieved through normalisation with respect to the internal standards and interpolation using 6-point calibration curves for each metabolite.

2.8. BH4 quantification by enzyme-linked immunosorbent assay (ELISA)

BH4 concentrations in serum samples were assessed by ELISA, using a commercial kit (Novus Biologicals, Colorado, USA), and following the manufacturer's instructions. The levels of BH4 were estimated by interpolation from a standard curve by colorimetric measurements at 450 nm on a plate reader (PHERAstar® *FSX*, Offenburg, Germany).

2.9. Cytokine analysis

Aliquoted serum samples were thawed and filtered 0.22 µm before being analysed in duplicate by Eve Technologies (Calgary, Alberta, Canada) using a Milliplex human high sensitivity T-cell discovery array 14-plex assay kit (HDHSTC14, Millipore), human soluble cytokine receptor array 14-plex (HDSCR14, Millipore) and a custom interleukin 37 (IL-37) kit (Millipore). Sample concentrations of the all the analytes were determined using a 7-point standard curve using the manufacturer's software.

2.10. Statistical analysis

Fisher's exact test was used to compare the proportion of male and female participants between the groups (Prism 8, GraphPad Software Inc.). All data were first analysed for the presence of multiple outliers using ROUT *t*-test (Q = 1%) in Prism 8 with outliers consistently excluded. The maximum number of outliers identified and removed in any of the 55 variables measured were 4 out of 30 datapoints for IL-37, sVEGFR3 and XA/KYN. One dataset had 3 outliers removed, 9 datasets had 2 outliers removed, 28 datasets had 1 outlier removed, and 14 had no outliers removed. Subsequently, the data were analysed for normality using the D'Agostino-Pearson normality test. Unpaired Student's t-test (for normally distributed data), or a Mann-Whitney *U* test (for non-normally distributed data) were used to test for statistically significant differences between the groups ($P < .05$ was considered significant). Where multiple analyses were conducted in a single assay (e.g. cytokine assays, HPLC) they were corrected for multiple comparisons using the Benjamini-Hochberg procedure with a false discovery rate of $q < 0.1$. The relationships of pain and psychological variables, and cytokine levels to KYN and BH4 metabolites were assessed using linear regression analyses.

2.11. Machine learning analysis

To determine the combined importance of all 67 variables (55 variables measured here, and 12 from a previous study on the same participants), as well as 4 variables of interest, in differentiating between 13 CRPS and 14 healthy control samples, we developed a machine learning pipeline and applied resampling. This approach utilises Python's built in machine learning library Scikit-learn (Buitinck et al., 2013). We split the data into training and validation datasets (60/40), resampling this split 500 times. We then ran 3 separate classification models (see section 2.11.2) before evaluating their performance using Area Under the Receiver Operator Curve (AUC) (Bradley, 1997). The AUC provides an aggregate measure of model performance. In this setting the AUC represents the probability that a randomly chosen CRPS participant is (correctly) rated or ranked with greater suspicion than a randomly chosen control participant (Hanley and McNeil, 1982).

2.11.1. Feature sets

The feature sets we selected were as follows:

Set 1: All features: 29 cytokines and soluble cytokines (see Table 2 and 3); 26 KYN and BH4 metabolites and enzymes (see Table 4, and supplementary Tables 2 and 3); 12 immune cell variables identified as being altered in CRPS: $CD4^+$ central memory T cell (Tcm) population, $CD4^+$T-bet^+ population, $CD4^+$T-bet^+ NFκB, regulatory T cell (Treg) population, Treg NFκB, Treg pPLCg2, $CD8^+$ Tcm population, $CD8^+$ Tcm pNFκB, $CD8^+$ Tcm pSTAT1, $CD8^+$ Tcm CD130, $CD1c^+$ myeloid dendritic cells (mDCs) population, $CD1c^+$ mDCS p-p38 (see Russo et al., 2019). Due to missing values for some variables for some samples, we excluded 5 samples, therefore, this set included 11 healthy controls and 11 CRPS participants.

Set 2: The absolute values of 4 features of interest (granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-37, $CD4^+CD45RO^+CCR7^+CD127^{lo}CD25^+FoxP3^+$ Treg cells (Treg population), $CD8^+CD45RO^+CCR7^+$ central memory T cells ($CD8^+$ Tcm population).

Set 3: The 4 features of interest engineered to binary variables based on observed thresholds. These thresholds were determined based on clustering of CRPS participants above or below the selected threshold for a particular feature. See below:

IL − 37 < 30 pg/mL

GM − CSF > 170 pg/mL

Treg population>5000 per mL/blood

$CD8^+$ Tcm population > 10,000 per mL/blood

2.11.2. Models

We developed a python (version 3.6.0) script (https://github.com/MikeAllwright23/Bioinformatics/blob/master/Cytokine%20ML-20200219.ipynb) using the following built in algorithms from Scikit-learn (version 0.22): (i) Logistic Regression; (ii) Decision Tree Classification; and (iii) Gradient Boosting (XGBoost). We computed the AUC value for each resampling of the data, for each model and each feature set and computed the average AUC per model per feature set as shown in Table 5. AUC has been validated as a single number measure for evaluating the predictability of learning algorithms (Jin and Ling, 2005).

3. Results

3.1. Demographic and clinical measures

This cohort largely overlaps with our previous mass cytometry study on immune cells (Russo et al., 2019), with the addition of 1 extra CRPS participant. Eleven participants in the CRPS group had symptoms for more than 6 months, and 4 participants had symptoms for 2–5 months (see supplementary Table 1). Symptom onset occurred following trauma; 7 suffered fractures, 5 had soft tissue injuries and 3 had undergone surgery, and there was an equal proportion of participants with upper and lower limbs affected. Severe pain was confirmed in the CRPS group, with a mean pain rating of 69/100 (VAS), and a mean score of 109/220 on the SF-MPQ-2, both significantly higher than the control group (both $U = 0$, $P < .001$, Table 1). According to the DASS21 severity ratings, the CRPS group had mild depression ($U = 36.5$, $P < .01$), moderate anxiety ($U = 30$, $P < .001$) and mild stress ($U = 21.5$, $P < .001$), all increased compared to the control group, which had normal scores on all ratings. Kinesiophobia, pain catastrophising, and reduced pain self-efficacy were also found in the CRPS group, with higher scores on the TSK and PCS, and lower scores on the PSEQ compared to the control group ($U = 14.5$, $U = 13.5$ and $U = 0$ respectively, all $P < .001$).

3.2. Cytokines and soluble cytokines

We analysed the serum levels of 15 cytokines. There was a significant decrease in the anti-inflammatory cytokine IL-37 in the CRPS group compared to healthy controls ($t_{24} = 2.184$, $P < .05$, Fig. 1D). No other cytokines showed a significant difference between groups (Table 2). However, a subset of CRPS participants had very high GM-CSF levels (Fig. 2A), and there was a trend towards higher TNF-α and lower IL-10 (Fig. 2B & C).

3.2.1. Relationships between cytokine levels and psychological measures

The serum protein level of TNF-α positively correlates with kinesiophobia in the CRPS group but not in the control group ($F_{1,12} = 5.56$, $R^2 = 0.30$, $P < .05$, Fig. 3A). IL-13 correlates with the anxiety score in the CRPS group ($F_{1,12} = 8.53$, $R^2 = 0.42$, $P < .05$, Fig. 3B). Soluble receptor for advanced glycation end products (sRAGE) correlates with both anxiety and stress scores in the CRPS group ($F_{1,13} = 6.14$, $R^2 = 0.32$ and $F_{1,13} = 6.21$, $R^2 = 0.32$ respectively, both $P < .05$, Fig. 3C, D).

3.3. Kynurenine and Tetrahydrobiopterin metabolites

We identified a significant decrease in TRP in CRPS participants compared to healthy controls, but no change in the rest of the KYN and BH4 metabolites or enzymes (Table 4, supplementary Tables 2 and 3).

3.3.1. Relationships between Kynurenine and Tetrahydrobiopterin metabolites, and pain and psychological measures

The DASS21 depression score significantly correlates with XA ($F_{1,13} = 13.32$, $R^2 = 0.51$, $P < .01$, Fig. 4A) and NEO ($F_{1,13} = 5.66$, $R^2 = 0.30$, $P < .05$, Fig. 4D), as well as the ratios of XA/KYN ($F_{1,13} = 7.45$, $R^2 = 0.36$, $P < .05$, Fig. 4B) and XA/3-HK ($F_{1,13} = 9.74$, $R^2 = 0.43$, $P < .01$, Fig. 4C) in the CRPS group, but not in the control group (Fig. 4).

XA and the XA/3-HK ratio also correlate with the DASS21 stress score ($F_{1,13} = 6.56$, $R^2 = 0.34$, $P < .05$ and $F_{1,13} = 16.88$, $R^2 = 0.56$, $P < .01$ respectively, Fig. 5A, B). The SF-MPQ-2 pain score

Table 1
Demographics, clinical and psychological measures in CRPS participants and healthy controls.

		Healthy controls (X ± SD; $n = 14$)	CRPS participants (X ± SD; $n = 15$)
Sex (female/male)		5/9	11/4
Age (years)		39.2 (25–60)	46.6 (19–70)
BMI (kg m^{-2})		28.3 (22–38)	33.2 (22–43)
CRPS type (I/II)			15/0
Limb affected (upper/lower)			7/8
CRPS triggering factors: (fracture/soft tissue injury/surgery)			7/5/3
Pain score (VAS 0–100)		3.6 ± 4.1	68.7 ± 13.4***
SF-MPQ-2 (0 − 220)		4.8 ± 7.4	109.4 ± 43.0***
DASS 21 (0–42)	Depression	2.7 ± 4.6	10.9 ± 10.1**
	Anxiety	3.0 ± 3.1	13.2 ± 8.1***
	Stress	5.9 ± 6.4	17.7 ± 7.4***
TSK (0–68)		29.1 ± 5.9	40.3 ± 5.1***
PCS (0–52)		3.2 ± 6.0	20.9 ± 10.5***
PSEQ (60–0)		57.4 ± 4.1	25.1 ± 11.4***

Abbreviations: VAS: Visual analogue scale; SF-MPQ-2: Short-form McGill Pain Questionnaire; DASS21: Short-form Depression, Anxiety and Stress Scales; TSK: Tampa Scale for Kinesiophobia; PCS: Pain Catastrophising Scale; PSEQ: Pain Self-Efficacy Questionnaire. ** $P < .01$, *** $P < .001$, Mann-Whitney U test.

Table 2
Cytokine protein levels in the serum of CRPS and healthy control groups.

Cytokine (pg/mL)	Healthy controls (X ± SD; n = 14)	CRPS participants (X ± SD; n = 15)
GM-CSF	87.3 ± 66.8	99.1 ± 89.7
IFN-γ	14.1 ± 8.4	12.2 ± 7.4
TNF-α	6.1 ± 2.2	7.3 ± 2.0
IL-1β	1.9 ± 1.0	1.7 ± 0.8
IL-2	5.4 ± 3.6	4.9 ± 2.9
IL-4	13.5 ± 8.7	11.5 ± 6.2
IL-5	1.9 ± 1.3	1.4 ± 0.7
IL-6	2.0 ± 1.4	1.3 ± 0.3
IL-8	7.7 ± 1.9	7.5 ± 3.2
IL-10	5.9 ± 3.7	4.8 ± 2.2
IL-12	3.7 ± 2.3	3.0 ± 1.1
IL-13	4.2 ± 2.5	4.3 ± 2.3
IL-17A	19.4 ± 11.3	15.4 ± 8.0
IL-23	516.0 ± 393.4	313.8 ± 141
IL-37	33.0 ± 7.8	27.4 ± 5.0*

Abbreviations: GM-CSF: Granulocyte-macrophage colony stimulating factor; IFN-γ**:** Interferon-γ**;** TNF-**α:** Tumor necrosis factor**-α;** IL: Interleukin.
* $P < .05$ unpaired t-test.

Table 3
Soluble cytokine protein levels in the serum of CRPS and healthy control groups.

Cytokine (pg/mL)	Healthy controls (X ± SD; n = 14)	CRPS participants (X ± SD; n = 15)
sCD30	48.4 ± 16.8	39.3 ± 32.4
sEGFR	54,017 ± 7897	55,679 ± 5288
sgp130	139,404 ± 22,538	132,726 ± 27,371
sIL-1RI	30.0 ± 6.3	29.0 ± 8.1
sIL-1RII	10,831 ± 2454	11,506 ± 2442
sIL-2Ra	752.4 ± 285.1	767.2 ± 265
sIL-4R	626.3 ± 255.2	542.1 ± 276.7
sIL-6R	21,978 ± 4370	21,902 ± 5619
sRAGE	24.2 ± 8.0	22.4 ± 10.1
sTNFRI	1222 ± 377.3	1101 ± 379.6
sTNFRII	5399 ± 1862	6277 ± 1803
sVEGFR1	116.3 ± 87.6	109.4 ± 100.4
sVEGFR2	19,338 ± 4986	21,133 ± 4273
sVEGFR3	1344 ± 1073	717 ± 293.1

Abbreviations: sCD30: soluble cluster of differentiation 30; sEGFR: soluble epidermal growth factor receptor; sgp130: soluble glycoprotein 130; sIL-R: soluble interleukin receptor; sRAGE: soluble receptor for advanced glycation end products; sTNFR: soluble Tumor necrosis factor; sVEGFR: soluble vascular endothelial growth factor receptor.

significantly correlates with BH4 ($F_{1,12} = 7.43$, $R^2 = 0.38$, $P < .05$, Fig. 5C). The KYN/TRP ratio correlates with the kinesiophobia score ($F_{1,13} = 5.73$, $R^2 = 0.31$, $P < .05$, Fig. 5D).

3.4. Machine learning analysis

The performance of all feature sets in each of the three classification models is indicated by the AUC values shown in Table 5. Feature set 1, which contained 67 features, including cytokines, soluble cytokines, KYN and BH4 metabolites, and immune cell variables, yielded AUC values ~0.60 across the three models, indicating that the ability to successfully distinguish CRPS participants from healthy controls was only a slightly better than chance. Feature set 2, which used 4 features of interest, GM-CSF, IL-37, Treg cell number and CD8 + central memory T cell number, was also poor at distinguishing CRPS participants from healthy controls, with AUCs ranging from 0.54 to 0.62. However, the third feature set, which reduced these four features to binary values based on pre-determined thresholds, was much more successful at distinguishing CRPS participants or healthy controls, with AUCs above 0.7 for all three models.

Table 4
Levels of KYN and BH4 pathway metabolites in the serum of CRPS participants and healthy controls.

Metabolite	Healthy controls (X ± SD; n = 14)	CRPS participants (X ± SD; n = 15)
TRP (μmol/L)	50.6 ± 7	41.8 ± 7.4**
KYN (μmol/L)	2.1 ± 0.5	2.0 ± 0.5
3-HAA (nmol/L)	17.0 ± 5.2	18.7 ± 5.8
AA (nmol/L)	10.4 ± 2.5	11.8 ± 5.4
KYNA (nmol/L)	24.6 ± 7.4	25.8 ± 10.4
3-HK (nmol/L)	10.6 ± 2.8	10.6 ± 4.1
XA (nmol/L)	15.5 ± 7.2	18.8 ± 11.2
PIC (nmol/L)	24.7 ± 4.9	25.6 ± 4.7
QUIN (nmol/L)	212.9 ± 71.3	193.1 ± 81.3
BH4 (nmol/L)	5.2 ± 3.3	4.7 ± 1.8
NEO (nmol/L)	25.4 ± 7.9	28.3 ± 5.4

Abbreviations: TRP: Tryptophan; KYN: Kynurenine; 3-HAA: 3-Hydroxyanthranilic acid; AA: Anthranilic acid; KYNA: Kynurenine acid; 3-HK: 3-Hydroxykynurenine; XA: Xanthurenic acid; QUIN: Quinolinic acid; PIC: Picolinic acid; BH4: Tetrahydrobiopterin; NEO: Neopterin. All data are presented as mean ± standard deviation (X ± SD). **$P < .01$, unpaired two-tailed Mann-Whitney U test.

Fig. 6 shows a diagrammatic representation of a decision tree classification based on 100 resamples of feature set 3. The average AUC of this model is 0.72, showing good prediction ability. Logistic Regression performed the best of the three validation models with an AUC of 0.79, implying far better than random ability to distinguish CRPS from healthy controls based on these four binary parameters. The performance of the Logistic Regression model using feature set 3 is shown in a typical ROC curve (Fig. 7).

Thus, feature set 3, consisting of only four binary variables, GM-CSF, IL-37, the number of Treg cells and CD8 + Tcm cells, could offer a simple set of biomarkers for distinguishing CRPS from healthy controls. However, we caution that given that the analysis has been performed on a relatively small cohort of 14 healthy controls and 13 CRPS participants, these findings should be considered preliminary and be validated with larger studies.

4. Discussion

This comprehensive analysis of the immune and metabolic status of CRPS subjects compared to healthy controls has yielded several novel results. We observed significant decreases in serum IL-37 and TRP in CRPS participants, and particularly high levels of GM-CSF in a subset of CRPS participants. Additionally, we identified several correlations between different KP and BH4 metabolites and CRPS symptoms and psychological metrics. Finally, utilizing machine learning analysis, we identified a set of four binary variables that may represent a simple set of biomarkers for distinguishing chronic CRPS from healthy controls. Interpretation of these preliminary findings is somewhat speculative but nevertheless potentially illuminating.

4.1. Low interleukin-37 levels in CRPS participants

We observed significantly lower levels of IL-37 in CRPS participants. IL-37 is a newly discovered cytokine with potent immunosuppressive and anti-inflammatory actions. Evidence of its importance in maintaining immune homeostasis, and its involvement in various disease processes, especially autoimmune and inflammatory ones, is fast accumulating (Shuai et al., 2015; Dinarello et al., 2016; Bello et al., 2018; Jia et al., 2018; Wang et al., 2018a; Wang et al., 2018b). IL-37 plays a pivotal role in providing tolerance to self in the face of excessive immune-triggered responses. The primary source of IL-37 in humans are monocytes and dendritic cells (Rudloff et al., 2017). IL-37 has been shown to suppress the adaptive immune response via induction of

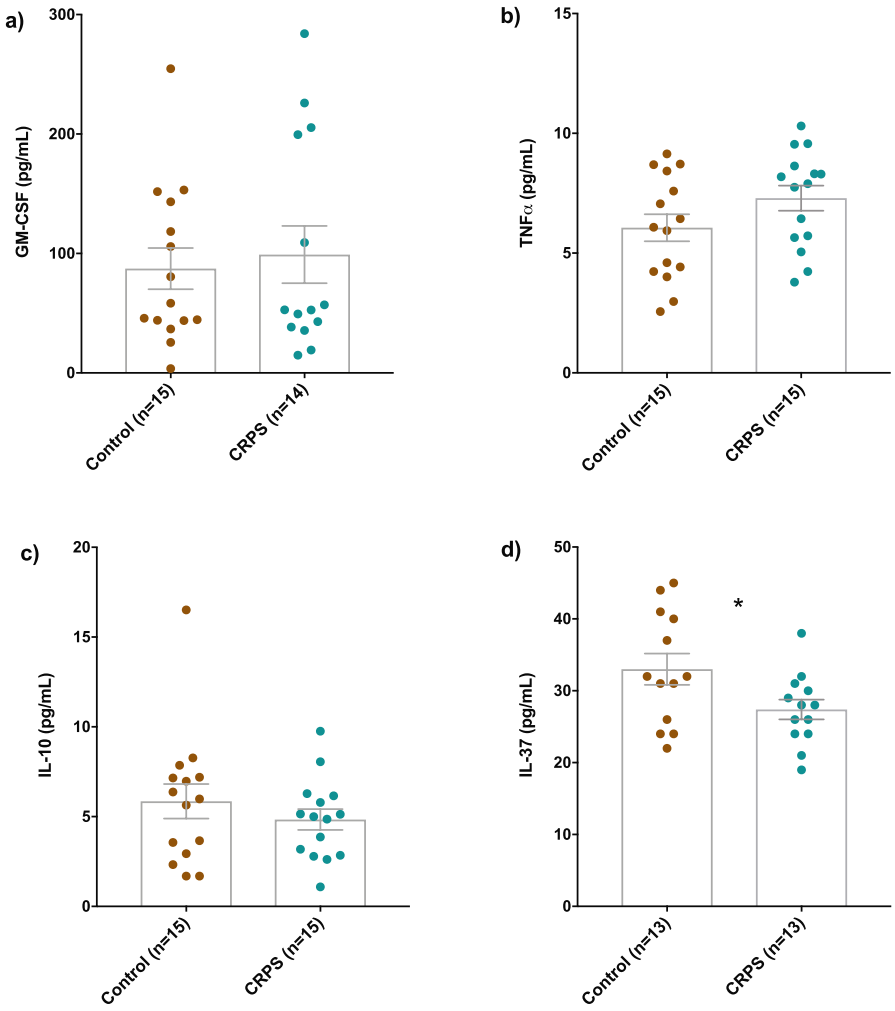

Fig. 2. Cytokine levels in the serum of CRPS and healthy control groups. (A) GM-CSF, (B) TNF-α, (C) IL-10 and (D) IL-37. Mean ± SEM. * $P < .05$ unpaired *t*-test.

tolerogenic dendritic cells, which results in increased IL-10 production, suppression of CD8+ T cell proliferation, and induction of Treg cells with enhanced suppressive activity (Luo et al., 2014; Wang et al., 2016; Liu et al., 2019). Interestingly, we found no difference in IL-10 levels, and previously found an increase in CD8+ T cells in CRPS (Russo et al., 2019).

One possible explanation for lower IL-37 levels lies in the protein sequence (Kang et al., 2015). The third major variant of IL-37, "Var2", is less potent due to preferential proteasome degradation, lower secretion, and rapid accumulation and clearance after stimulation (immune response) (Guo et al., 2017). Var2 inhibition of TNF-α and IL-6 is also reduced. Individuals with Var2 are at risk of being unable to mount as much of an anti-inflammatory response as others, and this might be reflected in the lower IL-37 levels observed in CRPS subjects who have gone on to a maintained inflammatory disease state.

4.2. Cytokines and CRPS symptoms

Although we did not find significant differences in other serum cytokine levels between groups, we did identify a subset of CRPS participants that displayed particularly high levels of GM-CSF. GM-CSF is a pleiotropic cytokine that has been shown to polarise macrophages towards an M1 pro-inflammatory phenotype (Hamilton, 2008) and sensitise peripheral nociceptors and cause sprouting of sensory nerve endings in the skin (Schweizerhof et al., 2009).

Previous studies have identified an increase in numerous pro-inflammatory cytokines including TNF-α, sIL-1RI, IL-1Ra, IL-2, sIL−2Ra, IL-7, IL-8, IFN-γ and sRAGE, and a decrease in anti-inflammatory cytokines, IL4 and IL-10, in blood, CSF, and blister fluid of CRPS patients (Alexander et al., 2005; Schinkel et al., 2006; Alexander et al., 2007; Uceyler et al., 2007; Parkitny et al., 2013). We observed a trend towards elevated TNF-α and reduced IL-10, supporting previous studies which suggest an imbalance of pro- and anti-inflammatory cytokines in CRPS.

Interestingly, levels of pro-inflammatory mediators TNF-α and sRAGE correlated with kinesiophobia, stress and anxiety, all major symptoms of CRPS.

4.3. Low TRP and KP metabolites in CRPS participants

We observed significantly lower TRP levels in CRPS participants compared to healthy controls. This is consistent with the work of Alexander et al., which was conducted in a much larger cohort of 160 CRPS subjects and 60 healthy controls (Alexander et al., 2013). They also showed that TRP level was inversely associated with disease duration. A decrease in TRP may indicate that it is being metabolised down the KP, however, we did not see a significant increase in KP metabolites, whereas Alexander et al. reported an increased KYN/TRP

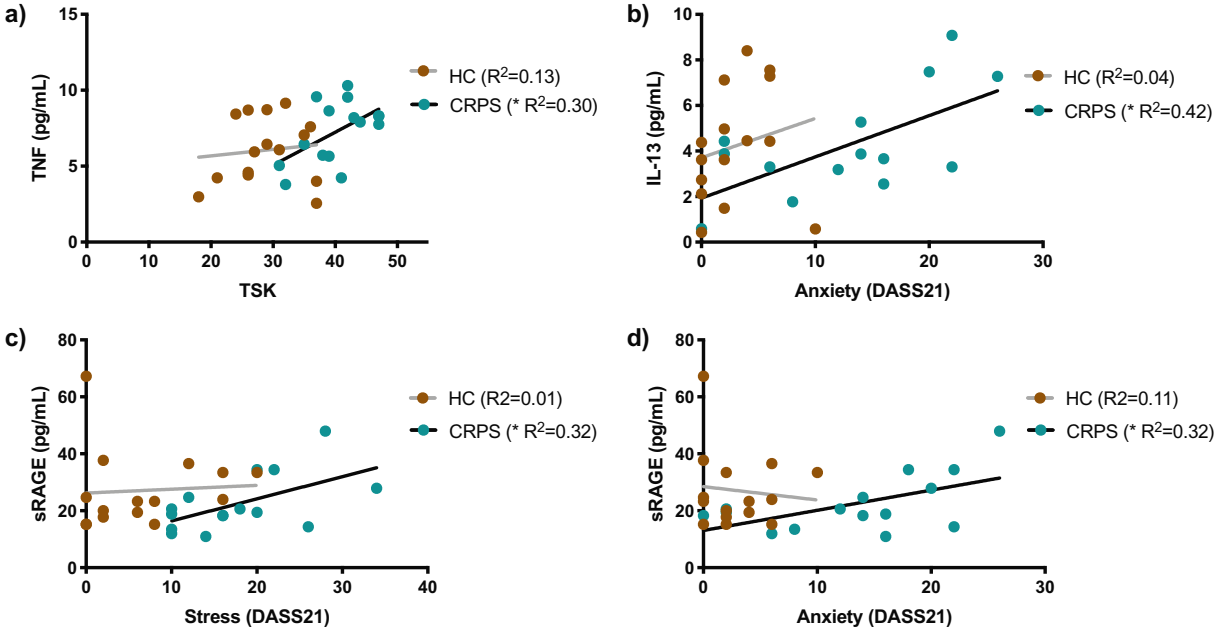

Fig. 3. The relationships between serum cytokine levels and psychological measures in CRPS and healthy control groups. (A) TNF-α and Tampa Scale for Kinesiophobia (TSK) score correlate in the CRPS group. (B) IL-13 and DASS21 anxiety score correlate in the CRPS group. (C) sRAGE and DASS21 stress score correlate in the CRPS group. (D) sRAGE and DASS21 anxiety score correlate in the CRPS group. * $P < .05$ & R^2 from linear regression analysis.

ratio, but not an increase in KYN, suggesting the decrease in TRP was driving the increased KYN/TRP ratio. Alexander et al. found that the KYN/TRP ratio was significantly correlated with overall pain level. Low TRP has been associated with low mood due to a reduction in serotonin production (Ruhé et al., 2007), which in addition to KP metabolites (discussed below), could be a contributing factor to the comorbid depression seen in CRPS. TRP is also the precursor for melatonin, which could be altered in CRPS (Srinivasan et al., 2012; Chen et al., 2016; Danilov and Kurganova, 2016; Yang et al., 2018).

Despite there being no significant differences in KP and BH4 pathway metabolites between groups, we found multiple significant correlations between metabolites and pain and psychological measures in CRPS participants. NEO, a well-known inflammatory biomarker, correlates with depression scores. There is mounting evidence that ongoing peripheral inflammation is linked to depression (Walker et al., 2014; Fiore and Austin, 2016). XA correlates with depression and stress scores, but it is one of the least well studied KYN metabolites (Roussel et al., 2016). Pre-clinical studies have reported that XA has analgesic properties (Heyliger et al., 1998). XA has been found to be decreased in cluster headache and increased in migraine (Curto et al., 2015a; Curto et al., 2015b). The KYN/TRP ratio, which is indicative of IDO activity and inflammation, correlates with kinesiophobia, a major symptom of

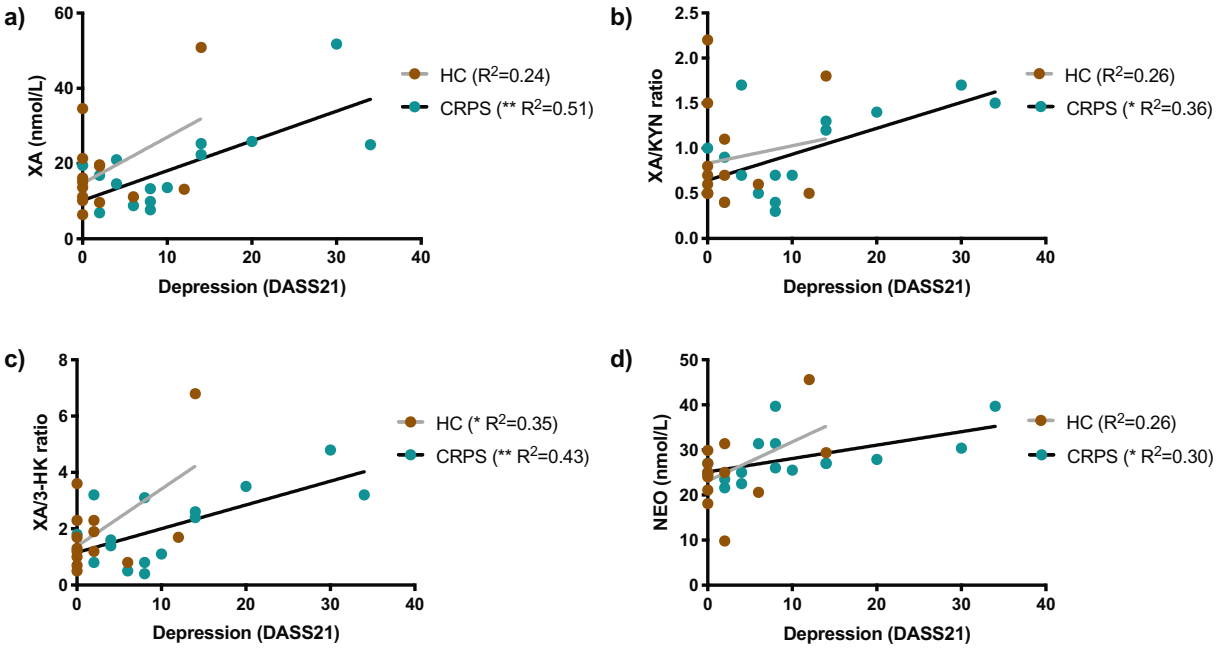

Fig. 4. The relationships between KYN and BH4 metabolites, and depression in CRPS and healthy control (HC) groups. (A) XA, (B) XA/KYN ratio, (C) XA/3-HK ratio and (D) NEO correlate with the DASS21 depression score in the CRPS group. * $P < .05$, ** $P < .01$ & R^2 from linear regression analysis.

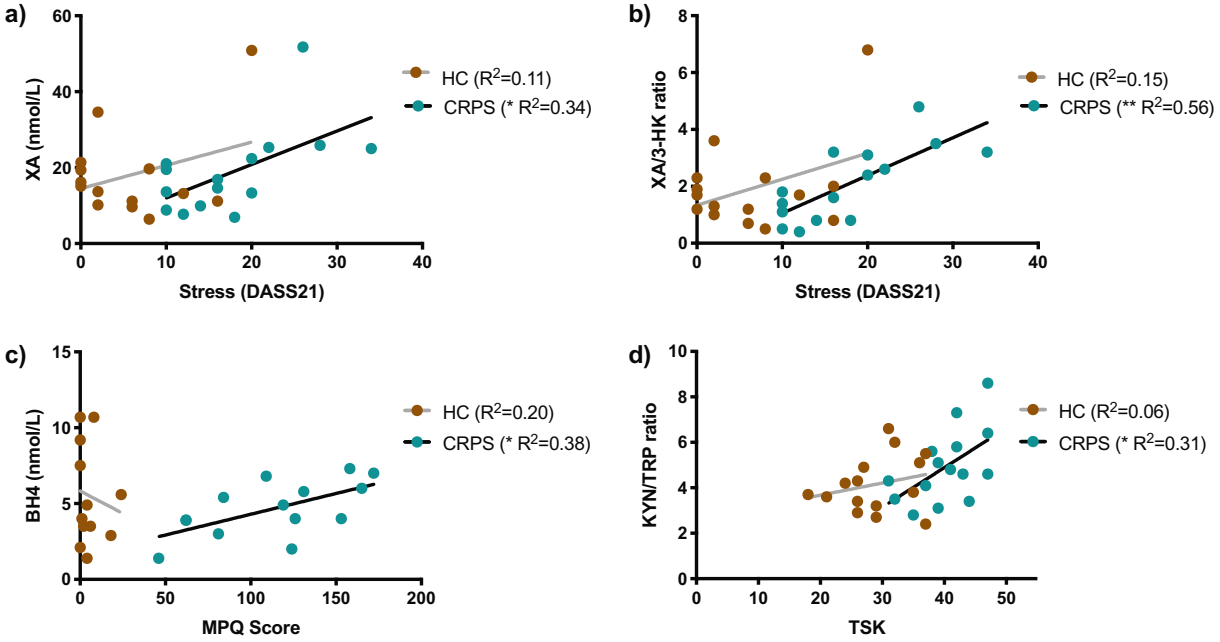

Fig. 5. The relationships between KYN and BH4 metabolites, and pain and psychological measures in CRPS and healthy control (HC) groups. (A) XA and the DASS21 stress score correlate in the CRPS group. (B) XA/3-HK ratio and the DASS21 stress score correlate in the CRPS group. (C) BH4 and the SF-MPQ-2 pain score correlate in the CRPS group. (D) KYN/TRP ratio and the TSK score correlate in the CRPS group. * $P < .05$, ** $P < .01$ & R^2 from linear regression analysis.

Table 5
Table of area under the ROC curve (AUC) performance by model and by feature set.

Model	Feature Set	AUC	Resamples
Logistic Regression	1	0.61	500
Decision Tree	1	0.57	500
Gradient Boosting	1	0.60	500
Logistic Regression	2	0.59	500
Decision Tree	2	0.54	500
Gradient Boosting	2	0.62	500
Logistic Regression	3	0.79	500
Decision Tree	3	0.72	500
Gradient Boosting	3	0.71	500

many chronic pain conditions. Interestingly, TNF-α, which potentiates IDO activity, also correlates with kinesiophobia. We also found a significant correlation between BH4 and the McGill pain score in CRPS participants. BH4 is an essential co-factor required for the production of dopamine, noradrenaline, serotonin, and nitric oxide, and therefore plays a role in mood and pain (nociceptive signalling) (Sumi-Ichinose et al., 2001; Latremoliere and Costigan, 2011). Several studies have shown that BH4 is increased following injury to the somatosensory system, causing increased pain sensitivity, and that blocking BH4 production provides analgesia in inflammatory and neuropathic pain models (Tegeder et al., 2006; Latremoliere and Costigan, 2011; Costigan et al., 2012; Latremoliere et al., 2015). Both the BH4 and KYN pathways have been associated with inflammatory-induced depression (Vancassel et al., 2018).

KYN enzymes QUIN and KYNA exert their neurological effects partly via NMDA receptors (Guillemin, 2012; Savitz, 2020), which are expressed in peripheral nerve fibres, spinal cord, and the brain, playing an important role in nociception and the pathogenesis of persistent pain (Petrenko et al., 2003; Gunn et al., 2020). Interestingly, there is increased expression of NMDA receptors on peripheral nociceptors during inflammation (Carlton and Coggeshall, 1999). The neurological effects of the KP could therefore be peripheral, however, since KYN and TRP can cross the blood brain barrier, there may also be some central effects

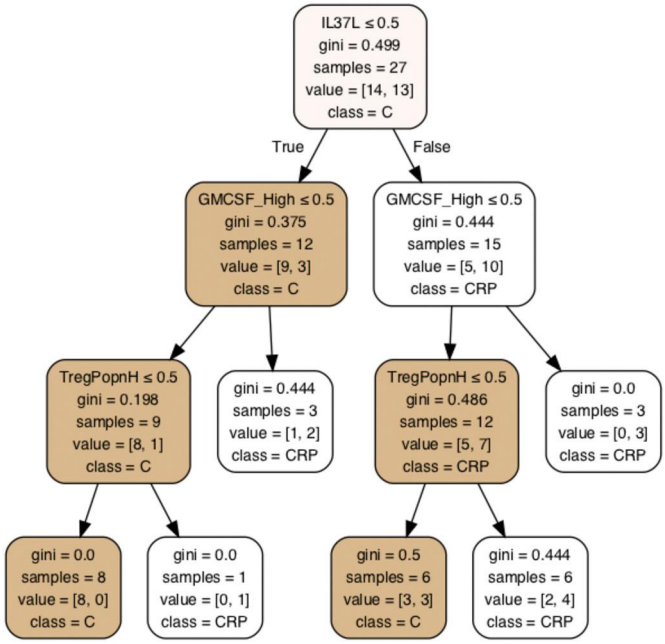

Fig. 6. Decision tree classification based on 100 resamples on feature set 3 (average AUC = 0.72).

(Savitz, 2020).

4.4. Machine learning findings

Machine learning provides new ways to assess large amounts of data or extract new insights into the pathophysiology of diseases (Li et al., 2018). We used machine learning algorithms to look for inputs with reasonable predictive capacity to distinguish chronic CRPS subjects from healthy controls. The best performance came from feature set 3, which combined IL-37 (low), GM-CSF (high), Treg cell number (high) and CD8 + T cell number (high) and gave an AUC of 0.79 (approaching

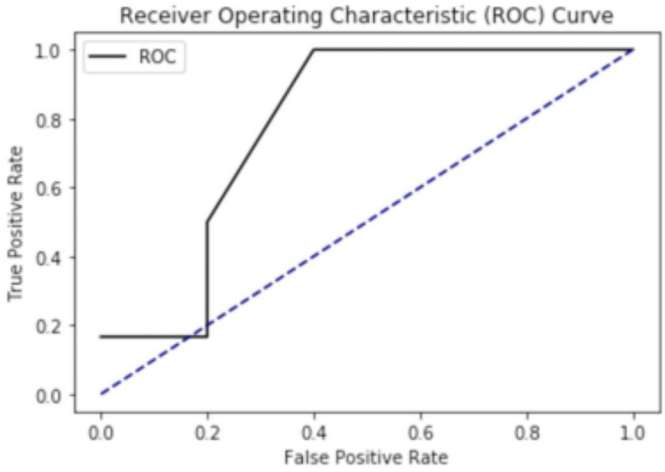

Fig. 7. Receiver operating characteristic (ROC) curve showing the typical performance of logistic regression on feature set 3. The AUC is the area under the ROC curve.

clinical usefulness).

The relevance of IL-37 and GM-CSF have been discussed above. Treg cells are a marker of anti-inflammatory activation and would be expected to be significantly elevated in a condition where pro-inflammatory changes dominate. CD8+ cytotoxic T cells are a marker of downstream adaptive immune activation and would be expected to be elevated in sustained immune activated conditions. In particular, they induce local accumulation of dendritic cells and antigen-specific CD8+ T cells (Min et al., 2010) and enhance dendritic cell cross-presentation (Greter et al., 2012; Zhan et al., 2012; Lee et al., 2015). Thus, these four inputs reflect a range of inherent immunological capability and immune system activation. That combined perturbations in these inputs away from normal values is found in CRPS subjects is mechanistically appealing.

4.5. Limitations

This study is limited by its small sample size. As a pilot investigation, validation of the results will require a larger sample, which will reduce the chance of type II error. A major limitation is that the controls were not gender-matched, with more females in the CRPS group. This possibly contributed to a disproportionate effect as the male and female immune systems differ, particularly in response to injury and pain processing (Klein and Flanagan, 2016). CRPS is more common in females than males at a ratio of 3:1, and although the proportion of females in our CRPS group was greater than in the healthy control group, this was not statistically significant ($P = .07$). Additionally, the serum samples were non-fasting, and metabolite analysis could have been influenced by recent dietary TRP consumption, though it is not clear whether TRP consumption would have differed between groups. We recommend a larger study with age and sex-matched controls, fasting before blood collection, and subgroup analysis of male and female data. It is also possible that our findings may be reflective of trauma unrelated to the development CRPS, and hence it may be worthwhile to compare CRPS patients to a group of patients who experienced similar injury or underwent similar surgery but did not develop CRPS. This would be most useful for investigations of acute CRPS patients. For chronic CRPS, healthy controls should be appropriate. Finally, while machine learning approaches are being refined over time, we cannot say if the variable set we have identified is likely to hold true for the greater CRPS population without performing a biomarker validation study.

4.6. Conclusion

Using a novel combined approach of targeted analysis, we found that reduced IL-37 and TRP, and increased Tregs, CD8+ T cells and GM-CSF may be critical to the inflammatory activation seen in CRPS. Several pro-inflammatory cytokines and KP and BH4 metabolites correlated with CRPS symptoms and disease severity, suggesting possible aetiological importance. The association of several immune markers and psychological variables suggests a link between cognitive and emotional responses and underlying immune markers. We believe that a coordinated analysis by a regional network or even a global CRPS population approach utilizing similar techniques combined with analysis of autoantibodies will yield more definitive understandings of the aetiological stages of this disease and allow validation of potential biomarker sets. There may be multiple mechanisms that can come into play for any one individual, but that results in the same CRPS condition, making single studies of a single variable difficult to interpret. Our findings can only inform future studies and be hypothesis generating, rather than definitive in nature.

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Appendix: Supplementary table 1. Individual participant demographics, clinical and psychological measures in CRPS group.

ID	Gender	Age	Height	Weight	BMI	VAS (0-100)	DASS21 (0-42)			SF-MPQ-2 (0-220)	PCS (0-52)	PSEQ (0-60)	TSK (0-68)	Time since onset (mths)	CRPS trigger	Affected limb	Symptom spread	Analgesic medication
							D	A	S									
1	F	19	157	76	30	81	2	12	18	165	38	26	44	14	Soft tissue injury	Left lower	Knee to foot	Acetylsalicylic acid, gabapentin, palmitoylethanolamide, phenoxybenzamine
2	M	22	169	75	26	65	2	0	16	119	20	14	37	16	Fracture	Right upper	Hand to mid arm	Paracetamol, codeine phosphate
3	F	48	169	65	22	68	14	22	22	109	18	20	32	8	Fracture	Left lower	Toes to fibula	Nil
4	M	37	165	115	42	63	4	8	10	62	8	38	39	5	Fracture	Left upper	Slightly	Pregabalin
5	M	32	190	88	24	74	20	26	28	84	40	10	43	4	Fracture	Left lower	No	Paracetamol, tapentadol
6	F	58	162	100	38	79	8	14	12	131	7	45	38	42	Surgery	Left lower	No	Nil
7	M	53	172	84	28	43	34	20	34	172	26	9	35	54	Fracture	Left upper	Hand to neck	Amitriptyline, paracetamol, tapentadol
8	F	44	160	111	43	98	0	2	10	62	20	12	41	138	Fracture	Right upper	No	Pregabalin, morphine sulfate pentahydrate
9	F	59	156	90	37	80	14	18	20	124	31	30	42	27	Surgery	Left lower	No	Paracetamol
10	F	61	166	97	35	65	8	16	14	126	12	25	31	282	Soft tissue injury	Right lower	No	Fentanyl
11	F	57	166	112	40	64	6	6	10	81	28	27	47	29	Fracture	Right lower	No	Paracetamol
12	F	53	175	76	25	60	8	2	20	49	11	39	47	2	Soft tissue injury	Left lower	No	Amitriptyline, pregabalin
13	F	25	166	101	37	79	4	14	16	153	19	23	47	6	Soft tissue injury	Right upper	Hand to shoulder	Diclofenac, gabapentin, tramadol
14	F	61	159	94	37	55	10	16	10	158	9	39	39	71	Soft tissue injury	Left upper	Hand to elbow	Nil
15	F	70	162	92	35	57	30	22	26	46	26	19	42	5	Surgery	Right upper	Thumb to shoulder	Acetylsalicylic acid, gabapentin
	Mean	46.6	166.3	91.7	33.3	68.7	10.9	13.2	17.7	109.4	20.9	109.4	40.3	46.9				
	SD	16.0	8.5	14.8	6.9	13.4	10.1	8.1	7.3	43.0	10.5	11.4	5.11	74.4				

Abbreviations: VAS: Visual analogue scale; SF-MPQ-2: Short-form McGill Pain Questionnaire; DASS21: Short-form Depression (D), Anxiety (A) and Stress (S) Scales; TSK: Tampa Scale for Kinesiophobia; PCS: Pain Catastrophising Scale; PSEQ: Pain Self-Efficacy Questionnaire.

Appendix: Supplementary table 2. Activity of KYN pathway enzymes in the serum of CRPS participants and healthy controls.

Enzymes	Expression	Healthy controls (X ± SD; n=14)	CRPS participants (X ± SD; n=15)
IDO1	100 × KYN/TRP	4.2 ± 1.2	4.9 ± 1.6
KMO	100 × 3-HK/KYN	0.5± 0.24	0.5± 0.29
KAT A	100 × KYNA/KYN	1.2 ± 0.3	1.3 ± 0.5
KAT B	100 × XA/3-HK	155.4 ± 84	207.7 ± 129
Total KAT	KAT A + KAT B	156.6± 84	209.0± 129
KYNU A	100 × AA/KYN	0.5 ± 0.12	0.6 ± 0.25
KYNU B	100 × 3-HAA/3-HK	175 ± 79	206.9 ± 105
Total KYNU	KYNU A + KYNU B	175.7 ± 79	207.5 ± 105

Abbreviations: IDO1: Indoleamine 2,3-dioxygenase; KMO: Kynurenine 3-monooxygenase; KYNU: Kynureninase; KAT: Kynurenine aminotransferases; 3-HAA: 3-Hydroxyanthranilic acid. All data are presented as mean ± standard deviation (X ± SD).

Appendix: Supplementary table 3. KYN pathway branches in CRPS and healthy control participants.

Pathway direction	Expression	Healthy controls (X ± SD; n=14)	CRPS participants (X ± SD; n=15)
KYNA production	100 × KYNA/KYN	1.2 ± 0.3	1.3 ± 0.5
AA production	100 × AA/KYN	0.5 ± 0.1	0.6 ± 0.2
XA production	100 × XA/KYN	0.62 ± 0.1	0.96 ± 0.4
3-HAA production	100 × 3-HAA/KYN	0.83 ± 0.2	1 ± 0.3
3-HAA : AA ratio	3-HAA/AA	1.614± 0.1950	1.714± 0.2218
PIC production	100 × PIC/KYN	0.088 ± 0.003	0.104 ± 0.02
QUIN production	100 × QUIN/KYN	49.2 ± 15	47.03 ± 18

Abbreviations: KYNA: Kynurenine acid; AA: Anthranilic acid; XA: Xanthurenic acid; 3-HAA: 3-Hydroxyanthranilic acid; PIC: Picolinic acid; QUIN: Quinolinic acid. All data are presented as mean ± standard deviation (X ± SD).

ORIGINAL RESEARCH ARTICLE: TETRAHYDROBIOPTERIN AS A KEY FACTOR PROMOTING COLITIS ABDOMINAL PAIN

(P3) Gastroenterology:
Tetrahydrobiopterin as a key factor promoting colitis abdominal pain.
Staats Pires, A.; Turnes, B. L.; Scheffer, D. L.; Menegassi, V. S.; Rizzatti, S. M.; Niero, L.; Guillemin, G.; Latini, A. (Final revisions).

This paper is under final revisions to be submitted in the journal Gastroenterology (impact factor 22.682). **ASP contributed to the conception of the study, sample collection and preparation, clinical database management, data acquisition and curation, writing the original draft and revising the article.** BLT contributed to the conception of the study, data acquisition, revising the article. DLS contributed to sample preparation and data acquisition. VSM contributed to participant recruitment and clinical database management. SMR contributed to sample collection, sample preparation and clinical database management. LN contributed to sample clinical database management. GJG contributed to the conception of the study, funding acquisition and revising the article. AL contributed to the whole conception of the study, project supervision, funding acquisition and revising the article.

Research Gap

Ulcerative colitis (UC) is characterized by chronic inflammation of the colon mucosal layer (Satsangi, Silverberg, Vermeire, & Colombel, 2006). The cause of this aberrant immune response remains largely unknown, but environmental risk factors have a role as well as host factors such as genetic susceptibility and gut microbiota (Silverberg et al., 2005). Clinical course of UC is characterized by alternating periods of symptoms presentation, which include hematochezia, diarrhea, faecal incontinence and abdominal pain (Satsangi et al., 2006).

The annual incidence of UC has steadily increased over the last decades worldwide, particularly in industrialized countries (Molodecky et al., 2012). Additionally, patient care in UC remains challenging and a cure for this disease is not currently available (Satsangi et al., 2006). The current treatment goal for UC is aimed at the maintenance of steroid-free disease remission (Magro et al., 2017). This target seems to be insufficient to completely abolish abdominal pain. A sizable proportion of UC patients still experience abdominal pain during disease remission (Fukuba et al., 2017; Fukuba et al., 2014; Ishihara et al., 2019), which negatively impacts patients' quality of life (Minderhoud, Oldenburg, Wismeijer, van Berge Henegouwen, & Smout, 2004).

The negative impact of persistent UC-related abdominal pain associated with the increased rate of opioids prescriptions for abdominal pain (Dorn, Meek, & Shah, 2011), underscoring the need for better understanding and management of UC-related abdominal pain. In this context, excessive activation of the tetrahydrobiopterin (BH4) pathway due to inflammatory activation has been characterised in different models of chronic pain (Fujita et al., 2019; Latremoliere et al., 2015; Tegeder et al., 2006).

Additionally, BH4 is a mandatory cofactor for the production of serotonin (Miwa, Watanabe, & Hayaishi, 1985) and changes in the BH4 levels can impact serotonin levels in the colon of mice (Kobayashi, Hasegawa, Kaneko, & Ichiyama, 1991). Serotonin is a critical signalling molecule in the colon and the activation of gut serotoninergic receptors have been implicated in visceral hypersensitivity (Kayser et al., 2007). Therefore, the measurement of BH4, serotonin and other biochemical markers in biological fluids from patients with UC may offer relevant information about the molecular mechanisms that might underlie the UC-related abdominal pain.

Key Results & Implications

The findings reported in our cross-sectional analysis of BH4 metabolism and clinical features in a UC cohort indicate that 1) abdominal pain is a prevalent symptom during active disease (85.7 %) and remission (47.8 %) stages of UC, and 2) an exacerbation of the BH4 metabolism may increase serotonin levels and ultimately contribute to abdominal pain in UC. Specifically, we observed *i)* higher levels of BH4 in the urine and plasma of UC participants compared to pain-free healthy volunteers (HV) participants. *ii)* The plasma levels of C-reactive protein (CRP; *i.e.*, an inflammatory marker) and zonulin (*i.e.*, a marker of increased intestinal permeability) were increased in UC-affected subjects, suggesting inflammation and intestinal microbiota as two possible sources of exacerbated BH4 metabolism. *iii)* Additionally, plasma rich platelet serotonin levels were positively correlated with urinary BH4 levels and pain levels in UC-affected patients. Given the fact that the rate-limiting enzyme of serotonin biosynthesis is dependent on the cofactor BH4, it is feasible that exacerbated BH4 metabolism may contribute to increased serotonin production. In turn, the increased serotonin levels can favour colonic hypersensitivity and abdominal pain in UC-affected individuals. Finally, *iv)* a significant increase in urinary BH4 levels were identified in UC patients with pain *vs* pain-free HV, but not in the UC patients without pain. This observation may support the hypothesis that the exacerbation of the BH4 metabolism is a key factor in the establishment of UC-related abdominal pain and that the urinary BH4 levels may be a feasible candidate to be further investigated as a biomarker of UC-related abdominal pain. Our clinical data suggest the importance of the BH4 metabolism exacerbation for UC-related abdominal pain. Additionally, our results suggest that urinary BH4 levels can be a low-invasive biomarker to detect/indicate the presence and severity of abdominal pain in UC. Our findings endorse further investigation regarding BH4 metabolism in UC-related abdominal pain.

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Tetrahydrobiopterin as a key factor promoting colitis abdominal pain

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ABSTRACT

Ulcerative colitis (UC) is a chronic inflammatory disorder of the colonic mucosa, which is marked by alternating periods of exacerbation and remission. Abdominal pain is a prevalent symptom during the development of the UC disease, and it is still present in 30 % of affected patients with negative endoscopic lesions and markers of inflammation, suggesting that other factors might also contribute to this symptomatology. Tetrahydrobiopterin (BH4) has traditionally been investigated due to its role as a cofactor for the synthesis of neurotransmitters and nitric oxide. Excessive activation of the BH4 pathway was recently characterized in different models of inflammatory and neuropathic pain. Based on that, we aim to identify whether the activation of the BH4 pathway contributes to abdominal pain in the UC. In a cross-sectional study, outpatients with a clinical diagnosis of UC (n=60) or age- and sex-matched healthy participants (n=55) were included. All the participants rated their abdominal pain in the last 3 months on a Visual Analogue Scale. Urine and blood samples were collected for further biochemical analysis. We identified increased levels of BH4 in urine and plasma of patients with UC that experience chronic abdominal pain. Overall, our results demonstrated an exacerbation of the BH4 metabolism in patients with UC-related abdominal pain. Additionally, increased BH4 urinary levels were positively correlated with serotonin plasma levels, while the last was positively correlated with abdominal pain levels. These findings indicate that changes in the biosynthesis of BH4 may impact serotonin availability and, ultimately, contribute to the abdominal pain associated with ulcerative colitis. Our research encourages further studies exploring the exact source of BH4 in colitis-related abdominal pain.

Abbreviations

BH4, Tetrahydrobiopterin;

BMI, Body mass index;

CRP, C-reactive protein;

DHPR, Dihydrofolate reductase;

DTE, Dithioerythritol;

EC, Extensive colitis;

ECC, Enterochromaffin cells;

GCH1, Gene that codified for the GTPCH;

GTPCH, GTP cyclohydrolase;

HPLC, High performance liquid chromatography;

HV, Healthy volunteers;

LC, Left side colitis;

PC, Proctitis;

SPR, Sepiapterin reductase;

TCA, Trichloroacetic acid;

TPH, Tryptophan hydroxylase;

UC, Ulcerative colitis;

VAS, Visual analogue scale.

1 Introduction

Ulcerative colitis (UC) is a chronic inflammatory condition that causes mucosal inflammation of the colon. The inflammation in UC characteristically commences in the rectum and extends proximally in a continuous manner to affect a variable extent of the colon or its entire mucosal surface. The clinical course of UC is characterised by alternating periods of remission and relapse (Satsangi, Silverberg, Vermeire, & Colombel, 2006). Currently, there is no cure for UC and the treatment goal is to maintain complete remission (*i.e.*, symptomatic and endoscopic remission) without corticosteroid therapy (Magro et al., 2017).

Symptoms of UC are dependent upon the extent and severity of the disease and include bloody diarrhoea, rectal bleeding, tenesmus, urgency, abdominal pain and faecal incontinence (LennardJones & Shivananda, 1997). More than 50 - 70 % of patients affected by colitis present pain as their initial symptom, or as a prevalent symptom during the development of the disease (Bielefeldt, Davis, & Binion, 2009). The fact that abdominal pain is still present in approximately 30 % of affected patients with negative endoscopic lesions and markers of inflammation, suggests that other factors might also contribute to this symptomatology (Fukuba et al., 2017; Fukuba et al., 2014; Ishihara et al., 2019; Minderhoud, Oldenburg, Wismeijer, van Berge Henegouwen, & Smout, 2004).

Tetrahydrobiopterin (BH4) has traditionally been investigated due to its role as a mandatory cofactor for phenylalanine metabolism and the synthesis of aminergic neurotransmitters and nitric oxide. The fine regulation of BH4 intracellular concentrations is the result of a balance among three biosynthetic pathways: the *de novo*, recycling and salvage pathways (for review see Ghisoni, Martins, Barbeito, & Latini, 2015). GTP cyclohydrolase (GTPCH) is the rate-limiting enzyme of the *de novo* BH4 pathway and at the transcriptional level is upregulated by a variety of pro-inflammatory mediators (Werner et al., 1990). Indeed, excessive activation of the BH4 pathway was characterized in different models of inflammatory and neuropathic pain (Fujita et al., 2019; Latremoliere et al., 2015; Tegeder et al., 2006). For example, the *de novo* pathway was upregulated in injured sensory neurons, inflamed tissues and immune cells that infiltrate the tissues in experimental pain models (Latremoliere et al., 2015). Specifically, expression and functional profiling have shown that enhanced GTPCH, sepiapterin reductase (SPR) and dihydrofolate reductase (DHPR) transcription and activity in sensory neurons and macrophages lead to increased BH4 levels, which results in greater pain hypersensitivity in rodents (Latremoliere et al., 2015; Tegeder et al., 2006). Additionally, a single BH4 intrathecal administration in *naïve* animals reduced pain thresholds, suggesting the nociceptive activity of BH4 itself (Tegeder et al., 2006).

Considering that UC is a chronic inflammatory disease and most of the affected individuals experience pain, we aimed to investigate in the present study whether the BH4 metabolism is involved in the physiopathology of UC-induced pain. Therefore, a better understanding of the pathophysiology and the recognition of markers in patients affected by UC-related abdominal pain may allow a more rational choice of therapeutic modalities.

2 Methods

2.1 Participants

Participants were recruited from University Hospital, Universidade Federal de Santa Catarina (Florianópolis Brazil) between July 2017 and Sept 2019. The study included both male and female outpatients aged 18–79 years old, with a definite clinical diagnosis of ulcerative colitis (UC; n=60) or age- and sex-matched healthy volunteers (n=55). Exclusion criteria included age less than 18 years; the presence of acute or chronic pain, other than colitis abdominal pain; any neurological, psychiatric or pain condition that could confound the study endpoints; and pregnancy. For the control group, further exclusion criteria included the presence of any acute or chronic pain. All participants from the UC group were taking medication to manage their disease.

2.2 Ethics approval and consent to participate

This study was approved by the Human Ethics Committee from Universidade Federal de Santa Catarina (#54297916.7.0000.0121). Authorisation for access to medical records, demographic information and participants who fit the inclusion criteria were granted by the University Hospital from Universidade Federal de Santa Catarina. Participation in the study was on a completely voluntary basis, and all participants signed informed consent. All demographic and medical information, as well as the samples were de-identified from the study team.

2.3 Pain profiling and samples collection protocol

All participants rated the average pain intensity over the last 3 months, from 0 to 10, on a visual analogue scale (VAS) during the outpatient appointment. On the same occasion, a non-fasted blood sample and a urine sample were collected (Figure 1A). Blood samples were then incubated in an EDTA tube at room temperature for 15 min and then centrifuged at 2,000 × *g* for 10 min. The supernatant was removed and stored in polypropylene aliquot tubes at -80°C until analysis. The urine samples were aliquoted in polypropylene tubes and stored at -80°C until analysis.

2.4 Sample processing

Plasma samples were deproteinised by adding 1 vol. of 5 % trichloroacetic acid (TCA) containing 6.5 mM dithioerythritol (DTE) and centrifuged at 16,000 × *g* for 10 min at 4ºC. Urine samples were deproteinised by adding 10 vol. of 5 % TCA containing 6.5 mM DTE and centrifuged at 16,000 × *g* at 4ºC for 10 min. After centrifugation plasma and urine supernatants were transferred to high performance liquid chromatography **(**HPLC) vials for analysis. All the samples were protected from light and kept on ice during the sample processing.

2.5 Tetrahydrobiopterin (BH4) quantification by HPLC

BH4 concentrations in plasma, urine and colon samples were determined by HPLC and quantified using a sequential electrochemical detection as previously described with some modifications (Latremoliere et al., 2015). The HPLC analysis was carried out in an HPLC system (Alliance e2695, Waters, Milford, USA) by using a Waters Atlantis dC-18 RP column (4.6 x 250mm, 5 µm; Waters, Milford, USA). The temperature of the column compartment was set at 35°C. The injection volume was 20 µL and the autosampler tray temperatures was set at 4ºC to prevent sample degradation. The flow rate was set at 0.6 mL/min with an isocratic elution of a mobile phase consisting of 6.5 mM sodium dihydrogen phosphate, 6 mM citric acid, 1 mM sodium octyl sulfate, 2.5 mM diethylenetriaminepentaacetic acid, 160 µM DTE and 12 % acetonitrile, pH 3.0. The identification and quantification of BH4 were performed by an electrochemical detector (Thermo Scientific - Dionex Coulochem III) with two-sensor electrochemical cells (+50 and +450 mV). The results were calculated by interpolation using a 6-point calibration curve and expressed as nmol/L, µmol/mmol creatinine or pmol/ mg of protein.

2.6 Platelet-Rich Plasma Preparation (PRP)

Blood samples were drawn from the median cubital vein and distributed into sterile vacutainer tubes containing 10% EDTA. The PRP was separate from whole blood by light spin centrifugation 120 × *g* for 10 min at room temperature. Under these centrifugation conditions, platelets remained suspended in the plasma (upper fraction, yellow-colored fluid), while the white and red cells were softly sedimented in the lower fraction. Using a pipette, was carefully collected the upper 25% of the PRP, to limit contamination by white and red cells, and placed into a fresh tube. Next, 10% of EGTA 100mM was added (10% v/v) to the PRP to prevent platelet activation and aggregation and immediately centrifuged at 700 × *g* for 15 min at room temperature. The supernatant was used as platelets poor plasma (PPP). The sediment (PRP) was washed with PBS + 10% of EGTA 100mM

and centrifuged at 700 × *g* for 10 min at room temperature. The final sediment was resuspended in 0.5 mL of PBS + 10% of EGTA 100mM. Finally, PRP and PPP were immediately added of one volume (1:1, v/v) of 5% trichloroacetic acid and centrifuged at 16,000 × *g* for 10 min at 4 °C. The supernatant was frozen and stored - 86 °C.

2.7 Serotonin quantification by HPLC

Serotonin were determined by HPLC (Alliance e2695, Waters, Milford, USA) and quantified by using electrochemical detection as previously described (Scheffer, Ghisoni, Aguiar, & Latini, 2019). Twenty microliters of the s PRP and PPP samples were analyzed in a 150 x 2.0 mm, 4µm, C18 column (Synergi Hydro, California, USA) and an isocratic elution of 90 mM sodium phosphate, 50 mM citric acid, 2.3 mM sodium 1-heptane-sulfonate, 50 µM ethylenediaminetetraacetic acid, 10 % acetonitrile, pH 3.0, with a flow of 0.20 mL / min. The results were expressed an ηmol/mL.

2.8 Creatinine determination

The urinary creatinine concentrations were determined using a commercial kit (Pointe Scientific Inc., Canton, United States), according to the manufacturer's technical recommendations.

2.9 Biochemical analyses

All further biochemical analyses including C-reactive protein (CRP), zonulin, liver function parameters and complete blood count were performed using the standard procedures and biochemical analysis at the University Hospital from Universidade Federal de Santa Catarina. The results were accessed in the medical record system from the hospital.

2.10 Physiological levels of biochemical markers

The reference physiological levels of biochemical markers for healthy adults that were reported here are based on published literature. Specifically, the physiological levels of BH4 in plasma and urine were extracted from The Human Metabolome Database (Wishart et al., 2007) and Canada-Canada et al., (2009), respectively. The physiological levels of CRP and zonulin were extracted from Sung et al. (2003) and Zang et al. (2014), respectively.

2.11 Statistical analysis

Fisher's exact test was used to compare the proportion of male and female participants between the groups. All the other parameters were first analysed for normality using the D'Agostino-Pearson normality test. Unpaired Student's T test (for normally distributed data), or a Mann-Whitney U test (for non-normally distributed data) were used to test for statistically significant differences between the groups. Unpaired and two-tailed tests were used for these group comparisons. Furthermore, the Colitis group was eventually subdivided into specific groups of interest and the Kruskal-Wallis test was performed followed by a pertinent *post-hoc* test. Pearson correlation coefficients were calculated to evaluate the association between pain and disease scores with biochemical markers. The $p < 0.05$ was considered significant for all the analyses and all the significant values were reported. All the statistical analysis was carried out with GraphPad Prism Software (Prism 9, GraphPad Software Inc.).

3 Results

3.1 Demographics and clinical measures

The demographics and clinical measures in the participants from ulcerative colitis (UC) and healthy volunteers (HV) groups are summarised in Table 1. The proportion of females was greater in both groups, represented by 65 % and 63.6 % of females in the UC and HV groups, respectively. The mean age in the UC and HV groups was 47.63 and 46.93 years, respectively. The percentage of non-smokers in the UC group was 93.9% (n=31) while the percentage of non-smokers in the HV group was 100% (n=7). The body mass index (BMI) in the UC group was higher in the healthy HV group, specifically, the mean BMI from UC and HV was 27.33 and 22.28 km/m^2, respectively. These mean BMI classified the UC and HV groups as overweight and normal weight, respectively (WHO, 2000).

The patients from the UC group presented a heterogenous disease extension. About half of the patients had extensive colitis (51.7 %, n=31), 38.3 % (n=26) had left side colitis and 10 % (n=6) had ulcerative proctitis, according to the Montreal criteria for disease distribution (Satsangi et al., 2006; Supplementary table 1). The average time of the disease in the UC group was 11 years, ranging from a minimum of 3 months to a maximal of 43 years of disease time. The average age at the diagnostic for the UC-affected participants was 36.68 years, ranging from 12 to 60 years.

Among the participants from the UC group, 23.3 % (n=14) described the presence of colitis-related signs and symptoms (*i.e.*, presence of disease activity) at the day of the appointment and samples collection. Accordingly, the mean partial Mayo score (Supplementary Table 2), which assesses the clinical disease activity, was 0.68 for the UC group (*i.e.*, clinical remission as score ≤

1). For the participants from the UC group that undergone colonoscopy exam, the mean endoscopic Mayo score (Supplementary Table 2) was 0.61 (*i.e.*, endoscopic remission as score ≤ 1) and the average total Mayo score (sum of the partial and endoscopic Mayo scores) was 1.11 (*i.e.*, disease remission as score ≤ 2). A total of 16.7 % (n=10) of the patients from the UC group had extraintestinal manifestation, including arthropathy (n=4), pyoderma gangrenosum (n=2), erythema nodosum (n=2) and primary sclerosing cholangitis (n=2). Among the participants from the UC group, 11.7 % (n=7) had the presence of disease complications, including opportunistic pulmonary infection with fungal agents (n=1) and hospitalization due to severe hematochezia and abdominal pain (n=6).

The overall mean visual analogue scale (VAS) score for abdominal pain in the last 3 months reported by the UC-affected subjects was 2.65, meeting the criteria for mild pain (Treede et al., 2019). The presence of pain was shown as follows in the participants of the UC group: absence of pain 43.3 % (n=26), mild pain, 15 % (n=9), moderate pain 31.7 % (n=19) and severe pain 10 % (n=6). All participants from the UC group were taking medication to manage the disease, specifically, 8.3 % (n=5) of the participants were using corticosteroid, 36.7 % (n=22) azathioprine, 58.3 % (n=35) oral mesalazine, 38.3 % (n=23) oral sulfasalazine, 21.7 % (n=13) topical aminosalicylates and 8.3 % (n=5) biologics.

Table 1. Demographics and clinical measures in ulcerative colitis (UC) affected individuals and healthy volunteers (HV)

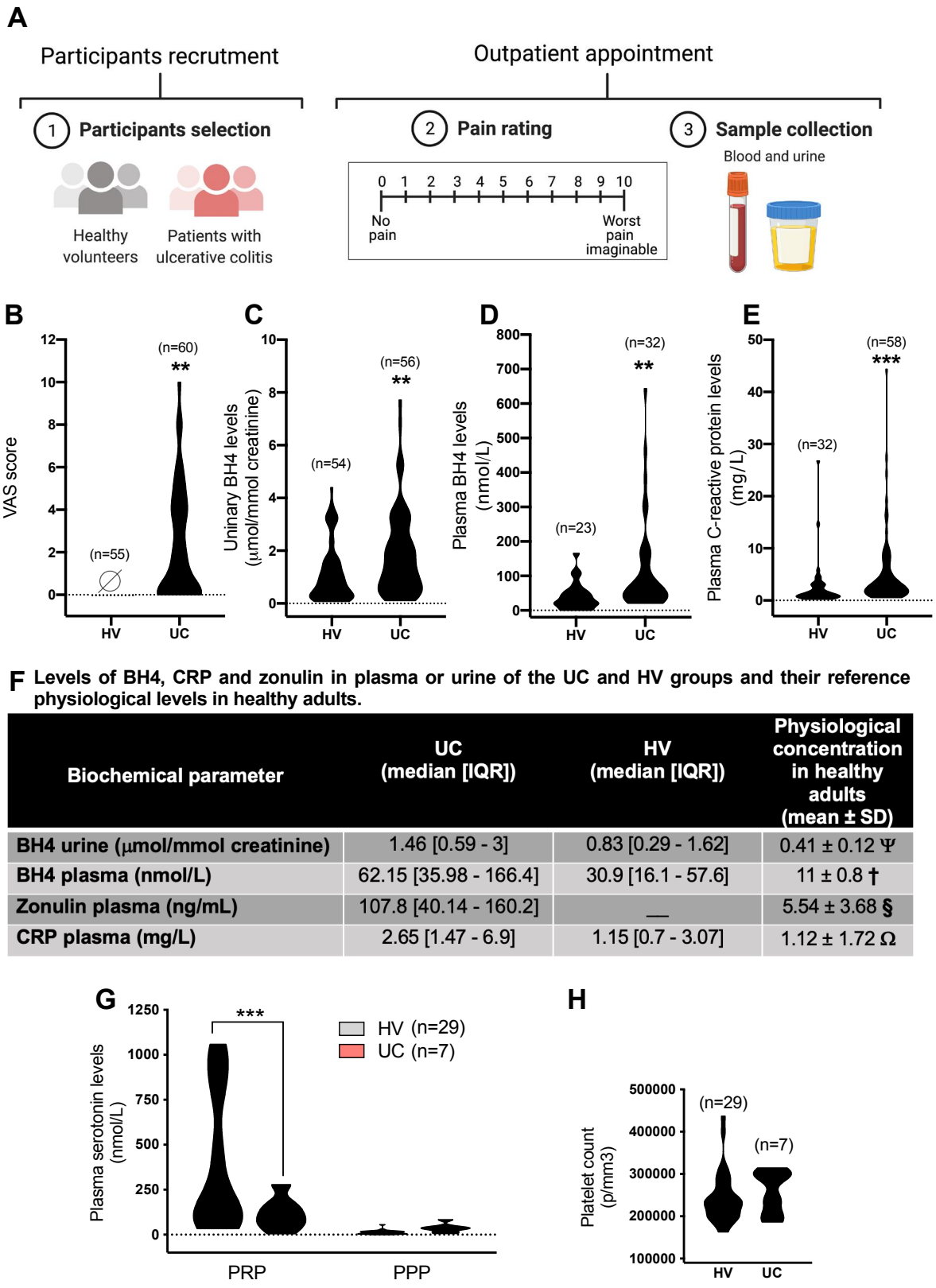
	HV (n=55)	UC (n=60)
Sex (Female)	(n=35) 63.6 %	(n=39) 65 %
Age (Years)	46.93 [18– 78]	47.45 [18 – 79]
Body mass index (km/m^2)	22.28 [18.73 – 26.7]	27.33 [18.89 – 43.28] **
Smoking habit (Non-smokers)	(n=7) 100%	(n=31) 93.9 %
Disease distribution (Montreal classification)		
Ulcerative proctitis	NA	(n=6) 10 %
Left side ulcerative colitis		(n=23) 38.3 %
Extensive ulcerative colitis		(n=31) 51.7 %
Disease time (Years)	NA	11 [0.25 – 43]
Age at the diagnostic (Years)	NA	36.68 [12 – 60]
Partial Mayo Score (0 - 9)	NA	0.68 [0 – 6]
Endoscopic Mayo Score (0 - 3)	NA	0.61 [0 – 2]
Total Mayo Score (0 - 12)	NA	1.11 [0 – 5]
Presence of disease activity (Yes)	NA	(n=14) 23.3 %
Presence of extraintestinal manifestation (Yes)	NA	(n=10) 16.7 %
Presence of disease complication (Yes)	NA	(n=7) 11.7 %
Pain score (VAS 0 –10)	Pain-free	2.65 [0 – 10] ***
Pain severity (VAS classification)		
No pain (VAS 0)	NA	(n=26) 43.3 %
Mild pain (VAS 1 -3)		(n=9) 15 %
Moderate pain (VAS 4 - 6)		(n=19) 31.7 %
Severe pain (VAS 7 -10)		(n=6) 10 %
Medication		
Corticosteroid	NA	(n=5) 8.3 %
Azathioprine		(n=22) 36.7 %
Oral mesalazine		(n=35) 58.3 %
Oral sulfasalazine		(n=23) 38.3 %
Topical aminosalicylates		(n=13) 21.7 %
Biologics		(n=5) 8.3 %

Abbreviations: HV, Healthy volunteers; UC, Ulcerative colitis; VAS, Visual analogue scale; NA, not applicable. Sex, smoking habit, disease distribution, presence of disease activity, presence of extraintestinal manifestation, presence of disease complication, pain severity classification and medication are expressed in percentage (%); Age, body mass index, disease time, age at the diagnostic, partial Mayo score, endoscopic Mayo score, total Mayo score and pain score are express as mean [range]. ** $p < 0.01$, *** $p < 0.001$ for unpaired two-tail Mann Whitney test.

3.2 Pain score and biochemical parameters

UC participants recruited in the present study presented a mean VAS score for abdominal pain of 2.65 (mild pain) in the last three months (U= 416, p<0.001, Figure 1B). The levels of tetrahydrobiopterin (BH4), C-reactive protein (CRP), and zonulin were quantified in the urine and/or plasma of UC subjects and HV (Figure 1). The levels of BH4 were higher in the urine (U= 1034, p<0.01, Figure 1C) and plasma (U= 182, p<0.01, Figure 1D) of the UC group compared to pain-free HV participants. The plasma levels of CRP, a marker of inflammation (Gabay & Kushner, 1999), were significantly higher in the UC group compare to the HV group (U= 542.5, p<0.001, Figure 1E). The plasma levels of zonulin, a marker of increased intestinal permeability (Caviglia et al., 2019; Sapone et al., 2006), were quantified in the participants from the UC group (Figure 1F). The zonulin levels in the UC group are above the cut-off values from healthy adults, indicating an increased intestinal permeability in the UC-affected subjects from the present study.

In addition, the levels of serotonin were measured in the platelet rich plasma fraction (PRP) (Figure 1G). The levels of serotonin were significantly lower in the PRP of UC subjects compared to healthy volunteers (U= 2, p<0.001, p<0.05, Figure 1G], while the platelet count was similar in both groups (Figure 1H).



F Levels of BH4, CRP and zonulin in plasma or urine of the UC and HV groups and their reference physiological levels in healthy adults.

Biochemical parameter	UC (median [IQR])	HV (median [IQR])	Physiological concentration in healthy adults (mean ± SD)
BH4 urine (µmol/mmol creatinine)	1.46 [0.59 - 3]	0.83 [0.29 - 1.62]	0.41 ± 0.12 Ψ
BH4 plasma (nmol/L)	62.15 [35.98 - 166.4]	30.9 [16.1 - 57.6]	11 ± 0.8 †
Zonulin plasma (ng/mL)	107.8 [40.14 - 160.2]	—	5.54 ± 3.68 §
CRP plasma (mg/L)	2.65 [1.47 - 6.9]	1.15 [0.7 - 3.07]	1.12 ± 1.72 Ω

Figure 1. Levels of the tetrahydrobiopterin (BH4) and other biochemical markers in ulcerative colitis (UC) subjects and pain-free healthy volunteers (HV). (A) Schematic representation of the study design. In this cross-sectional study patients with UC and HV were recruited. At the outpatient appointment, the abdominal pain score was rated using the visual analogue scale (VAS), and the biological samples were collected. (B) Pain score measured by VAS in the UC group. (C) BH4 in the urine and (D) the plasma of UC and HV groups. (E) C-reactive protein (CRP) in the plasma of UC and HV groups. (F) Table with the values of BH4, CRP and zonulin in the urine or plasma from the UC group and the reference physiological values

for healthy adults. The values are presented as median [IQR]. The data of reference physiological levels for healthy adults is derived from the literature (Canada-Canada et al., 2009; Sung et al., 2003; Wishart et al., 2007). (G) Serotonin levels in the platelet rich plasma (PRP) and platelet poor plasma (PPP) from UC and HV groups. (H) Platelet count in UC and HV groups. ** $p < 0.01$, *** $p < 0.001$, unpaired two-tailed Mann-Whitney U test.

Ψ Data from Canada-Canada (2009) Determination of marker pteridins and biopterin reduced forms, tetrahydrobiopterin and dihydrobiopterin, in human urine, using a post-column photoinduced fluorescence liquid chromatographic derivatization method. Analytica Chimica Acta. 2009; 648 (2009): 113-122.

† Data from The Human Metabolome Database (Wishart et al., 2007); available at https://hmdb.ca/metabolites/HMDB0000027.

§ Data from Zang et al. (2014) Circulating zonulin levels in newly diagnosed Chinese type 2 diabetes patients. Diabetes Res Clin Pract. 2014; 106(2): 312-318.

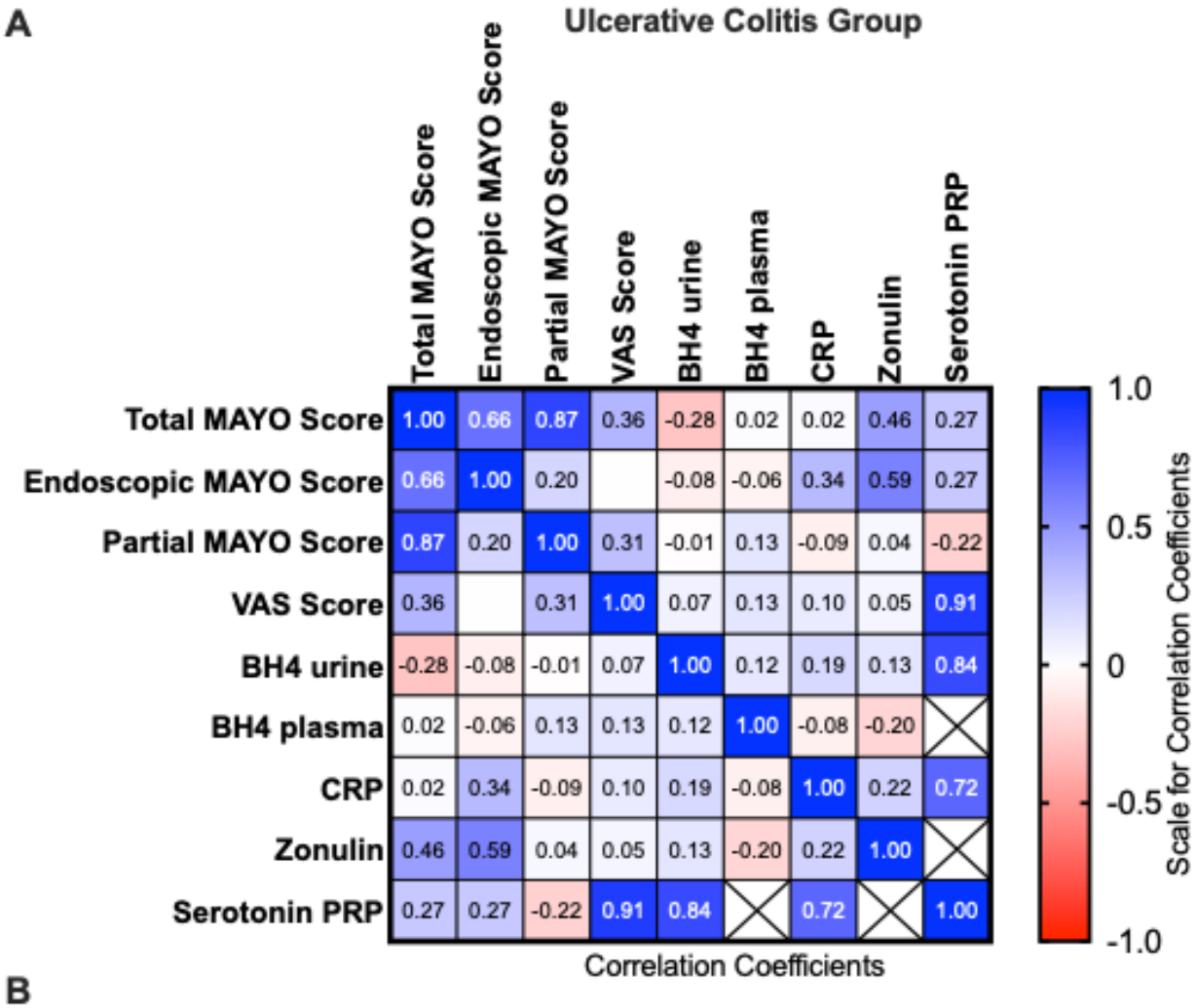
Ω Data from Sung et al. (2003) High Sensitivity C-Reactive Protein as an Independent Risk Factor for Essential Hypertension. American Journal of Hypertension; 2003, 16: 429–433

∅, Pain-free. Abbreviations: UC, Ulcerative colitis; HV, Healthy volunteers; VAS, Visual analogue scale; BH4, tetrahydrobiopterin; CRP, C-reactive protein; IQR, interquartile range; PRP, platelet rich plasma; PPP, platelet poor plasma.

3.3 Pain scores correlated with clinical disease severity and plasma serotonin

Correlation between the disease and pain scores with the biochemical markers was performed, Pearson's correlation coefficients and associated *p*-values are shown in Figure 2. The total Mayo score was positively correlated with the partial and the endoscopic Mayo scores (r= 0.87, $p < 0.001$ and r= 0.66, $p < 0.01$, respectively, Figure 2A and B). Significant positive correlations were observed between the pain (*i.e.*, VAS score) and disease (*i.e.*, partial Mayo score) scores (r= 0.31, $p < 0.05$, Figure 2A and B). The VAS score and the levels of serotonin in the PRP were positively correlated (r= 0.91, $p < 0.01$, Figure 2A and B), while the serotonin PRP levels were positively correlated with the urinary BH4 levels (r= 0.84, $p < 0.05$, Figure 2A and B). Some of the parameters that were significantly correlated in Pearson's correlation analysis are represented in a scatterplot graph (Figure 2C).

A



B

Parameter 1	Parameter 2	Pearson r	P value
Total Mayo Score	*Partial Mayo Score*	0.87	*** $p < 0.001$
Total Mayo Score	*Endoscopic Mayo Score*	0.66	** $p < 0.01$
VAS Score	*Partial Mayo Score*	0.31	* $p < 0.05$
VAS Score	*Serotonin PRP*	0.91	** $p < 0.01$
Serotonin PRP	*BH4 urine*	0.84	* $p < 0.05$

C

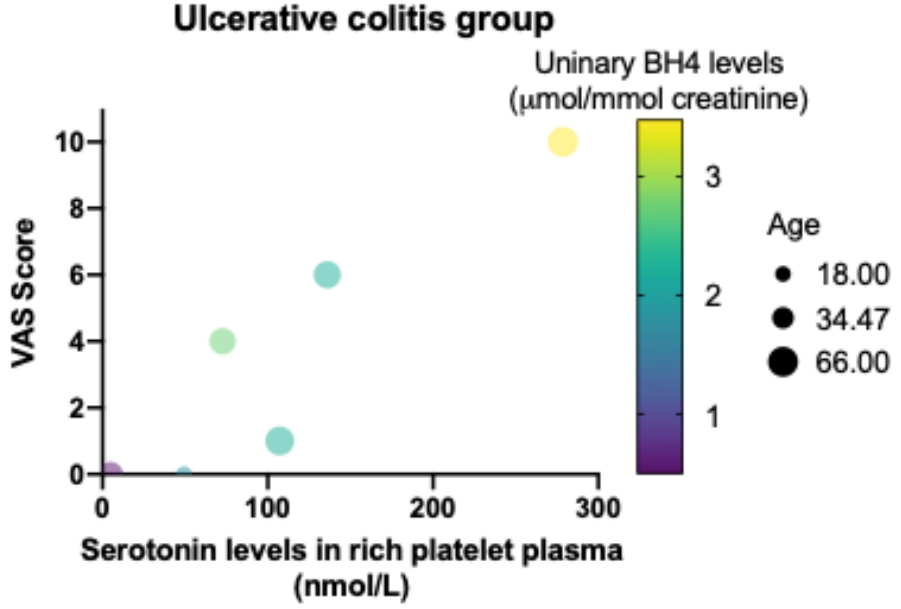


Figure 2. Pearson's correlation between the disease/pain scores and biochemical markers. (A) Heatmap of the correlation matrix showing the correlation between the disease/pain scores and biochemical markers. The number in each cell represents the correlation coefficient, the blue colour indicates a positive correlation, and the red colour indicates a negative correlation. (B) Pearson r and P values statistically significant were displayed in the table. (C) Scatterplot representation of the visual analogue scale (VAS) Score (Y axis) and serotonin levels in platelet rich plasma (PRP) (X axis). The yellow colour indicates a high concentration of urinary BH4, and the purple colour indicates a low concentration of urinary tetrahydrobiopterin (BH4). The size of the bubbles represents the age of the population. Abbreviations: UC, Ulcerative colitis; VAS, Visual analogue scale; BH4, tetrahydrobiopterin; CRP, C-reactive protein; PRP, platelet rich plasma.

3.4 Characterisation of biochemical markers according to the pain profile of UC subjects

Given the heterogeneous prevalence of abdominal pain in the UC-affected subjects from the present study (Table 1, sections Pain score and Pain severity; Approximately half of the UC-affected patients reported absence of abdominal pain), the UC group was subdivided into groups of subjects with or without pain (Figure 3A-C). The BH4 levels in the urine were higher in the UC-affected patients with pain compared to the pain-free HV [H=8.877, $p<0.05$; Figure 3A]. The levels of CRP in the plasma were higher UC-affected patients with and without pain compare to the pain-free HV [H=11.09, $p<0.01$ for UC no pain, $p<0.05$ for UC pain; Figure 3C]. When the UC-affected patients experiencing pain were further subdivided according to pain severity (*i.e.*, mild, moderate, or severe; Figure 3D-F), the UC-affected patients with severe pain presented higher levels of BH4 in the urine compared to the pain-free HV [H=11.71, $p<0.05$; Figure 3D]. While the UC-affected patients without pain presented higher CRP plasma levels compared to the pain-free HV [H=13.41, $p<0.01$; Figure 3F].

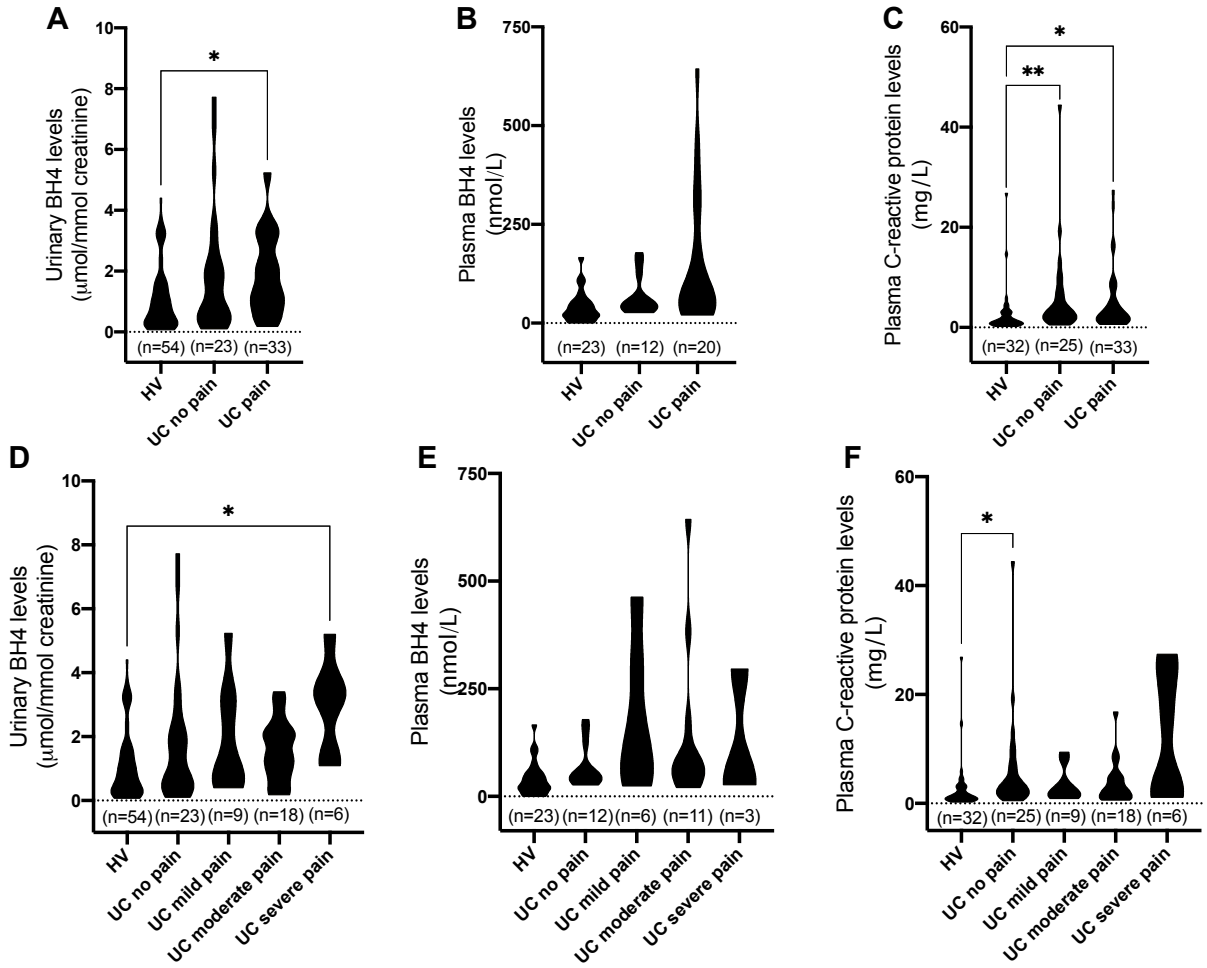


Figure 3. Biochemical markers in pain-free healthy volunteers (HV), ulcerative colitis (UC) participants with or without abdominal pain. (A) Urinary and (B) plasma levels of BH4 in the pain-free HV, UC no pain and UC pain groups. (C) CRP plasma levels in the HV, UC no pain and UC pain groups. (D-F) Levels of the biochemical markers in pain-free HV, UC participants without pain or with different pain severity. (D) Urinary and (E) plasma levels of BH4 in the HV, UC no pain, and UC mild, moderate and severe pain groups. (F) CRP plasma levels in the HV, UC no pain, and UC mild, moderate and severe pain groups. * $p < 0.05$, ** $p < 0.01$, Kruskal-Wallis test followed by the *post hoc* test of Dunn. Abbreviations: UC, Ulcerative colitis; HV, healthy volunteers; BH4, tetrahydrobiopterin; CRP, C-reactive protein.

3.5 Characterisation of biochemical markers according to the disease activity profile of UC subjects

The clinical course of UC is marked by alternating periods of active disease and remission based on the presence or absence of disease-related manifestations (*i.e.*, increased stool frequency, rectal bleeding and colonic mucosal inflammation), respectively (Satsangi et al., 2006). To investigate the relationship between the clinical disease activity status (remission or active disease) with the peripheral biochemical markers and pain scores, we divided the UC participants into remission and active UC subjects (classification according to the partial Mayo score; Supplementary Table 2). The VAS score, the CRP plasma levels and the BH4 levels in the plasma and the urine were accessed (Figure 4). The mean VAS score for pain was 2.13 and 4.35 for the UC participants in

remission and active disease, respectively (Figure 4A). The proportion of patients with abdominal pain was 47.8 % and 85.7 % in the UC remission and active disease groups, respectively (Figure 4A). The pain score was significantly higher in the UC remission and active disease groups compare to HV [H=34.78, p<0.001 for UC remission, p<0.0001 for UC active disease; Figure 4B]. In addition, the VAS score was higher in the UC active disease compare to the UC remission group [H=34.78, p<0.05; Figure 4B]. The BH4 levels in the urine were higher in the UC remission and active disease groups compared to the HV [H=26.65, p<0.0001 for UC remission, p<0.01 for UC active disease; Figure 4C]. The BH4 plasma levels were higher in the UC remission group compare to the HV [H=10.09, p<0.05; Figure 4D]. The plasma levels of CRP were higher in the UC remission group compare to the HV [H=10.92, p<0.01; Figure 4E].

A

	VAS pain score (mean [range])	Proportion of subjects with abdominal pain (%)
UC remission	2.13 [0 - 10]	47.8
UC active disease	4.35 [2.75 - 8]	85.7

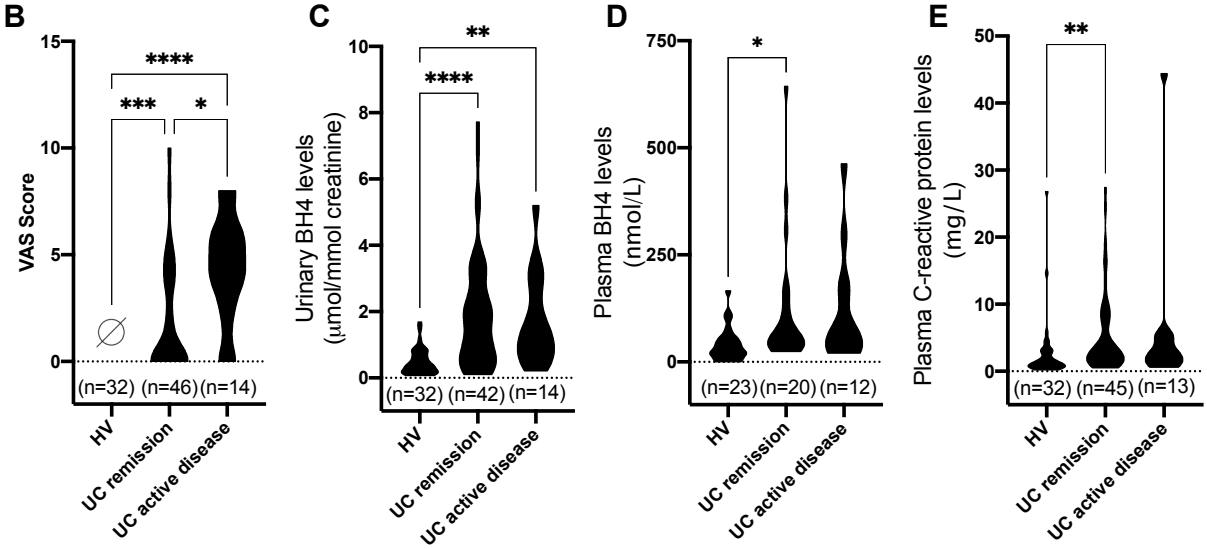


Figure 4. Levels of the biochemical markers in pain-free healthy volunteers (HV), ulcerative colitis (UC) participants in remission and active disease. (A) Table with the visual analogue scale (VAS) scores for abdominal pain and proportion of subjects with abdominal pain in the UC remission and active disease groups. (B) VAS pain scores in UC remission and active disease groups. (C) BH4 in the urine and (D) plasma of HV, UC remission and active disease groups. (E) plasma levels of CRP in the HV, UC remission and active disease groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Kruskal-Wallis test followed by the *post hoc* test of Dunn. ∅, Pain-free. Abbreviations: HV, Healthy volunteers; UC, Ulcerative colitis; VAS, Visual analogue scale; BH4, tetrahydrobiopterin; CRP, C-reactive protein.

3.6 Characterisation of biochemical markers according to the disease distribution in UC subjects

The UC can be classified as proctitis, left-sided colitis, and extensive colitis according to the maximal macroscopic extent of colonic inflammation observed at colonoscopy (classification

based on the Montreal Score; Supplementary Table 1). Given the heterogeneous extension of colon inflammation in the UC-affected patients from the present study (Table1, disease distribution section), the UC group was further divided into proctitis (PC), left side (LC) and extensive colitis (EC) groups to access the influence of the disease extension on the peripheral biochemical markers and pain levels. The VAS score for pain, the CRP plasma levels and the BH4 levels in plasma and urine were accessed in the HV, PC, LC and EC groups (Figure 5). VAS score was significantly higher in the PC, LC and EC groups when compared to pain-free HV participants [H=28.43, p<0.01 for PC, p<0.0001 for LC, p<0.001 for EC; Figure 5A]. Urinary BH4 levels were higher in the PC, LC and EC groups when compared to the HV [H=27.84, p<0.01 for PC, p<0.001 for LC, p<0.0001 for EC; Figure 5B]. The BH4 levels in the plasma were higher only in the EC group compare to the HV [H=15.23, p<0.01; Figure 5C]. The CRP levels in the plasma were higher only in the LC colitis group when compared to the HV [H=10.72, p<0.05; Figure 5D].

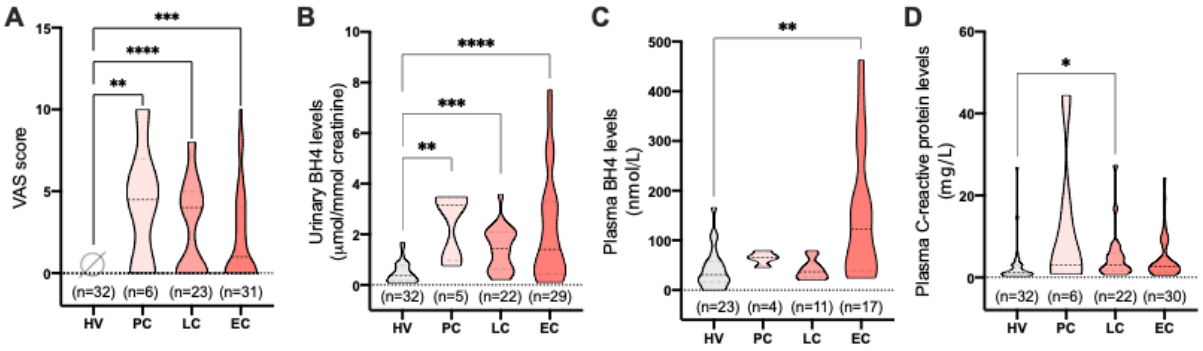


Figure 5. Biochemical markers in pain-free healthy volunteers (HV), and participants affected by different ulcerative colitis extension. (A) Pain score measured by using the visual analogue scale (VAS) in HV, proctitis (PC), left side colitis (LC) and extensive colitis (EC) groups. (B) Urine and (C) plasma levels of BH4 in HV, PC, LC and EC groups. (D) Plasma levels of C-reactive protein (CRP) in HV, PC, LC and EC groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Kruskal-Wallis test followed by the *post-hoc* test of Dunn. ∅, Pain-free. Abbreviations: HV, Healthy volunteers; PC, Proctitis; LC, Left side colitis; EC, Extensive colitis; VAS, Visual analogue scale; BH4, tetrahydrobiopterin; CRP, C-reactive protein.

4 Discussion

UC is a chronic inflammatory disease that affects the mucosal layer of the colon. The clinical course of UC is marked by intercalated periods of remission and relapse (Satsangi et al., 2006). During the period of active disease, UC-affected patients experience colonic mucosal inflammation alongside increased stool frequency, rectal bleeding, among other symptoms that reflect the disease severity (LennardJones & Shivananda, 1997). Abdominal pain is a common symptom during the UC disease course and, it has been reported to be frequently present even during the period of disease remission and absence of colonic inflammation (Fukuba et al., 2017; Fukuba et al., 2014; Ishihara et al., 2019; Minderhoud et al., 2004). Hence, it could be hypothesised

that other factors dissociated from active colonic inflammation might play a role in the establishment of UC-related abdominal pain. This prompted us to investigate whether the BH4 metabolism is involved in the physiopathology of UC-related pain. The findings reported here indicate that an exacerbation of the BH4 metabolism may increase serotonin levels and ultimately contribute to visceral pain hypersensitivity. The exact source of increased BH4 production in UC-affected patients could be derived from *i)* inflammatory activation and/or *ii)* the gut microbiota.

Abdominal pain in UC can be defined as a characteristic visceral pain of an organic pathology. During the disease course of UC, local factors such as altered paracellular permeability of the intestinal wall and the balance between the submucosal immune system and the gut microflora may participate in *i)* local immune stimulation and *ii)* release of bacteria-derived metabolites, both of which can contribute to sensitisation of primary afferents (Hering, Fromm, & Schulzke, 2012). Inflammatory and other classes of mediators are released and can participate in the sensitisation of primary afferent endings during the UC course. These mediators include pro-inflammatory cytokines (*i.e.*, TNFα, IL-1β and IL-6), substance P, nerve growth factor, cholecystokinin, serotonin, among others (Delafoy, Raymond, Doherty, Eschalier, & Diop, 2003; Sabate, Gorbatchef, Flourie, Jian, & Coffin, 2002; Tack et al., 2006). Data regarding the peripheral concentration of zonulin and CRP indicate increased intestinal permeability and inflammation in the UC cohort of patients that present mild chronic abdominal pain. These results reflect two key pathophysiologic features that have been reported in UC (Bai et al., 2020; Buning et al., 2012). Indeed, lower urinary lactulose/mannitol ratio as a proxy of increased intestinal permeability has been previously observed in UC-affected patients and the barrier defect was associated with increased serum concentration of pro-inflammatory cytokines, including TNFα, IL-1β and IL-6, as well as with increased CRP serum levels (Bai et al., 2020). Systemic endotoxemia, a circulating bacterial marker used as a surrogate of increased intestinal barrier permeability (Barclay & Scott, 1987), was reported to be present in 28∼88 % of patients with UC and was correlated with disease extent and plasma TNF-α (Gardiner et al., 1995).

Active inflammation can also upregulate the transcription of the gene encoding the rate-limiting enzyme of the BH4 *de novo* biosynthetic pathway (*GCH1)* and ultimately increase the BH4 levels. Indeed, excessive activation of the BH4 pathway was characterized in different experimental models of inflammatory pain (*i.e.*, intraplantar and intraarticular injection of complete Freund's adjuvant in mice) by showing increased *GCH1* expression and GTPCH activity in infiltrating macrophages, with increased content of BH4 in inflamed tissues (Latremoliere et al., 2015). This is also in agreement with our earlier observations showing a positive correlation between plasma levels of IL-1β with BH4 plasma levels in patients affected by long-term diabetic neuropathic pain (Staats Pires et al., 2020). In addition, it cannot be ruled out that microbiome abnormalities, which are part of the UC pathophysiology, can contribute to the exacerbation of

BH4 metabolism. This hypothesis is supported by the fact that intestinal microbiota from humans and mice contains BH4 generating bacteria (from the *Actinobacteria phylum*), which contribute to an age-dependent rise in BH4 tissue levels of mice (Belik et al., 2017). Accordingly, it was recently reported that the BH4 derived from the gut microbiota can affect the host physiology (*i.e.*, social behaviour) in a mice model of autism (Buffington et al., 2021). We also observed increased plasma zonulin levels, a marker of increased intestinal permeability, in UC-affected patients. Noteworthy, increased intestinal permeability is also described in patients with UC during remission (Buning et al., 2012). Therefore, it is feasible to propose that the exacerbation of the BH4 metabolism reported in UC-affected patients can also be derived from the gut microbiome. In the current study, the levels of BH4 in the urine and plasma were increased in UC patients with mild chronic abdominal pain. These results suggest that UC patients with abdominal pain present a systemic exacerbation of the BH4 metabolism, which can be related at least in part to immune system activation (*i.e.*, increased CRP levels) and/or exogenous BH4 due to increased intestinal permeability (*i.e.*, increased zonulin levels).

This study set out with the aim of assessing the importance of BH4 for abdominal pain reported by UC-affected patients. In the current study, the UC group reported mild abdominal; however, the heterogeneous prevalence and severity of pain within this group prompted us to further divide the UC-affected patients according to the presence and/ or severity of pain to investigate the BH4 levels. A significant increase in urinary BH4 levels was identified in UC patients with pain *vs* free-pain healthy volunteers (HV), but not in the UC patients without pain. Accordingly, the group of UC patients with severe pain (but not mild or moderate pain) had significantly higher levels of urinary BH4 related to HV. These observations may support the hypothesis that the exacerbation of the BH4 metabolism is a key factor in the establishment of UC-related abdominal pain. The fact that increased CRP plasma levels were observed in UC patients regardless of the presence or absence of abdominal pain, raises the question of whether active inflammation is the main responsible for abdominal pain in UC. The majority of UC patients suffering from acute flares will experience pain, which typically improves as disease activity decreases (Coates et al., 2013). In agreement, we observed that 85.7 % of the UC patients during the active disease present abdominal pain (Figure 4A). However, a sizable percentage of UC patients (47.8 %; Figure 4A) continue experiencing pain symptoms despite resolved inflammation and achieving what appears to be clinical remission (Bielefeldt et al., 2009). Likewise, in the present study the UC patients during the active disease experience on average higher abdominal pain levels when compared to patients during the disease remission. However, the UC patients in disease remission still experience high levels of abdominal pain (scores of 9 or 10 on the VAS for abdominal pain; Figure 4A and B). Additionally, the urinary BH4 levels were the only biochemical marker that was increased in patients during the active disease and remission *vs* HV (Figure 4C).

While CRP was solely increased in UC remission *vs* HV (Figure 4E). These findings strengthen our hypothesis that the BH4 may be a key player in the pathophysiology of UC-related abdominal pain. In addition, these results suggest that the urinary BH4 levels may be a feasible candidate to be further investigated as a biomarker of UC-related abdominal pain.

The link between BH4 and pain was discovered through the identification of a haplotype allele of *GCH1* which was associated with reduced transcription of the *GCH1* gene and pain scores in patients (Tegeder et al., 2006). This relationship led our group to study BH4 metabolism in inflammatory and neuropathic mouse models of pain (Latremoliere et al., 2015), and now we extended our studies by adding further information on abdominal pain in humans. Mechanistically, the molecular pathways by which BH4 induces chronic pain is not fully elucidated but we have proposed to be related to its role as a cofactor for the synthesis of aminergic neurotransmitters and nitric oxide (Latini et al., 2018; Latremoliere et al., 2015; Tegeder et al., 2006). Here we explored a possible link between the exacerbation of the BH4 metabolism with increased serotonin levels in the induction of abdominal pain, given the importance of serotonin in gastrointestinal sensation (Camilleri, 2002). Serotonin is synthesised from the essential amino acid tryptophan by the enzyme tryptophan hydroxylase (TPH). TPH requires BH4 as a mandatory cofactor for the production of serotonin (Miwa, Watanabe, & Hayaishi, 1985). The enterochromaffin cells (ECC) from the gut produce the majority of the body's serotonin content that is further released in the gastrointestinal tract (Yano et al., 2015). Overflowing serotonin from ECC is taken up and concentrated into platelets from the blood. Therefore, the serotonin levels in PRP are a surrogate of the serotonin gut content (Tamir, Payette, Huang, Liu, & Gershon, 1985). In the present study, increased PRP serotonin was significantly associated with higher abdominal pain scores within the UC-affected patients. Serotonin is a critical signalling molecule and the activation of gut serotoninergic receptors, especially 5-HT_3 receptors, have been implicated in visceral hypersensitivity (Kayser et al., 2007). In agreement, 5-HT_3 antagonists (alosetron and cilansetron) decrease hyperalgesia and abdominal pain in inflammatory bowel syndrome patients (Lacy, Nicandro, Chuang, & Earnest, 2018; Tack et al., 2006). Given the fact that the rate-limiting enzyme of serotonin biosynthesis (*i.e.*, TPH) is dependent on the cofactor BH4 (Ghisoni et al., 2015), changes in the BH4 levels can impact serotonin production. In fact, the systemic inhibition of BH4 synthesis reduces serotonin levels in the colon of mice (Kobayashi, Hasegawa, Kaneko, & Ichiyama, 1991). Noteworthy, PRP serotonin levels were positively correlated with the urinary BH4 levels in UC-affected patients of our study, whilst the former was positively correlated with pain levels. Thus, it is feasible that exacerbated BH4 metabolism is positively correlated with increased serotonin production which favours colonic hypersensitivity contributing to abdominal pain in UC-affected individuals.

The current treatment goal for UC is to maintain steroid-free disease remission (Magro et al., 2017), but this approach seems to be insufficient to completely abolish the abdominal pain related to this disease. Consequently, the persistence of abdominal pain represents a burden for UC patients, as well as a challenge for clinicians and researchers. To put it into perspective, a lack of understanding about the molecular mechanism of UC-related abdominal reflects an increased rate of opioids prescriptions for abdominal pain (*e.g.*, it doubled in the US from 1997 to 2008) (Dorn, Meek, & Shah, 2011). The use of opioids for abdominal pain has not been proved to be beneficial and, instead, it can even worsen the condition, *e.g.*, the narcotic bowel syndrome (Grunkemeier, Cassara, Dalton, & Drossman, 2007). Other high-risk side effects of opioids misuse are addiction and even death for overdose (NIDA, 2020). Therefore, the development of effective and safe management of UC-related abdominal pain requires a better understanding of the cellular and molecular mechanisms of this condition. Our findings represent a novel proposal of molecular alteration in UC subjects that experience abdominal pain. Although no direct cause-and-effect relationship has been confirmed, our clinical data suggest the importance of the BH4 metabolism exacerbation for UC-related abdominal pain. It is still not clear if or how the exacerbation of the BH4 metabolism detected in this study could account for the establishment of UC-related abdominal pain. However, based on previous observations and the findings reported here, increased intestinal permeability and/or inflammatory activation may contribute to elevated levels of BH4, which could ultimately increase the production of serotonin, enhancing visceral sensitivity. Additionally, the urinary BH4 levels seem to be a feasible low-invasive biomarker for the presence and severity of abdominal pain in UC. New therapeutic strategies may arise from the progressing identification of molecular prognostic markers and characterization of the molecular basis of UC-related abdominal pain. In the long run, the results that were present in this study can be put forward in potential therapeutic targets for UC-related abdominal pain.

4.1 Study limitations

The present cross-sectional study included only outpatients with mild disease severity and does not allow us to exclude the possibility that the regulation of BH4 metabolism may evolve over time and can be related to the stage and severity of the disease. Indeed, it is well known that chronic pain is associated with disease severity in UC and other health-related disorders that might appear in a time-dependent manner (*i.e.*, anxiety, depression), all of which could influence the BH4 metabolism and serotonin levels. It would be of prime importance to assess, in a longitudinal study, the possible temporal changes in BH4 and serotonin levels linked with the process of pain chronification. In addition, the inclusion of hospitalised patients with a more severe disease condition would allow a better understanding of the participation of BH4 in the pathophysiology of UC-related abdominal pain.

5 Conclusion

This is the first report showing exacerbated BH4 metabolism in human body fluids of UC-affected patients with abdominal pain. The increased serotonin plasma levels positively correlated with urinary BH4 levels, and the former positively correlated with abdominal pain levels. These observations indicate that changes in the biosynthesis of BH4 may impact serotonin availability and, ultimately, contribute directly to the abdominal pain associated with UC. Our research encourages further studies exploring the exact source of BH4 in UC-related abdominal pain.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

ASP contributed to the conception of the study; sample collection and preparation; clinical database management; data acquisition and curation; writing the original draft; revising the article. BLT contributed to the conception of the study; data acquisition; revising the article. DLS contributed to sample preparation and data acquisition. VSM contributed to participant recruitment and clinical database management. SMR contributed to sample collection and preparation; clinical database management. LN contributed to clinical database management. GJG contributed to the conception of the study; funding acquisition; revising the article. AL contributed to the whole conception of the study; project supervision; funding acquisition; revising the article.

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9 Data Availability Statement

The datasets generated for this study are available on request to the corresponding author.

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Supplementary Content - Tetrahydrobiopterin as a Key Factor Promoting Colitis Abdominal Pain

Appendix: Supplementary table 1. Montreal classification of extent of ulcerative colitis (UC) adapted from (Satsangi et al., 2006).

Term	Distribution	Description
E1	Ulcerative proctitis	Involvement limited to the rectum (that is, proximal extent of inflammation is distal to the rectosigmoid junction)
E2	Left sided UC (distal UC)	Involvement limited to a proportion of the colorectum distal to the splenic flexure
E3	Extensive UC (pancolitis)	Involvement extends proximal to the splenic flexure

Adapted from Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: Controversies, consensus, and implications. Gut. 2006;55(6):749-753.

Appendix: Supplementary table 2. Mayo scoring system for assessment of ulcerative colitis activity.*

Stool frequency† (Subscore, 0 to 3)
0 = Normal no. of stools for this patient 1 = 1 to 2 stools/day more than normal 2 = 3 to 4 stools/day more than normal 3 = 5 or more stools/day more than normal
Rectal bleeding‡ (Subscore, 0 to 3)
0 = No blood seen 1 = Streaks of blood with stool less than half the time 2 = Obvious blood with stool most of the time 3 = Blood alone passes
Findings on endoscopy (Subscore, 0 to 3)
0 = Normal or inactive disease 1 = Mild disease (erythema, decreased vascular pattern, mild friability) 2 = Moderate disease (marked erythema, absent vascular pattern, friability, erosions) 3 = Severe disease (spontaneous bleeding, ulceration)
Physician's global assessment§ (Subscore, 0 to 3)
0 = Normal 1 = Mild disease 2 = Moderate disease 3 = Severe disease

Adapted from Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. N Engl J Med 1987;317:1625–1629.

* The Mayo score ranges from 0 to 12, with higher scores indicating more severe disease.

† Each patient serves as his or her own control to establish the degree of abnormality of the stool frequency.

‡ The daily bleeding score represents the most severe bleeding of the day.

§ The physician's global assessment acknowledges the three other criteria, the patient's daily recollection of abdominal discomfort and general sense of wellbeing, and other observations, such as physical findings and the patient's performance status.

Appendix: Supplementary table 3. Complete blood count in ulcerative colitis (UC) and healthy volunteers (HV) groups.

Metabolite	HV (median [IQR]; n=32)	UC (median [IQR]; n=60)	Reference range
RBC (mi/mm3)	4.46 [4.16 - 4.8]	4.47 [4.04 - 4.76]	Male: 4.3 - 5.9 Female: 3.5 - 5.5
Hb (g/dL)	13 [12.33 - 13.98]	13.4 [12.3 - 14.18]	Male: 13.5 - 17.5 Female: 12.0 - 16.0
Ht (%)	38.5 [36.55 - 40.63]	40.15 [37.5 - 42.43]	Male: 41 - 53 Female: 36 - 46
MCV (u2 fl)	87.65 [84.88 - 89.73]	90.55 [86.9 - 95.3] **	80-100
MCH (pg)	29.15 [28.63 - 30.73]	30.1 [28.88 - 31.8]	25.4 - 34.6
MCHC (g/dl)	33.95 [33.23 - 34.55]	33.3 [32.48 - 34.13] **	31 - 36
RDW	12.7 [12.5 – 13]	12.95 [12.7 – 13.75] *	10.2–14.5
WBC (p/mm3)	7315 [6453 - 8253]	6630 [5340 - 8180]	4500 - 11000
Seg p/mm3	4251 [3591 - 4767]	3842 [3047 - 5111]	-
Lymphocytes (p/mm3)	2448 [2085 - 3021]	1981 [1491 - 2570] **	-
Monocytes (p/mm3)	546.2 [467.6 - 646]	558.5 [428.3 – 660]	-
Eosinophils (p/mm3)	127.2 [70.8 - 215.1]	132.3 [89.48 - 249]	-
Basophils (p/mm3)	20.8 [16.43 - 35.93]	29.25 [18.75 – 40.3]	-
Platelet (p/mm3)	233500 [202750 -274750]	242500 [204000 - 284750]	150000-400000
MPV	10.1 [9.7 - 10.9]	10.1 [9.6 - 11.1]	-

Abbreviations: HV, Healthy volunteers; UC, Ulcerative colitis, RBC, Red blood cells; Hb, Haemoglobin; Ht, Haematocrit; MCV, Mean corpuscular volume; MCH, Mean corpuscular haemoglobin; MCHC, Mean corpuscular haemoglobin concentration; RDW, Red cell distribution width; MPV, Mean platelet volume. All data are presented as median [IQR]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for unpaired two-tail Mann Whitney test.

Appendix: Supplementary table 4. Levels of liver function markers in ulcerative colitis (UC) and healthy volunteers (HV) groups.

Metabolite	HV (median [IQR]; n=32)	UC (median [IQR]; n=60)
ALB (g/dL)	3.9 [3.8 – 4.2]	3.9 [3.7 – 4.1]
AST (U/L)	19.5 [16 - 24]	20 [16 – 25.25]
ALT (U/L)	25.5 [19.5 - 30.75]	27 [21.75 – 33.25]
GGT (U/L)	28 [24 - 38.75]	29.5 [23 - 49]
ALP (U/L)	69 [57 – 78]	75 [61 - 90]

Abbreviations: HV, Healthy volunteers; UC, Ulcerative colitis, ALB, Albumin; AST, Aspartate transaminase; ALT, Alanine transaminase; GGT, Gamma-glutamyl transferase; ALP, Alkaline phosphatase. All data are presented as median [IQR].

CHAPTER 4

DISCUSSION & CONCLUSION

The overarching aim of this thesis was to investigate relevant biochemical pathways (KYN and BH4 pathways) as sources of biomarkers for chronic pain. This chapter will discuss and synthesise findings from the three clinical studies investigated in this thesis. Future directions for research, clinical implications, and limitations will also be presented. Finally, a general conclusion for this thesis will be outlined.

4 DISCUSSION & CONCLUSION

4.1 DISCUSSION

Chronic pain is a highly prevalent and costly health problem, yet our understanding of the condition is limited, existing treatments are largely ineffective and precipitate serious adverse effects (Painaustralia, 2019). Additionally, clinical subjective ratings to evaluate the intensity or symptoms of chronic pain have played a major role in poor diagnosis and treatment (Dworkin et al., 2005; Treede et al., 2019). Given the subjective nature of current pain assessments, limited efficacy of treatment options, and risks associated with chronic pain therapy, the need for objective and quantitative data to assist with chronic pain management has never been greater.

The purpose of this thesis was to explore the potential utility of KYN and BH4 pathways-related metabolites as biomarkers of chronic pain. Collectively, the results from this thesis suggest that KYN and BH4 metabolites can be quantified in human body fluids, in a low-invasive manner, as markers of chronic pain and its related features. The arising data reveals that BH4 and KYN/Trp ratio were directly or indirectly associated with pain intensity across the cohort of chronic pain participants studied. While other KYN and BH4 pathways metabolites were correlated with chronic pain features and symptoms, such as depression, stress and kinesiophobia.

This thesis explored for the first time the potential utility of KYN and BH4 pathways-related metabolites as biomarkers of chronic pain. Specifically, our results showed an increased KYN/Trp ratio in the plasma of subjects with DNP, which was positively correlated with pain intensity. Trp is an essential amino acid that can be metabolised into KYN, the pivotal metabolite from the KYN pathway (Guillemin, Smythe, Takikawa, & Brew, 2005). One of the enzymes that catabolises this first metabolic step is IDO1, which is upregulated by pro-inflammatory factors (Takikawa, Yoshida, Kido, & Hayaishi, 1986). Accordingly, the ratio between the product (*i.e.*, KYN) and its precursor (*i.e.*, Trp) indicates an upregulation of the KYN pathway and has been largely assessed as a marker of inflammatory activation (Badawy & Guillemin, 2019). Our results suggest the KYN/Trp ratio as a marker of pain intensity in DNP. Another study also previously reported an association between the KYN/Trp plasma ratio and pain rating in temporomandibular disorders myalgia (Barjandi et al., 2020), supporting our findings. Additional literature showed increased plasma KYN/Trp ratio in other cohorts of chronic pain subjects, including chronic low back pain (Kim et al., 2012) and CRPS (Alexander

et al., 2013). However, the correlation between the KYN/Trp ratio with pain intensity was not assessed in these studies.

A growing body of preclinical evidence has demonstrated that the activation of the KYN pathway contributes to chronic pain hypersensitivity (Huang et al., 2016; Kim et al., 2012). Increased expression of IDO1 was observed in lymphoid tissue of mice with mechanical pain hypersensitivity induced by a chronic viral infection (Huang et al., 2016); while the pharmacological inhibition of IDO1 (1-methyl-tryptophan [1-MT]; oral administration) reduced the virus-induced mechanical pain hypersensitivity (Huang et al., 2016). Increased plasma KYN/Trp ratio was also observed in the chronic arthritis rat model that develops persistent pain hypersensitivity after the complete Freund's adjuvant injection in the tibiotarsal joint (Kim et al., 2012). The pharmacological inhibition of IDO1 (1-MT; intraperitoneal administration) attenuated the persistent mechanical hyperalgesia observed in these animals (Kim et al., 2012). This evidence strengthens the correlation between KYN activation and chronic pain, suggesting that the increased KYN/Trp ratio and its correlation with pain levels observed in our study may also reflect an underlying pathophysiological mechanism pertaining to chronic pain in the DNP condition.

Another result derived from this thesis is the positive correlation between BH4 plasma levels with pain scores in subjects with CRPS. BH4 is traditionally known as an essential cofactor for the catalytic activity of phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase (TPH) and all nitric oxide synthase isoforms (Thöny, Auerbach, & Blau, 2000). Recently, pathophysiological roles for exacerbated production of BH4 have been described, causing pain, increasing the aggressiveness of the immune system and the progression of the symptoms of chronic diseases, including, chronic pain, asthma, multiple sclerosis, ulcerative colitis, rheumatoid arthritis, cognitive impairment (Cronin et al., 2018; Fujita et al., 2019; Latremoliere et al., 2015). High CD8+ T cell number was one of the four variables from the machine learning approach developed in our study that distinguished CRPS subjects from healthy controls. Of note, BH4 is essential for CD8+ T cell expansion and their capacity to infiltrate tissue and precipitate T-cell-mediated immune response (Cronin et al., 2018). This is an important link given the positive association between BH4 plasma levels with pain scores and, perhaps, suggests a putative role for BH4 in the neuro-immune crosstalk that underlying CRPS.

BH4 levels were also increased in the urine of patients with UC-related abdominal pain in one of our clinical studies. Although we could not observe a direct correlation between the urinary concentration of BH4 and pain intensity in subjects with UC, urinary BH4 levels were

positively correlated with serotonin plasma levels, and the latter with pain intensity in UC subjects. Noteworthy, BH4 is a mandatory cofactor for the enzyme THP and, therefore, for the production of serotonin (Ghisoni, Martins, Barbeito, & Latini, 2015). Serotonin is a critical signalling molecule in the gut, where the activation of serotoninergic receptors has been implicated in visceral hypersensitivity (Kayser et al., 2007). Indeed, clinical evidence demonstrates that serotonin antagonist agents decreased hyperalgesia and abdominal pain in inflammatory bowel syndrome patients (Lacy, Nicandro, Chuang, & Earnest, 2018; Tack et al., 2006). Therefore, our results support a possible role for BH4-enhanced serotonin production in the process of persistent intestinal hypersensitivity of UC. Altogether, the studies presented here suggest that KYN and BH4 pathways and their various biologic active metabolites can be proposed as quantitative markers of chronic pain severity. Additionally, and in agreement with the data presented, it is feasible that KYN and BH4 pathways are involved in the mechanisms underlying chronic pain. Hence, KYN and BH4 pathways-related metabolites can be proposed as markers that inform about the pathophysiological mechanisms responsible for chronic pain.

Recent years have witnessed increasing evidence suggesting a role for inflammation in the induction and maintenance of chronic pain (Gao & Ji, 2010; Kawasaki, Zhang, Cheng, & Ji, 2008). Inflammation is a well-controlled physiological process that serves to promote regeneration and healing, but chronic pain may emerge as a maladaptive mechanism if the resolution of inflammation is disturbed (Christianson et al., 2011). Experimental data and clinical evidence point to an immune pathogenesis for chronic pain (Rojewska, Makuch, Przewlocka, & Mika, 2014; Shi, Gelman, Lisinicchia, & Tang, 2012). Likewise, the recruitment of the immune system after tissue damage initiates a cascade of molecular and cellular processes culminating in the production of pro-inflammatory cytokines that, in turn, activate the KYN and BH4 pathways (Latremoliere et al., 2015; Maganin et al., 2021).

The *de novo* BH4 pathway can be massively upregulated by inflammatory stimuli, such IFN-γ, TNF-α, IL1-β, GM-CSF and IL-8 (Werner et al., 1990). These inflammatory mediators will greatly increase the transcription of *GCH1* (the gene that codifies for the first enzyme in the *de novo* BH4 biosynthetic pathway) but not 6-pyruvoyl tetrahydrobiopterin synthase (the second enzyme in the *de novo* BH4 biosynthetic pathway) creating a pseudo-metabolic blockage with production and accumulation of the by-product neopterin (for a review see Ghisoni et al., 2015). Neopterin has been used for decades as an indicator of the presence of inflammation/activation of the immune system (Chittiprol et al., 2010; Hagberg et al., 2010). Similarly, inflammatory stimuli greatly activate IDO1, the rate-limiting enzyme of the KYN pathway (Takikawa et al., 1986), establishing the KYN/Trp ratio as a marker for IDO1 and

inflammatory activation (Badawy & Guillemin, 2019). Ultimately, the activation of KYN and BH4 pathways can generate bioactive metabolites with pro-nociceptive activity that have been related to chronic hypersensitivity in preclinical models of chronic pain (Latremoliere et al., 2015; Maganin et al., 2021).

Accordingly, our data showed increased levels of neopterin and KYN/Trp ratio, two inflammatory biomarkers from the BH4 and KYN pathways, respectively, in DNP subjects. In addition, pro-inflammatory mediators, such as GM-CSF and IL-8, were also increased in the plasma of subjects with DNP. Furthermore, a positive correlation between the KYN pathway metabolites (KYN and picolinic acid) with the pro-inflammatory TNF-α, and the BH4 with IL-1β was observed in DNP. Additionally, our results showed a reduction of the anti-inflammatory cytokine IL-37 in the plasma of CRPS subjects. Whereas the inflammatory marker CRP was increased in the plasma of subjects affected by UC-related abdominal pain. Altogether, the results derived from this thesis support the idea that increased levels of KYN and BH4 metabolites occur due to elevated pro-inflammatory cytokines, which could be involved in the pathogenesis of chronic pain conditions.

Some of the KYN and BH4 metabolites correlated with chronic pain features such as emotional functioning and kinesiophobia in our cross-sectional analyses. Specifically, *i)* plasma KYN/Trp ratio was positively associated with kinesiophobia, *ii)* plasma xanthurenic acid levels were positively correlated with depression and stress scores, and *iii)* neopterin levels were positively correlated with depression scores in CRPS subjects. An overlap of markers for chronic pain and emotional functioning has been previously reported (Niculescu et al., 2019). Niculescu *et. al.*, (2019) identified that most of the gene signature biomarkers identified for chronic pain overlap with biomarkers for suicide and other psychiatric disorders (Niculescu et al., 2015).

Previous clinical evidence showed that KYN/Trp ratio was associated with depressive symptoms, reduced motivation and pessimism in elderly subjects; While markers of BH4 pathway alteration (*i.e.*, increased neopterin and phenylalanine) correlated with neurovegetative symptoms, including sleep disturbance, fatigue and sickness symptoms in the same elderly population (Capuron et al., 2011). Additionally, a preclinical study reported that the depressive-like behaviour observed in a neuropathic pain mice model was associated with skewing of the KYN pathway balance toward the production of neurotoxic metabolites in the hippocampus (Laumet et al., 2017). Therefore, our observation that KYN- and BH4-related metabolites are also correlated with chronic pain symptoms, such as depression, stress and kinesiophobia, is not surprising. Suggesting that inflammation-induced KYN and BH4 pathways alterations can

be a common pathophysiological mechanism of chronic pain and its related symptoms or comorbidities. Overall, the cross-sectional evidence drawn from the studies presented in Chapter 3 showed that metabolites from the KYN and BH4 pathways were correlated with chronic pain and its symptoms and can be used as potential biomarkers of chronic pain.

4.2 RESEARCH IMPLICATIONS

The contemporary clinical practice for chronic pain relies on over-simplistic diagnosis and treatment with no mechanistic considerations (Tracey, Woolf, & Andrews, 2019). The current gold standard diagnostic tool for chronic pain is patient's report, which describes this condition by its severity, anatomical location, and duration, but does not reflect any neurobiological cause (Tracey et al., 2019). Accordingly, the current treatment for this disease rarely targets pathophysiological mechanisms and simply aimed at symptom relief (Woolf, 2010). Consequently, clinical outcomes for chronic pain conditions remain disappointingly poor, and prevalence and morbidity-related health care costs are unacceptably high (Gaskin & Richard, 2012; Painaustralia, 2019). Our analysis of KYN and BH4- related metabolites indicates atypical biochemistry in patients with chronic pain. The panel of metabolites from biochemical pathways analysed in our cross-sectional studies evaluates molecules that can regulate nerve health, synaptic connections, inflammation, redox state, neurotransmitter production as well as nociceptive function (Cronin et al., 2018; Guillemin, 2012; Latini et al., 2018; Maganin et al., 2021). Therefore, abnormalities in these biomarkers can provide information about key processes possibly involved in neuropathic, nociplastic and inflammatory chronic pain states. Clarification regarding the biochemical basis of chronic pain conditions can guide adequate treatment, increasing safeness and likelihood of success in chronic pain management (Woolf, 2010).

Our investigation of metabolites from KYN and BH4 pathways in three different cohorts and their correlation to chronic pain features have shown, to our knowledge for the first time, that *i)* measurement of metabolites from KYN and BH4 pathways could be used to objectively assess pain severity in chronic pain conditions; *ii*) metabolites from the KYN and BH4 pathways represent possible biomarkers of molecular mechanisms that underlying chronic pain conditions and its symptoms and, therefore, *iii)* the biomarkers we proposed could also be used to assess response to future therapeutic interventions aiming the modulation of the KYN and BH4 biochemical pathways.

We provided objective data implicating pathological changes in two relevant biochemical pathways (*i.e.*, KYN and BH4 metabolisms) underlying chronic pain in patients. An objective indication of the underlying neurobiology of chronic pain disorders can provide information to assist with the diagnosis, treatment, and monitoring of chronic pain (Tracey et al., 2019). Interestingly, KYN and BH4 metabolisms are targeted in current preclinical and clinical research for chronic pain drug development. Synthetic inhibitors recently developed targeting the activity of sepiapterin reductase to reduce exacerbated BH4 levels have been tested in preclinical chronic pain models (Fujita et al., 2019; Latremoliere et al., 2015). Moreover, chronic pain pharmacotherapy targeting the KYN pathway has recently reached Phase 1 (clinical trial IDs: NCT01483846 and NCT3212430). Further clinical studies should be carried out to investigate the role of KYN and BH4 pathways in chronic pain, which could strengthen the case for therapeutically targeting them in this disease.

4.3 LIMITATIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

In the previous chapter, limitations of clinical studies were acknowledged and discussed individually and, therefore, the limitations presented here are those relevant to this thesis as a whole. An important limitation includes the fact that only data at one time point were evaluated in the cross-sectional analyses. The timing of biomarker analysis can be critical, and most biomarkers discussed above can potentially change during the disease course, fulfilling the criteria for chronic pain biomarkers for a limited period (Mayeux, 2004). Additionally, the concentration of biomarkers may vary in patients, due to adaptation to a chronic pain state, to comorbidities and related administration and combination of pharmacological agents that cause alterations in metabolic processes or due to age-related changes in metabolism (Mayeux, 2004). To address these issues, the time-course of each potential biomarker would need to be determined within the respective pain state to identify the time point at which correlation with the pathophysiological clinical state. Overall, it is essential that the findings described in Chapter 3 to be tested in longitudinal cohort studies with a within-subject design to evaluate long-term outcomes for pain intensity, chronic pain-related symptoms, and biomarkers.

Some of the proposed biomarkers in the present study have been previously reported to be increased in the nervous system and body fluids of animals submitted to chronic pain models (Latremoliere et al., 2015; Maganin et al., 2021). In addition, the inhibition of the KYN and BH4 pathways induce analgesic-like effects in preclinical model of chronic pain (Latremoliere et al., 2015; Maganin et al., 2021). Accordingly, we suggest that the KYN and BH4 biomarkers

proposed in our research can be a reflection of underlying molecular mechanisms pertaining to the chronic pain conditions investigated. However, we cannot readily differentiate with our cross-sectional observational design if these putative biomarkers are a reflection of molecular mechanisms underlying chronic pain or if they are reflections of compensatory mechanisms and associated comorbidities. Therefore, the causal relationship between KYN and BH4 pathways alterations with chronic pain deserves further investigation. In this research we aimed to uncover chronic pain biomarker candidates from the KYN and BH4 pathways and a full biomarker validation will require further research investigation.

In summary, this project has been original in exploring relevant biochemical pathways for chronic pain. The project was designed from its inception through the literature review presented in Chapter 2, then, we for the first time presented an analysis of the KYN and BH4 pathways profile in patients affected by chronic pain. Based upon the arising data we proposed that the metabolites from both biochemical pathways can be targeted as biomarkers for chronic pain. Given the negative impact of untreated chronic pain on quality of life and the current lack of objective measures to determine the appropriateness of treatment, the importance of approaches such as ours cannot be overstated.

4.4 CONCLUSION

- KYN and BH4 metabolites can be quantified in human body fluids, in a low-invasive manner, as markers of chronic pain conditions and their related features;

- Exacerbation of the KYN and BH4 metabolisms are observed in chronic pain conditions, including DNP, CRPS and UC-related abdominal pain;

- Inflammation contributes to the pathological production of metabolites from the KYN and BH4 pathways in chronic pain;

- KYN and BH4 pathways alterations reflect underlying mechanisms of chronic pain that can be objectively assessed in patients.

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