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Mechanisms of Action of Manuka Honey as a Potential Therapeutic Agent for Bladder Pain Syndrome/Interstitial Cystitis: Clinical and Laboratory Studies

by

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Thesis for the degree of PhD

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Abstract

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Introduction: Bladder Pain Syndrome/Interstitial cystitis (BPS/IC) is a debilitating chronic inflammatory condition. It significantly reduces patients' quality of life, work productivity and represents a huge burden on health reserves. Current treatments are failing patients. Neurogenic inflammation through the interaction between mast cells and the bladder nociceptive sensory nerve endings is thought to be implicated. Mast cells response to the neuropeptide substance P (SP) is dependent on the expression of MRGPRX2, a recently identified receptor for SP. However, its expression by resident bladder mast cells has not been elucidated. Manuka honey has anti-inflammatory and antibacterial properties. Thus, it might be a useful adjuvant therapy for BPS/IC. The aims of this study were to investigate the link between neurogenic inflammation in the pathophysiology of BPS/IC and explore the potential anti-inflammatory effects of Medihoney and Manuka honey extract on inhibiting mast cell degranulation.

Materials and Method: Using immunohistochemistry, mast cell numbers and MRGPRX2 expression were evaluated in formalin-fixed paraffin-embedded bladder biopsies obtained from BPS/IC patients. Sections free of inflammation were used as controls. Human LAD2 cells were stimulated by SP. Subsequently, degranulation and pro-inflammatory cytokine release, with or without pre-incubation with Medihoney or Manuka honey extract, were assessed using β-hexosaminidase assay and electrochemoluminescent assay. In addition, phospho MAPK array was used to assess the associated intracellular signalling events.

Results: Upon both tryptase and chymase staining, mast cell numbers were significantly higher in the BPS/IC biopsies vs controls (166.1 ± 21.02 vs 72.64 ± 9.150 cells/mm^2) and (155.7 ± 23.86 vs 59.83 ± 9.153 cells/mm2), respectively. Nevertheless, the percentage MRGPRX2 expression on the mast cells was significantly higher in the BPS/IC biopsies compared to controls (68% VS 28% co-expression, respectively). These findings strongly support a

positive role for neurogenic inflammation in the pathophysiology of BPS/IC. Interestingly, Medihoney and Manuka honey extract strongly and dose-dependently inhibited LAD2 cell degranulation with a maximum of 96% inhibition at 4% concentration. This was accompanied by strong inhibition of the intracellular signalling events well known to underly mast cell degranulation, including the phosphorylation of Akt, ERK1/2 and STAT3. Interestingly, the substance P-induced mast cell activation was accompanied by the activation of the kinases WNK1, GSK3α/β and PRAS40, which was inhibited upon pre-incubation with Medihoney or the Manuka extract. To our knowledge, the activation of the later 3 kinases is a novel finding and the exact roles of these kinases in mast cell activation is yet to be explored.
Conclusion: Medihoney and the Manuka honey extract inhibited SP-induced mast cell degranulation. This strongly supports an anti-inflammatory role of these natural agents against the mast cell driven neurogenic inflammation, which is believed to be highly involved in the pathophysiology of BPS/IC. Our findings suggest that Medihoney and Manuka honey extract could potentially be an effective adjuvant intravesical therapy for BPS/IC.

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1 Glossary of Terms

Akt – Protein kinase B

APF – Antiproliferative factor

BPS/IC – Bladder Pain Syndrome/Interstitial Cystitis

CREB – Cyclic AMP Response Element Binding Protein

CT- computed tomography

EGF - epidermal growth factor

ERK1/2 – Extracellular Signal regulated Kinase 1/2

ESSIC - European Society for the Study of Interstitial Cystitis

FFPE- Formalin Fixed Paraffin Embedded

GAG – glycosaminoglycans

GSK 1/2/3 – Glycogen Synthase Kinase 1/2/3

HB-EGF - heparin-binding epidermal growth factor-like growth factor

ICS – International Continence Society

IVP-intravenous pyelogram

KI-67 - Marker of Proliferation Ki-67

LP – Lamina Propria

MC_T – Mast cell $_{Tryptase}$

MC_{TC} – Mast cell $_{Tryptase\ Chymase}$

MRGPRX2 – Mast cell related G Protein-coupled Receptor X2

mTORC1 - mammalian target of rapamycin C1

NGF – Nerve Growth Factor

NIDDK - National Institute of Diabetes, Digestive and Kidney diseases

NK-1 – Neurokinin-1

OAB - Overactive bladder

OLS - O'Leary Sant questionnaire

PRAS40 - proline-rich AKT substrate of 40 kDa

PSA - prostate-specific antigen

PUF - Pelvic Pain and Urgency/Frequency questionnaire

RSK 1/2/3 p90 ribosomal S6 kinase 1/2/3

SCF - Stem Cell Factor

SP - Substance P

STAT3 - Signal transducer and activator of transcription 3

SUFU-Society for Urodynamics and Female Urology

ZO-1 – Zona occludens 1

TNF-α – Tumour Necrosis Factor Alpha

TUNEL- Terminal deoxynucleotidyl transferase dUTP nick end labeling

WNK - lysine deficient protein kinase 1

2 List of publications and conference presentations

2.1 Publications

(a) Medical-Grade Manuka Honey Inhibits Mast Cell Degranulation through Downregulation of Protein Kinase-B/Akt Phosphorylation: Potential Intravesical Agent in the Management of Interstitial Cystitis/Bladder Pain Syndrome? (Submitted).

(b) Mast cells in the urinary bladders of interstitial cystitis/bladder pain syndrome patients express the Mast cell Related G-Protein coupled Receptor X2 (MRGPRX2): Potential role in the pathogenesis of neurogenic inflammation (Under preparation).

2.2 Conference presentations

2.2.1 Podium presentations

(a) Abdelwahab O.K., Abadalkareem R.S., Lau L.C., Ewings S.M., Yusuh M., Garba K., Bodey K., Markham H., Walls A.F., Birch B.R.P., Lwaleed B.A. (2022) 'Medical-Grade Manuka Honey inhibits mast cell degranulation by downregulating Protein Kinase-B (Akt) Phosphorylation: Potential role as intravesical agent in the treatment of interstitial cystitis/bladder pain syndrome', International Continence Society. Vienna, 2022. (Podium, 15 minutes) Best urogynaecology and Female & Functional Urology.

(b) Abdelwahab, O.K., Markham, H., Yusuh, M., Garba K., Bodey, K., Lopez, M.A. M., Lau, L., Johnston, D., Walls, A., Birch, B., Lwaleed, B. (2022) ‘Mast cells in the urinary bladders of interstitial cystitis/bladder pain syndrome patients express the Mast cell Related G-Protein coupled Receptor X2 (MRGPRX2): Potential role in the pathogenesis of neurogenic inflammation’ International Continence Society. Vienna, 2022. (Short oral Podium, 7.5 minutes).

(c) Abdelwahab O.K., Abadalkareem R.S., Lau L.C., Ewings S.M., Yusuh M., Garba K., Bodey K., Markham H., Walls A.F., Birch B.R.P., Lwaleed B.A. (2020) 'Medical-Grade Manuka Honey inhibits mast cell degranulation by downregulating Protein Kinase-B (Akt) Phosphorylation: Potential role as intravesical agent in the treatment of interstitial cystitis/bladder pain syndrome', British Association of

Urological Surgeons. Birmingham. Journal of Clinical Urology, pp. 20-22. Available at: https://journals.sagepub.com/doi/10.1177/2051415820963006.

(d) Abdelwahab O.K., Abadalkareem R.S., Lau L.C., Ewings S.M., Yusuh M., Garba K., Bodey K., Markham H., Walls A.F., Birch B.R.P., Lwaleed B.A. (2020) '293 - Medical-grade Manuka honey inhibits mast cell degranulation by downregulating protein kinase-B (Akt) phosphorylation: Potential role as intravesical agent in interstitial cystitis/ bladder pain syndrome', European Urology Open Science, 19, pp. e499-e500.

(e) Abdelwahab, O.K., Markham, H., Yusuh, M., Garba K., Bodey, K., Lopez, M.A. M., Lau, L., Johnston, D., Walls, A., Birch, B., Lwaleed, B. (2021) 'P0002 - Mast cells in the urinary bladders of interstitial cystitis/bladder pain syndrome patients express the Mast cell Related G-Protein coupled Receptor X2 (MRGPRX2): Potential role in the pathogenesis of neurogenic inflammation', European Urology, 79, pp. S2-S3.

3 Declaration of Authorship

Print name: Omar Abdelwahab

Title of thesis: Mechanisms of Action of Manuka Honey as a Potential Therapeutic Agent for Bladder Pain Syndrome/Interstitial Cystitis: Clinical and Laboratory Studies

I declare that this thesis and the work presented in it is my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at the University of Southampton;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission;

Signature: **Date:** 13 / 06 / 2022

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5 Chapter one: Introduction and Background

5.1 Bladder Pain Syndrome

5.1.1 Definition and Evolution

Bladder pain syndrome/Interstitial cystitis (BPS/IC) is a chronic debilitating inflammatory disease of the urinary bladder that significantly lowers the life quality of its patients. Hanash and Pool first described the disease in 1969. They termed the disease Interstitial Cystitis (IC) and defined it as a lower urinary tract disease associated with severely reduced bladder capacity and characterised by a characteristic bladder ulcer (Hunner's Ulcer) (Hanash and Pool (1969)). Such a diagnosis was only restricted to symptomatic patients who had a severely reduced bladder capacity and the characteristic Hunner's Ulcer on bladder cystoscopy with hydro-distension. Hunner's Ulcer was defined as small circumscribed reddish bladder mucosal area with small vessels radiating towards a central scar (Hunner, 1915).

In 1978, Messing and Stamey highlighted the fact that patients with both the characteristic bladder ulcers and severely reduced bladder capacity accounted only for a classic type of the disease. However, there are many more common cases with longstanding lower urinary tract symptoms and no evidence of any urinary tract infection (Sterile non-infected urine), which accounted for the non-Classic (non-ulcer) type of the disease. They also concluded that the cystoscopic finding of small petechial haemorrhages (glomerulations) on hydro-distension were pathognomonic for the diagnosis of Interstitial cystitis (Messing and Stamey, 1978).
In 1987 and 1988, the National Institute of Diabetes Digestive and Kidney Diseases (NIDDK) of the United States of America established robust criteria for the diagnosis of Interstitial Cystitis (IC). Over 90% of patients who met these criteria were believed by both researchers and clinical experts to have Interstitial Cystitis, which suggests an important role for these criteria for research purposes. However, it also became apparent that strict adherence to the NIDDK criteria resulted in the misdiagnosis of more than 60% of patients who were believed to have interstitial cystitis by experts (Hanno *et al.*, 1999).

In 2002, the International Continence Society (ICS) renamed the disease as Painful Bladder Syndrome, which was defined as "the complaint of suprapubic pain, related to

bladder filling accompanied by other urinary symptoms, such as daytime and night-time frequency, in the absence of proven urinary infection or other obvious abnormality on biopsy". They confined the term Interstitial Cystitis to Painful Bladder Syndrome cases with typical cystoscopic and histological features (Abrams *et al.*, 2002).

The currently accepted definition was established by the European Society for the Study of Interstitial Cystitis (Préfontaine *et al.*). They proposed the term Bladder Pain Syndrome/Interstitial Cystitis (BPS/IC) and defined it as "chronic pelvic pain, pressure or discomfort perceived to be related to the urinary bladder, accompanied by at least one of the lower urinary symptoms including urge to void, frequency and nocturia". Such diagnosis is confirmed after exclusion of a wide group of confounding diseases, including urinary tract infection, as the main factors responsible for the disease condition (van de Merwe *et al.*, 2008; Dasgupta and Tincello, 2009; Wyndaele, Van Dyck and Toussaint, 2009; Richter *et al.*, 2010; Nordling, Fall and Hanno, 2012). Such a definition was accepted by the International Consultation of Incontinence in order to include all patients with bladder pain (Hanno *et al.*, 2009).

In 2009, the Society for Urodynamics and Female Urology (SUFU) defined the BPS/IC as "an unpleasant sensation (pain, pressure, discomfort) perceived to be related to the urinary bladder, associated with lower urinary tract symptoms for more than six weeks duration, in the absence of infection or other identifiable causes".

5.1.2 BPS/IC a major health problem

The multifactorial nature of the disease, the lack of an accurate diagnostic tool, in addition to a large number of confounding diseases, which are much more common than BPS/IC make the establishment of an accurate diagnosis of BPS/IC a very challenging and complicated process. All of the questionnaires established to measure the disease prevalence rates depend on the patient's perception and expression of the clinical symptoms, which can vary from one patient to another in case of BPS/IC. Additionally, the difference between the symptom questionnaires, which include the O'Leary Sant questionnaire (OLS) and Pelvic Pain and Urgency/Frequency questionnaire (PUF) result in differences in the augmented incidence and prevalence rates due to the sensitivity and specificity differences between these questionnaires (Parsons *et al.*, 2002; Vella, Robinson and Cardozo, 2015). As such, it is difficult to establish accurate prevalence rates

for the disease. Despite the huge numbers of studies attempting to establish accurate prevalence and incidence rates of BPS/IC, they remain difficult to interpret, analyse and compare. Some of these reports depend on non-verified patient filled questionnaires, while others depend on physician diagnosis of BPS/IC symptoms without exclusion of the confounding diseases (Nickel, 2006).

However, all the prevalence indicators suggest that BPS/IC is not a rare condition but a common disease that affects a significant proportion of the United States population of both sexes, most commonly women (Tirlapur *et al.*, 2013; Held, Hanno and McCormick, 2018).

5.1.3 Prevalence rates of BPS/IC

Recent epidemiologic studies have revealed progressively increasing prevalence rates of the disease (Curhan *et al.*, 1999; Mishra, 2015; Vella, Robinson and Cardozo, 2015). In 1999, an epidemiologic study by Curhan and colleagues concluded that the prevalence rate of the BPS/IC among American women ranged from 52 to 67 per 100,000 women (Curhan *et al.*, 1999). A more recent study by Clemens et al revealed a disease prevalence rate of 158 per 100000 women and 28 per 100000 men in the United States (Clemens *et al.*, 2005). In a Canadian study, 2.8% of 8712 patients attending urology clinics were diagnosed as BPS/IC patients, with a female to male ratio of 8:1 (Nickel *et al.*, 2005b). Recently, the prevalence rate of the disease has been progressively increasing, especially with increasing life stresses. Berry and colleagues concluded that from 3.3 to 7.9 million of United States women above the age of 18 years have BPS/IC (Berry *et al.*, 2011). Recent estimates reveal a high BPS/IC prevalence rate of 197 per 100,000 women (Mishra, 2015; Vella, Robinson and Cardozo, 2015).

BPS/IC lowers the life quality and work productivity of patients, and results in a significant increase in the consumption of the health care reserves, which imposes a heavy burden on the national economy of all countries around the world (Robinson *et al.*, 2011; Hakimi *et al.*, 2017; Held, Hanno and McCormick, 2018).

5.1.4 Classification of BPS/IC

5.1.5 Diagnosis of BPS/IC

5.1.5.1 Symptoms

5.1.5.1.1 Pain

BPS/IC is characterised by a chronic sterile bladder inflammation and urothelial barrier defect, leaving the bladder tissue abnormally hypersensitive to even minimal urine volumes. This results in abnormal pain perception, which can vary between the sensation of pressure, discomfort or severe pain, on non-noxious stimuli, including minimal urine volume. Such pain forces the patient to go rapidly to the bathroom to void in order to avoid the exaggeration of the bladder pain, which is appreciated by the patient as an urge to void. It also leads to increased frequency of both daytime voiding sessions (Frequency) and night-time voiding sessions (Nocturia).

Pelvic pain is the predominant symptom in the clinical picture of BPS/IC (Warren *et al.*, 2006). Not only does it predispose to the other accompanying symptoms, but also its intensity negatively affects the patients' general condition. In the early stages of BPS/IC or in the mild form of the disease, pain may be replaced by the sensation of pressure, burning or discomfort that forces the patient to void (Hanno *et al.*, 2011; Cox *et al.*, 2016). According to the International Association for the Study of Pain (IASP), pain is defined as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage". Due to their association with bladder tissue damage, the sensations of pressure, burning, sharp or discomfort experienced in the milder forms of the disease can be considered as pain, despite not being described as such (van de Merwe *et al.*, 2008). This evidence adds to the concept of pain as the main clinical symptom in the clinical picture of BPS/IC patients. Suprapubic pain is usually described by patients, however, the pain can be referred to other areas including the perineum, medial aspect of the thigh, urethra, vagina, inguinal area infra pubic region, lower abdomen and lower back (Hanno *et al.*, 2011; Fall, Logadottir and Peeker, 2014; Cox *et al.*, 2016).

The nature of pain and its timing in relation to bladder filling and menstruation can aid the physician greatly in making a differential diagnosis of BPS/IC. Pain that occurs only during voiding is most likely to arise by the contact between the urine and the vulva or urethra, which is strongly suggestive of vulvar/urethral disorders. The symptoms of BPS

may be aggravated a few days before menstruation; however, in endometriosis such aggravation occurs during menstruation. Several factors can result in aggravation of the symptoms of BPS/IC patients resulting in periods of worsening of the patients' general condition (flares). These factors include sexual intercourse, menstruation, and stress. Additionally, some types of food and drinks can intensify the patients' symptoms. These include tomatoes, coffee, alcohol, carbonated drinks, citrus fruits and spicy foods (Cox *et al.*, 2016).

5.1.5.1.2 The Urge to Void

The International Continence Society has defined urgency as "a compelling need to urinate which is difficult to postpone." Urgency is a major symptom of Overactive Bladder Syndrome (OAB), a major confounding condition for BPS/IC. In OAB, urgency describes a sudden compelling desire to pass urine by the patient to avoid getting wet. According to ESSIC, the term urgency is not included in the symptoms of BPS/IC, and has been replaced by the term persistent urge to void. In BPS/IC, the patient experiences a strong compelling desire to void to avoid intensifying the pain, a condition described as the Urge to void (van de Merwe *et al.*, 2008; Hanno *et al.*, 2011).

5.1.5.1.3 Frequency in BPS/IC patients

In BPS/IC, frequency is the most common symptom in the patients' clinical presentation, being found in nearly 90% of the patients (Tincello and Walker, 2005; Ito *et al.*, 2007). Marked frequency can be observed in OAB patients as well as BPS/IC patients. Consequently, frequency alone cannot be relied upon to make a diagnosis of BPS/IC and must be studied as a part of the complete patient clinical picture; however, it can be very useful for monitoring the clinical response to treatment.

5.1.5.2 Clinical Findings in Bladder Cystoscopy

5.1.5.2.1 Hunner's lesion (ulcer)

Hunner's ulcer is a characteristic lesion (Hunner, 1915; Hanno *et al.*, 2018) that includes dehiscence of the urinary bladder mucosa and submucosa following bladder hydro-distension, and consequently, it is not a true ulcer (van de Merwe *et al.*, 2008; Fall,

Logadottir and Peeker, 2014). It is found in less than 10% of BPS/IC cases, which constitute the ulcerative type of BPS/IC (classified as BPS/IC type 3C by the ESSIC see later). By way of contrast, more than 90% of BPS/IC cases do not show Hunner's type lesions on cystoscopy with hydro-distension. These constitute the non-ulcerative type of BPS/IC.

5.1.5.2.2 The Glomerulations

These are mucosal bleeding points (petechiae) seen in the bladder after hydro-distension, which can be graded as shown in the following table:

Table 1: Glomerulations on bladder hydro-distension in BPS/IC patients (van de Merwe *et al.*, 2008)

Glomerulations grade	1	2	3
Definition	Minimal submucosal petechial haemorrhages	a large submucosal bleed (ecchymosis)	diffuse submucosal bleeding, on hydro-distension
Positive sign for BPS/IC	-	+	+

Glomerulations of both grade (2), which is a large submucosal bleed (ecchymosis), and grade (3), which is a diffuse submucosal bleeding on hydro-distension, are considered positive signs for BPS/IC according to the ESSIC.

5.1.6 Classification of BPS/IC

5.1.6.1.1 According to the histopathological findings in BPS/IC bladder biopsies, patients are classified into

- Histologically normal, which is referred to as A.
- Histologically inconclusive, which is referred to as B.
- Histologically positive for BPS/IC, which is referred to as C. In such case, the bladder biopsies show inflammatory infiltrates and/or detrusor mastocystosis (> 28 mast cells per mm^2) and/or granulation tissue and/or intra fascicular fibrosis (van de Merwe *et al.*, 2008; Nordling, Fall and Hanno, 2012).

According to the ESSIC classification each BPS/IC case is classified by two symbols, the first refers to the cystoscopic finding (1, 2 or 3), and the second refers to the histopathologic finding (A, B or C). The symbol (X) can replace any of the two symbols if the corresponding technique (biopsy histological examination or Cystoscopy with hydro distention) has not been performed.

Table 2: ESSIC Classification of the types of BPS/IC according to the findings at cystoscopy with hydro-distension and of biopsies (van de Merwe *et al.*, 2008)

	Cystoscopy with Hydrodistension			
Biopsy	Not done	Normal	Glomerulations[a]	Hunner's lesion[b]
Not done	XX	1X	2X	3X
Normal	XA	1A	2A	3A
Inconclusive	XB	1B	2B	3B
Positive[c]	XC	1C	2C	3C

a refers to grade (2-3) glomerulations (submucosal petechial haemorrhages).

b refers to Hunner's lesion (ulcer).

c refers to positive histology (inflammatory cell infiltrates and/or detrusor mastocystosis and/or granulation tissue and/or intrafascicular fibrosis on bladder histology).

Table 3 Confounding diseases for BPS/IC (van de Merwe *et al.*, 2008).

Confounding disease	**Excluded or diagnosed by [a]**
Carcinoma and carcinoma in situ	Cystoscopy and biopsy
Infection with Common intestinal bacteria	Routine bacterial culture
Chlamydia trachomatis, Ureaplasma urealyticum, Mycoplasma hominis, Mycoplasma genitalium, Corynebacterium urealyticum, Candida species	Special cultures
Mycobacterium tuberculosis	Dipstick; if ''sterile'' pyuria culture for M. tuberculosis

Herpes simplex and human papilloma virus	Physical examination
Radiation	Medical history
Chemotherapy, including immunotherapy with cyclophosphamide	Medical history
Anti-inflammatory therapy with tiaprofenic acid	Medical history
Bladder-neck obstruction and neurogenic outlet obstruction	Uroflowmetry, ultrasound, and medical history
Bladder stone	Imaging or cystoscopy
Lower ureteric stone	Medical history and/or haematuria: upper urinary tract imaging such CT or IVP
Urethral diverticulum	Medical history, physical examination and imaging
Urogenital prolapse	Medical history and physical examination
Endometriosis	Medical history, physical examination and Laparoscopy
Vaginal candidiasis	Medical history and physical examination
Cervical, uterine, and ovarian cancer	Physical examination
Incomplete bladder emptying (retention)	Post-void residual urine volume measured by ultrasound scanning
Overactive bladder	Medical history and Urodynamics
Prostate cancer	Physical examination and PSA
Benign prostatic obstruction	Uroflowmetry and pressure-flow studies
Chronic bacterial prostatitis	Medical history, physical examination, culture
Chronic non-bacterial prostatitis	Medical history, physical examination, culture
Pudendal nerve entrapment	Medical history, physical examination, nerve block may be diagnostic
Pelvic floor muscle-related pain	Medical history, physical examination

CT = computed tomography.

IVP = intravenous pyelogram.

PSA = prostate-specific antigen.

a = the diagnosis of a confounding disease does not necessarily exclude a diagnosis of BPS.

5.1.7 Pathophysiology of BPS/IC

The exact pathophysiology of BPS/IC remains unclear and needs much exploration and is often referred to as multifactorial. However, multiple theories have been recently proposed. These theories include urothelial barrier defects, mast cell degranulation, chronic inflammation, neurogenic inflammation, and autoimmune processes (Ke and Kuo, 2015; Patnaik *et al.*, 2017b).

5.1.7.1 Urothelial barrier defect and aberrant urothelial differentiation programme in BPS/IC

5.1.7.1.1 The normal urinary bladder Urothelium

The bladder urothelium plays a vital role as an impermeable barrier against harmful urinary constituents including cations, pathogens and urea. To perform such a function, the urothelial cells undergo a characteristic differentiation programme that allows the single-layered basal urothelial cells to divide into two to three layers of intermediate cells, which differentiate into the mature apical (Umbrella cells). The later cells are fully-differentiated, which in turn allows the cells to perform their main function as a urothelial permeability barrier. These Umbrella cells produce cell adhesion molecules (Cadherins), tight junctional proteins (Such as ZO-1 and occludin), crystalline plaque proteins (Uroplakins) and the glycosaminoglycans (GAG), which enable the apical urothelial cells to form a strong impermeable barrier against harmful urinary constituents. (Acharya *et al.*, 2004; Riedel *et al.*, 2005; Esko, Kimata and Lindahl, 2009; Keay, Birder and Chai, 2014; Hurst *et al.*, 2015; Ke and Kuo, 2015).

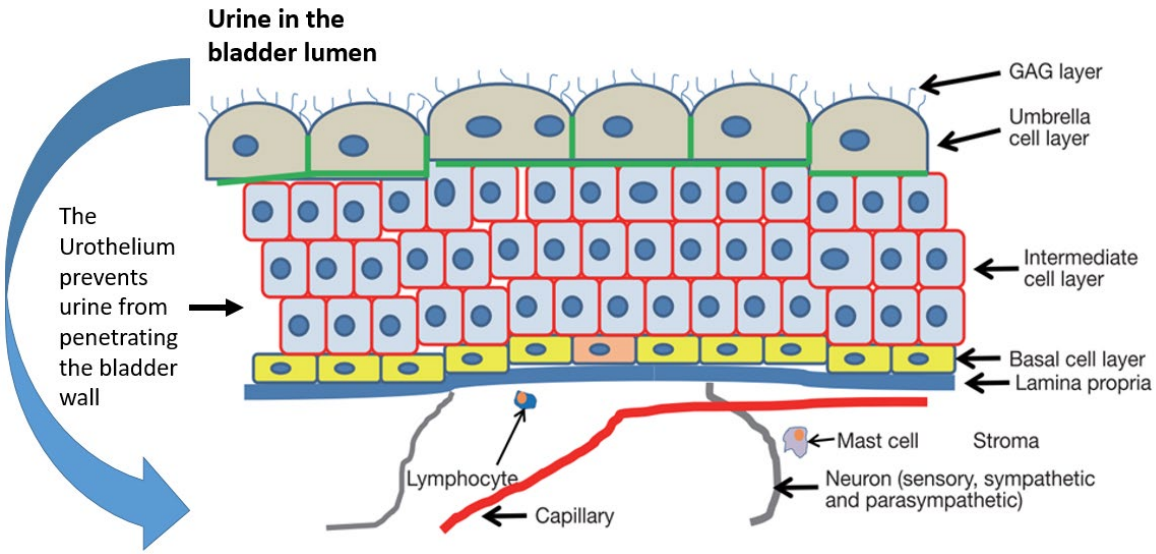

Figure 1: Urinary Bladder Urothelium adapted from (Hurst *et al.*, 2015).

5.1.7.1.2 Defective Urothelium in BPS/IC patients

One of the cardinal signs seen on the histopathological examination of the bladders of BPS/IC patients is urothelial thinning and denudation with or without ulceration (Lynes W. L. *et al.*, 1990; Hurst *et al.*, 2015). Such defective and denuded urothelium has been associated with increased urothelial permeability as demonstrated through the potassium sensitivity test, allowing K^+ and other harmful urinary constituents to penetrate the bladder tissue and depolarize sensory afferent nerve fibres (Parsons, Zupkas and Parsons, 2001; Parsons *et al.*, 2005; Parsons, 2007;2011; Hurst *et al.*, 2015).

Adding to the total (ulcerative BPS/IC) or partial (non-ulcerative BPS/IC) denudation of the bladder urothelium, histopathological examination of bladder biopsies from BPS/IC patients has revealed an abnormal urothelial differentiation program. Such an aberrant programme is characterised by increased apoptosis, decreased proliferation rate and decreased expression of various epithelial markers essential for maintaining the normal impermeability of the bladder urothelium (Hauser *et al.*, 2008; Shie and Kuo, 2009;2010; Liu *et al.*, 2012b; Shie, Liu and Kuo, 2012; Lee and Lee, 2013).

Many studies have demonstrated the increased apoptosis and decreased cell-proliferation rate of BPS/IC urothelial cells compared to controls. KI-67 and TUNEL immunofluorescence staining of the bladder biopsies have revealed significantly increased numbers of apoptotic cells and decreased urothelial proliferation in IC (Shie and

Kuo, 2009;2010). In BPS/IC bladder biopsies, urothelial TUNEL staining co-localized with phospho-p38 mitogen-activated protein kinase (MAPK), an inflammation marker, suggesting a contributing role of inflammation to urothelial apoptosis. Additionally, protein array analysis has revealed increased levels of the pro-apoptotic proteins, phospho P53 and cleaved caspase 3, together with the pro-inflammatory proteins, TNF-α and phospho-p38α (Shie, Hsin and Kuo, 2012). In 2013, Lee et al., demonstrated significantly increased numbers of apoptotic urothelial cells and activated mast cells in the biopsies from both ketamine-induced and non-ulcerative IC patients compared to controls (Lee, Jiang and Kuo, 2013). Activated mast cell release their granular contents, including chymase, which can induce urothelial cell apoptosis (Choi *et al.*, 2016).

In BPS/IC bladder biopsies, the expression of the tight junction-associated protein ZO-1 is generally reduced and localized to the cytoplasm or absent, instead of the normal distribution pattern in the tight junctions outlining the cells. Also, in BPS/IC biopsies, uroplakin was seen to be distributed throughout the urothelium instead of the normal distribution pattern seen on the luminal surface of the umbrella cells. The cell adhesive molecule E-cadherin, normally localized to the tight junctions outlining the cells in a similar fashion to ZO-1, was generally overexpressed and abnormally distributed in the cytoplasm in BPS/IC. This was explained as an early response to urothelial damage (Slobodov *et al.*, 2004).

Immunofluorescence staining of the urothelial cells explanted from BPS/IC bladder biopsies showed significant decreases in the expression of the proteins associated with tight junction formation compared to normal urothelial cells. Levels of ZO-1, occludin, α1-catenin, vimentin and α2-integrin proteins were decreased by 79.6% ± 0.1%, 84.1% $\pm$ 0.14%, 85.8% $\pm$ 0.1%, 97.5% $\pm$ 0.01% and 58.7% $\pm$ 0.04% in IC cells compared to their normal counterparts, respectively. At the same time, E-cadherin levels were significantly increased by 55.2% $\pm$ 0.1% (Zhang *et al.*, 2005) (fig. 2). Many other studies have shown markedly reduced levels of E-cadherin in BPS/IC biopsies compared controls (Shie and Kuo, 2010; Liu *et al.*, 2012b; Lee, Jiang and Kuo, 2013). Such evidence supports the previous explanation of increased BPS/IC E-cadherin levels as being an early response to urothelial injury.

The aberrant differentiation programme of BPS/IC is characterized by abnormal expression of the Glycosaminoglycan molecules in the bladder urothelium (Hurst, Moldwin and Mulholland, 2007; Lucon *et al.*, 2014). Immunohistochemical staining of BPS/IC bladder biopsies has revealed a defective GAG layer in the bladder urothelium, both on the luminal surface and the basement membrane, together with abnormal distribution of the urothelial chondroitin sulphate as shown in figure 2. (Hurst *et al.*, 1996; Slobodov *et al.*, 2004).

5.1.7.1.3 Ant proliferative factor (Krapf and Goldstein) and the defective bladder urothelium in BPS/IC

A defective and less proliferative bladder urothelium has been suggested as a major factor contributing to the pathophysiology of BPS/IC. The urothelial cells from bladder biopsies of BPS/IC patients show a significantly slower proliferation rate when compared to controls after explantation in a defined cell culture medium without any growth factors (Keay *et al.*, 2004). More interestingly, the bladder urothelial cells of BPS/IC patients exclusively produce a small frizzled 8-related peptide molecule called Anti-proliferative factor (Krapf and Goldstein) (Keay *et al.*, 2004). The urinary levels of both APF and epidermal growth factor (Simpson *et al.*) were significantly higher in BPS/IC than controls, while those of heparin-binding epidermal growth factor-like growth factor (HB-EGF) in patients were significantly lower than controls (Erickson *et al.*, 2002; Keay *et al.*, 2004; Zhang, Li and Kong, 2005).

APF treatment induced a marked decrease in the proliferation rate of primary cultured normal human urothelial cells. This was accompanied by significant decrease in expression of tight junctional proteins, which are mainly responsible for normal urothelial impermeability (Zhang, Li and Kong, 2005).

Interestingly, APF treatment of primary cultured normal human urothelial cells induced a marked increase in the number of the tetraploid and hyper tetraploid cells, compared to mock APF-treated cells, indicating blocking of the G2/M phases of the cell cycle (Rashid *et al.*, 2004).

5.1.7.2 Neurogenic inflammation theory

In BPS/IC bladders, the damaged urothelial cells and smooth muscle cells produce stem cell factor (SCF) (Theoharides, Kempuraj and Sant, 2001; Sant *et al.*, 2007b). The latter is responsible for the extravasation and migration of mast cells progenitors, originally circulating in the blood stream, which then localize in the perivascular spaces in the bladder wall. Additionally, SCF induces mast cell differentiation and maturation and increases mast cell responsiveness to degranulation factors such as SP (Tsai *et al.*, 1991; Pang *et al.*, 1996a; Da Silva, Reber and Frossard, 2006). Such is the scientific basis for using stem cell factor for inducing growth and maturation of in vitro cultured mast cells.

The defective permeability barrier function of the bladder urothelium in BPS/IC patients allows infiltration of harmful urine constituents, which can then interact with bladder afferent sensory nerve terminals in the lamina propria. These harmful urine constituents, including K^+, depolarize bladder afferent nerve fibres, which release neuroactive substances including neuropeptides such as substance P (SP) and calcitonin gene related peptide (CGRP). Acting on their specific receptors, these substances initiate and orchestrate many events that culminate in the development of chronic inflammation. These events include arteriolar dilatation and plasma extravasation (Subramanian, Gupta and Ali, 2016). SP activates mast cells to degranulate and release pro-inflammatory mediators (Pang *et al.*, 1996a; Theoharides *et al.*, 1998; Tancowny *et al.*, 2006; Kulka *et al.*, 2008; Tore and Tuncel, 2009; Tancowny *et al.*, 2010; Manning *et al.*, 2016).

Upon activation, mast cells undergo two reactions, one is immediate and the other is late. The immediate reaction is a process called degranulation. In such a process, various previously-synthesized granule-stored pro-inflammatory mediators are released outside of the cells by exocytosis. The previously-synthesized granule-packed mediators include biogenic amines such as histamine and serotonin, serine proteases such as tryptase, chymase, carboxypeptidase and cathepsin G, and proteoglycans such as heparin, in addition to the cytokine TNF-α. The post-activation late reaction of mast cells is in the form of de-novo synthesis and release of specific pro inflammatory mediators, a process that can take from 18 to 24 hours. These mediators include cytokines such as IL-6, TNF-α, IL-4, NGF, IL-10, IL-17a and IFN-γ, chemokines such as MCP-1, RANTES, IL-8, CXCL9, CXCL-10, CXCL-11 and arachidonic acid metabolites such as LTC4 and PGD2 (da Silva, Jamur and

Oliver, 2014). Cytokines, biogenic amines and proteoglycans cause vasodilatation, increase vascular permeability and increase the production of adhesion molecules by vascular endothelial cells (Fitzgerald *et al.*, 2006). Chemokines attract blood circulating leukocytes e.g. eosinophils, neutrophils, lymphocytes, macrophages and mast cells into the inflammation site (White, Bhangoo and Miller, 2005). Cytokines and chemokines are involved in the pathogenesis of pathological pain in chronic pain conditions (Zhang and An, 2007). Proteases and biogenic amines interact with and cleave receptors on sensory nerve fibre membranes, resulting in depolarization of the sensory nerve terminals and further production of neuropeptides such as SP and CGRP from the nerve terminals, which further activate mast cells. (Sant and Theoharides, 1994; Steinhoff *et al.*, 2000; Theoharides, Kempuraj and Sant, 2001; Batler *et al.*, 2002; Sant *et al.*, 2007a; Subramanian, Gupta and Ali, 2016).

These events create a vicious cycle, which culminates in the initiation of and/or maintenance of chronic inflammation characteristic of the disease. (Jiang *et al.*, 2013; Gonzalez, Arms and Vizzard, 2014; Logadottir *et al.*, 2014a; Logadottir *et al.*, 2014b; Ke and Kuo, 2015; Liu and Kuo, 2015).

The interaction between neuropeptides, including SP, and mast cells has been proposed by many studies to play a key role in the generation and maintenance of the neurogenic inflammation responsible for the pathophysiology of BPS/IC (Suzuki *et al.*, 1995; Pang *et al.*, 1996a). Immunohistochemistry of BPS/IC bladder biopsies has revealed higher numbers of mast cells. These mast cells lie in juxtaposition to SP-positive nerve fibres (Pang *et al.*, 1996a). The density of SP-positive nerve fibres was shown to be much greater in BPS/IC biopsies compared to controls and located mainly in the submucosa in close proximity to mast cells, (Pang *et al.*, 1995). Finally, the levels of the encoding mRNA for the neurokinin-1 (NK-1) receptor, a specific SP receptor, are markedly elevated in BPS/IC bladders (Marchand, Sant and Kream, 1998). These data provide strong evidence for a potential substance P-mast cell interaction and present this neuroimmune interaction and SP-induced mast cell degranulation as major contributing factors to the pathophysiology of the BPS/IC.

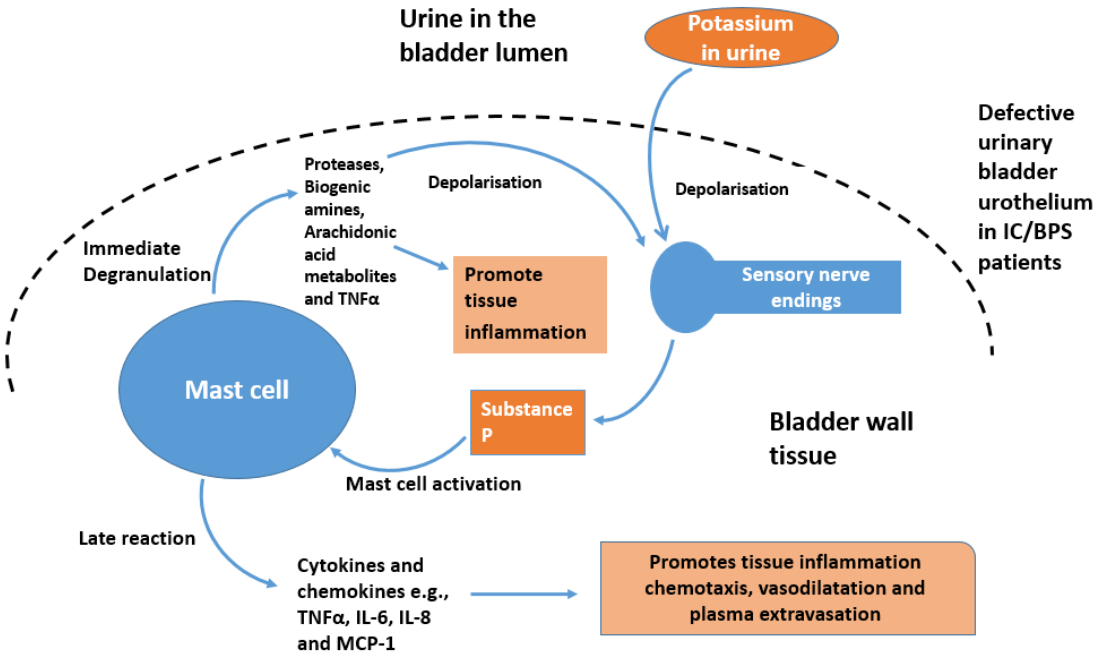

Figure 2: Schematic representation of the role of neurogenic inflammation in the pathophysiology of BPS/IC.

5.1.7.2.1 SP and mast cells

Several in vitro studies were established to investigate the role of substance P as an inducer of human mast cell activation. SP (10 µM/L) treatment for 18 hours induced a 6-fold increase in the production of the pro-inflammatory chemokine MCP-1 (Castellani *et al.*, 2009a).

Moreover, neuropeptides including Vasoactive Intestinal Peptide (VIP) and SP were able to induce activation and degranulation, assessed by β-hexosaminidase release, of both LAD2 mast cells and $CD34^{+}$ progenitor cell-derived mast cells. This neuropeptide-induced mast cell activation resulted in the production of pro-inflammatory mediators including chemokines CCL-3 (MIP-1α), CCL-4 (MIP-1β), CCL-2 (MCP-1), IL-8 (CXCL-8), CCL-5 (RANTES) and inducible protein-10 (CXCL-10), in addition to the cytokines TNF-α, IL-3, VEGF and Granulocyte-Macrophage colony-stimulating factor (Kulka *et al.*, 2008; Castellani *et al.*, 2009b; Patel and Theoharides, 2017; Taracanova *et al.*, 2017; Green *et al.*, 2019). These proteins (Cytokines and Chemokines) are well known to promote and maintain tissue damage and the inflammatory process (Pang *et al.*, 1996a; Kulka *et al.*, 2008; Castellani *et al.*, 2009a).

Substance P induces mast cell degranulation by activating its G-protein coupled receptors (which will be discussed later), resulting in increased G-protein signalling pathway activity characterized by the activation of various signalling proteins including G-protein α subunits, Phosphoinositide-3 kinase (PI3K), Inositol triphosphate (I3P) and Protein Kinase A (Kulka *et al.*, 2008). The signalling pathway mTOR has been recently implicated in Substance P-induced mast cell activation and de novo cytokine synthesis (Patel and Theoharides, 2017).

5.1.7.2.1.1 Mast cell receptors responsible for the substance P-mediated effect

Neurokinin-1 receptors (NK-1) are members of the rhodopsin-like G-Protein coupled receptors, continuously expressed by peptidergic neurons (releasing neuropeptides) in both the peripheral and central nervous systems, in addition to various immune cells including mast cells (Douglas and Leeman, 2011). NK1-Rs have been suggested as the high affinity Substance P receptors responsible for mediating the effects of Substance P in both the peripheral and central nervous systems (Manthy, 2002).

In animal pain models, NK-1 receptor blockers have been shown to inhibit both IgE and SP-induced nociception and inflammation. In a rat model of complex regional pain syndrome, induced by tibial fracture, the significantly increased regional accumulation, maturation and degranulation of mast cells, in addition to their co localisation with SP-positive nerve fibres, were mediated through the NK1-R and inhibited by the injection of the selective NK-1 receptor blocker LY303870. Such effects inhibited injury-induced afferent sensitization and nociception (Li *et al.*, 2012). In a mouse model of stress (Starvation plus SP injection), NK-1R blocker CP99994, injected immediately before stress, was able to reduce the numbers of the degranulated mast cells in the FFPE skin sections (Erin *et al.*, 2004).

In RBL-2H3 rat mast cells, NK-1 receptor knock down using short interference RNA resulted in a significant inhibition of IgE/FcεRI-mediated mast cell activation, indicated by a marked decrease in intracytoplasmic Ca^{++} mobilisation, MCP-1 protein production and levels of the phosphorylated mitogen-activated protein kinases (MAPK) (Fang *et al.*, 2012).

NK-1 receptor expression in mouse bone marrow-derived mast cells was significantly increased by co-culture with IL-4 and SCF, both of which were upregulated in inflammatory conditions such as interstitial cystitis. This was also associated with enhanced SP-induced mast cell degranulation, an effect that was partially blocked by the NK-1 receptor blocker RP67580 only in lower SP doses, but not in higher doses of SP, implying the existence of an alternative SP signalling pathway (van der Kleij *et al.*, 2003).

NK-1 knock out mice sensitized with dinitrophenyl (DNP) human serum albumin failed to develop bladder inflammation upon Intravesical sensitizing antigen challenge despite the post-NK-1 knock out increased mast cell count and positive mast cell degranulation (Saban and Saban, 2000). The Neurokinin-1 receptor antagonist Aprepitant, an FDA-approved drug, was able to reduce pelvic pain, urinary frequency (fewer and smaller urine spots) and bladder inflammation in an experimental autoimmune mouse model similar to interstitial cystitis. This was associated with reduced bladder mast cell counts and reduced expression of cytokines TNF-α and IL-1β (Liu *et al.*, 2019). The above data reveal a successful role for NK-1 receptor blockers in the inhibition of the Substance P-induced mast cell degranulation and activation in animal models.

In Humans, the role of the NK-1 receptors have been controversial. NK-1 receptor antagonists were successful in the control of various adverse effects induced by Substance P signalling. They are also used successfully for the control of chemotherapy-induced nausea and vomiting by blocking the effect of SP on NK-1Rs in the vomiting centre of the brain stem (Aziz, 2012). The NK-1R blocker Vestipitant was also seen to be effective in the treatment of the wake after sleep syndrome (insomnia) (Ratti *et al.*, 2013). In LAD2 human mast cells line, NK-1 receptor antagonists L-733,060 and CP-96345 were able to partially inhibit Substance P, and IL-33 (and their combination)-induced TNF-α release (Taracanova *et al.*, 2017).

However, other studies have introduced evidence undermining the role of the NK-1Rs in the induction of mast cell activation and degranulation. Saban et al., demonstrated that SP-induced mast cell degranulation was independent of NK-1 receptors (Saban, 2002). Recently, mast cell-related G protein-coupled receptor x2 (MRGPRX2) has been identified as a member of the G protein coupled receptors, which is over expressed consistently on

the surface of human mast cells in chronic allergic and inflammatory conditions such as severe asthma and chronic urticaria (Balzar *et al.*, 2011; Fujisawa *et al.*, 2014; Manorak *et al.*, 2018). In such conditions, MC_{TC} (a mast cell subtype, which will be discussed later) is the predominant mast cell subtype (Balzar *et al.*, 2011).

MRGPCRX2 receptors are expressed on the surfaces of connective tissue mast cells (MC_{TC}), in addition to LAD2 cells, but not on the surface of the mucosal mast cells (MC_T) (Mast cell subtypes will be described in page 45)(Subramanian *et al.*, 2011; Fujisawa *et al.*, 2014; Ali, 2016a). In Humans, MRGPRX2 receptors have been demonstrated to mediate pseudo allergic (non IgE-dependant) mast cell activation. Such can be induced by basic secretagogues including neuropeptides (substance P and homokinin-1), eosinophil peroxidase, host defence peptides such as (Human β-defensins) and cathelicidin (LL-37), in addition to drugs such as atracurium (skeletal muscle relaxant), icatibant (Bradykinin B2 receptor antagonist), mastoparan and antibiotics such as vancomycin and fluoroquinolones (Subramanian *et al.*, 2011; McNeil *et al.*, 2015; Ali, 2017).

Depletion of MRGPRX2 using lentivirus short interference RNA in explanted skin mast cells resulted in inhibition of degranulation with decreased histamine and PGD-2 release, while the selective NK-1 blocker protein CP-96345 failed to inhibit degranulation via a histamine release assay (Fujisawa *et al.*, 2014). In a recent study, Substance P (1μM for 24 hours) induced the release of the cytokines TNF-alpha, IL-8 and GM-CSF, in addition to the chemokines CCL-2 (MCP-1), CCL-3 (MIP-1α), and CCL-4 (MIP-1β) by LAD2 cells. The release of these mediators was significantly inhibited after MRGPRX2 depletion, while pre incubation with the potent NK-1 receptor blocker SR140 333 had no effect on the release of these pro-inflammatory mediators (Green *et al.*, 2019).

The pseudo allergic activation and degranulation of the human LAD2 mast cells by basic secretagogues, such as SP and compound 48/80, in addition to the antibiotic ciprofloxacin was inhibited by the tripeptide QWF, a dual antagonist for both NK-1R and MRGPRX2. However, the NK-1R antagonists L733060 and Aprepitant, which lack antagonistic properties against MRGPRX2, failed to inhibit the basic secretagogue-induced mast cell activation and degranulation (Azimi *et al.*, 2016).

Even in animal models, recent reports support the role of MRGPRX2 rather than NK-1R in Substance P signal transduction. In mouse models, depletion of MRGPRb2, the mouse analogue of MRGPRX2, resulted in marked decrease in both thermal and mechanical hypersensitivity 24 hours after hind paw incision, which was associated with significant decreases in the number of leukocytes recruited to the site of the incisional injury. Interestingly, substance P injection into mice hand paws resulted in significant recruitment and increase in the numbers of $CD45^{+}$ leukocytes including neutrophils and monocytes in the hind paw biopsies of both wild-type and neurokinin-1 receptor-depleted mice, while MCRGPRb2-depleted mice failed to develop this response (Green *et al.*, 2019).

BPS/IC is a chronic inflammatory disease of the urinary bladder in which mast cell-SP interaction has been considered a major engine driving the persistence of bladder inflammation. Immunohistochemistry of BPS/IC bladder biopsies revealed significant increases in the numbers of both mast cell subtypes (MC_T and MC_{TC}), see page 45, and both were closely positioned around blood vessels and SP^{+ve} nerves (Pang *et al.*, 1995; Pang *et al.*, 1996b; Malik *et al.*, 2018a). Such an intimate positional relationship between SP-producing never fibres and mast cells raises the possibility of the involvement of MRGCRX2-dependant mast cell activation in the pathophysiology of the disease.

5.1.7.2.2 Mast cell heterogeneity in the responsiveness to substance P

Despite the previous evidence of mast cell /substance P interaction in BPS/IC bladders, these evidences should be cautiously interpreted, and require further investigations to confirm its responsibility for bladder inflammation in BPS/IC patients. Mast cells display a great deal of heterogeneity in their receptor expression, which reflects on their responsiveness to variable secretagogues.

For example, there are differences in the expression of the substance P receptor MRGPCRX2 between mast cells residing in different tissues. Both skin and synovial mast cells express the receptor, which is associated with good responsiveness to substance P, while the pulmonary and cardiac mast cells neither express the receptor nor respond to substance P (Varricchi *et al.*, 2019). Intestinal mast cell explanted from both healthy

individuals and Crohn's disease patients failed to respond to substance P nor expressed the substance P receptor NK-1 (Bischoff *et al.*, 2004).

These differences depend mainly on the microenvironment surrounding these tissue resident mast cells including the cytokines, chemokines and growth factors produced by the surrounding tissue cells in addition to the pathological agents. These environmental factors affects migration, maturation, growth, survival, SCF dependence, receptor expression and mediator release (da Silva, Jamur and Oliver, 2014).

For example, the expression of the substance P receptor NK-1 is significantly increased by murine bone marrow derived mast cell upon co-culture with IL-4 and SCF, which was associated with increased substance P responsiveness (van der Kleij *et al.*, 2003).
In vitro, cross phenotyping of mast cells has been observed upon changing the cell culture environment. Culturing bone marrow-derived mast cells with TGF-β, IL-9, IL-3 and SCF (stem cell factor) yielded mucosal mast cells phenotype (MC_T), while culturing these cells with IL-4, IL-3 and SCF resulted in connective tissue mast cell phenotype (MC_{TC}) (Benedé *et al.*, 2018).

Interestingly, the expression of mast cell receptors can be altered in different pathological conditions. For example, the pulmonary mast cells, which normally do not express substance P receptors, expressed the MRGPCRX2 in lung samples from severe asthma patients. The receptor expression was associated with marked increase in mast cell numbers (MC_{TC} subset) (see page 43) and both increased with the severity of asthma (Balzar *et al.*, 2011; Manorak *et al.*, 2018).

Another example is the cutaneous mast cells, where there is a significant increase in the numbers of $MRGPCRX2^{+ve}$ mast cells in skin samples from chronic urticaria patients when compared to healthy individuals (Fujisawa *et al.*, 2014). In rectal mucosa samples from irritable bowel syndrome (IBS) patients, both the numbers of the mast cells and the substance P levels are significantly elevated when compared samples from healthy controls (Sohn *et al.*, 2013).

In the urinary bladders of BPS/IC patients, the numbers of both mast cell subtypes, MC_T and MC_{TC} (see page 43) are significantly increased compared to controls (Malik *et al.*, 2018a). As previously described, MRGPCRX2 is expressed on the surfaces of MC_{TC}. For our knowledge, the expression of this receptor on the urinary bladder mast cells in BPS/IC patients has not been investigated, and studying this expression might add valuable data for better understanding of the pathophysiology of the disease and might yield a new therapeutic target.

From the above evidence, we can conclude that there is a potential interaction between substance P and mast cells in the urinary bladders of BPS/IC patients, but this evidence needs further confirmation. Such confirmation can be achieved through the Immunohistochemical analysis of the expression of the substance P receptor MRGPCRX2 on the surfaces of the bladder mast cells in BPS/IC patients, which is an important part of the proposed study.

5.1.7.3 Allergic Inflammation Theory

A fundamental role for mast cells and IgE antibody interaction in the pathogenesis of anaphylaxis and other acute allergic conditions has been demonstrated and explained (Amin, 2012; Galli and Tsai, 2012). However, this interaction has recently been demonstrated to contribute to a wide range of chronic allergic and inflammatory conditions (Sismanopoulos *et al.*, 2012; Theoharides *et al.*, 2012). Additionally, abnormally elevated mast cell counts have been detected in various chronic allergic and inflammatory conditions, such as bronchial asthma (Metcalfe *et al.*, 2016), atopic dermatitis (Kawakami *et al.*, 2009), irritable bowel syndrome (Zhang, Song and Hou, 2016) and interstitial cystitis (Peeker *et al.*, 2000a; Malik *et al.*, 2018a).

In both resting and activated conditions, mast cells constantly express FCεRI, a cell surface receptor with high affinity for IgE (Krishnaswamy G. and Chi, 2006). Naive B-lymphocytes produce IgM and IgD antibodies. Upon exposure to allergens, CD^{4+} Th-2 lymphocytes produce IL-4, a cytokine that interacts with and induces B-lymphocyte maturation. Consequently, an antibody class switch occurs with the resultant production of IgE by the mature B-cells. These IgE antibodies bind to their high affinity receptors FCεRI on the surface of mast cells (Prussin and Metcalfe, 2006; Krystel-Whittemore, Dileepan and

Wood, 2015). Upon allergen exposure, polyvalent allergen cross links two FCεRI-bound IgE molecules resulting in receptor aggregation and activation of cell signalling pathways culminating in mast cell degranulation and resultant release of pro-inflammatory mediators (Krishnaswamy G. and Chi, 2006; Galli and Tsai, 2012; da Silva, Jamur and Oliver, 2014; Krystel-Whittemore, Dileepan and Wood, 2015).

In BPS/IC, although mast cell activation is strongly believed to be induced by the interaction between mast cells and the bladder sensory afferent nerve fibres (neurogenic inflammation), recent evidence supports a contribution of allergic inflammation to the pathophysiology of the disease, especially in its ulcerative form. Yamada demonstrated a significant association between allergy and BPS/IC in young patients between 20 and 40 years of age (86%), while only 19% of the older patients demonstrated allergy markers (serum IgE above 250 IU/ml and positive IgE RAST). This was associated with less improvement in the patients' clinical picture following hydro-distension (Yamada, 2003).

In 2016, tryptase and IgE double Immunofluorescence staining of bladder biopsies revealed positive staining for IgE in more than 90% of ulcerative IC and ketamine-induced cystitis biopsies, while only 5% of non-ulcerative IC and 36% of cases of bacterial cystitis showed IgE immunoreactivity (Jhang *et al.*, 2016). This was accompanied by elevated serum IgE levels (above 200 IU/ml) in both ulcerative IC and ketamine-induced cystitis (Jhang *et al.*, 2014;2016). Additionally, abnormal serum IgE levels (above 200 IU/ml) were detected in 22 out of 200 (nearly 10%) BPS/IC patients, but not in controls (stress urine incontinence), which might account for the less frequent ulcerative subtype of BPS/IC patients (ulcerative subtype is nearly 10% of the cases of BPS/IC) (Jhang and Kuo, 2015).

In 2012, a case report of a severe refractory case of BPS/IC was associated with marked eosinophilia and markedly elevated serum IgE, and only combined therapy with the immunosuppressive agents prednisolone and tacrolimus (A drug that inhibits intracellular calcineurin, suppressing the release of IL-2, which promotes the development and maturation of T-lymphocytes) improved the clinical symptoms (Kaneko *et al.*, 2012).

Given the symptom flares frequently experienced by BPS/IC patients following ingestion of certain various types of food and beverages (including Tea, coffee, carbonated

beverages, soda, alcohol, tomatoes, citrus fruits, artificial sweeteners and spicy foods) (Shorter *et al.*, 2007), IgE-FcεRI mediated allergic inflammation may be responsible for the chronic bladder inflammation seen in a significant proportion of BPS/IC patients.

Urinary tract infection and BPS/IC

It is well-established concept that urinary tract infections (Parsons *et al.*) have little role to play in the pathophysiology of BPS/IC, where traditional antibiotic regimens fail to control the patients' symptoms. However, recent reports highlighted that the use of traditional culture media and short incubation periods results in underdiagnosis of UTI in a significant proportion of IC/BPS patients (Aydogan *et al.*, 2019; Malde and Sahai, 2020). Such evidence might indicate the possibility of an underestimated role of urinary tract infections either in the pathophysiology of BPS/IC or triggering the BPS/IC flares or both.

Genetic element of BPS/IC

Since the etiopathological origin of BPS/IC remains a major urological mystery, genetic elements might underlie susceptibility to BPS/IC (Neuhaus, Berndt-Paetz and Gonsior, 2021). BPS/IC occurs more significantly in monozygotic siblings compared to their dizygotic counterparts, while the risk of BPS/IC incidence is 17 times higher in the first-grade relatives (Warren *et al.*, 2004). Moreover, the risk of BPS/IC-associated conditions such as fibromyalgia and constipation is significantly higher in near relatives. Interestingly, recent report highlighted BPS/IC-related alterations in chromosome 3, in addition to other alterations in chromosomes 4, 9 and 14, which strongly support the notion of BPS/IC genetic predisposition (Allen-Brady *et al.*, 2018).

Comprehensive view of the etiopathology of BPS/IC

Based on the above evidence, the etiopathology of BPS/IC can be explained by genetic predisposition. In these individuals, an untreated urinary tract infection with a subsequent chronic bladder inflammation might tigger the symptoms of BPS/IC. The above-mentioned genetic alterations might give an explanation to the aberrant differentiation programme of the defective BPS/IC urothelium characterised by the unique production of APF. Such defect provides an optimal environment for chronic non-resolving bladder tissue inflammation, including the mast cell-related neurogenic inflammation, in addition to repeated UTIs. In individuals with BPS/IC genetic predisposition and a concurrent IgE-mediated allergic conditions such as AD and asthma, allergen exposure might potentially trigger BPS/IC flares. This is supported by the fact that

BPS/IC frequently co-occurs with atopic conditions such as asthma, atopic dermatitis and allergic keratoconjunctivitis.
Importantly, the frequent co-incidence of BPS/IC with autoimmune disorders such as rheumatoid arthritis, ulcerative colitis, fibromyalgia and non-allergic forms of asthma (Kujala *et al.*, 2021), which strongly support the role of a genetically predisposed auto-immune reaction in the pathophysiology of BPS/IC.
Overall, despite that BPS/IC is a clinical diagnosis based on specific symptoms and signs displayed by the patient, the underlying pathophysiology behind that clinical picture is very complicated and multifactorial and can be different between one patient and the other. Despite that genetic elements might underlie susceptibility to the disease, the triggering factors for the development of the disease might be different between different patient subgroups.

5.1.8 Treatment of Bladder Pain Syndrome

Bladder Pain Syndrome is a chronic bladder inflammatory condition that is generally difficult to diagnose and treat (Bosch and Bosch, 2014b). Several treatment guidelines have been established to treat such a complicated condition (Hanno *et al.*, 2011; Cox *et al.*, 2016). The first lines of treatment of the disease include patient education, dietary modification, and stress management, in addition to physiotherapy of the pelvic muscles. Such conservative treatment lines are essential in all cases and can be effective in conjunction with oral medications, especially in the mild non-ulcerative patients (Han *et al.*, 2018).

One of the cardinal signs seen on the histopathological examination of the bladders of BPS/IC patients is urothelial thinning and denudation with or without ulceration (Lynes W. L. *et al.*, 1990; Hurst *et al.*, 2015). For diagnostic and phenotyping purposes, bladder cystoscopy with hydrodistension at 100 cm H_2O pressure is performed. According to the findings of the cystoscopy with hydrodistension, patients are classified into ulcerative (Hunner type) BPS/IC, where the urothelium is characterised by single or multiple reddened mucosal areas (Hunner's lesion) with small vessels radiating towards a central scar, fibrin deposit or coagulum, which is artificially ulcerated on hydrodistension (Hunner's ulcers) (Hunner, 1915; van de Merwe *et al.*, 2008; Hanno *et al.*, 2018). The

other subtype is the non-ulcerative BPS/IC, where the Hunner's lesions are absent and the urothelium remains intact on hydrodistension.

Ulcerative (Hunner type) patients represent the more sever inflammatory form of the disease and considered a different entity and their treatment should be more focused on the urinary bladder. In addition to the initial conservative lines, further lines of treatment include oral medications, intravesical treatments, and surgical treatment (Hanno *et al.*, 2011; Belknap, Blalock and Erickson, 2015; Cox *et al.*, 2016). Oral treatment includes painkillers, antihistamines, pentosane polysulphate sodium (PPS), quercetin ~~(flavonoid)~~ and the immunosuppressant agents like cyclosporine A. Intravesical agents include dimethyl sulfoxide (DMSO), heparin, chondroitin sulphate, hyaluronic acid, alkalinized lidocaine ~~(sedative)~~ and intravesical PPS.

Intravesical botulinum toxin A (BOTX A) injection is one of the surgical treatment lines, which help to improve the clinical condition of the patients (Chiu *et al.*, 2016). In a small prospective study of 13 BPS/IC patients, 6 from the U.S and 7 from Poland, 100 to 200 IU of Botulinum toxin A were injected into 20 to 30 sites of the bladder submucosa, including the floor and trigone, under cystoscopic guidance (Smith *et al.*, 2004). Symptoms of the patients were assessed pre- and post-operatively by the O'Leary-Sant ICSI and ICPI, in addition to the VAS for pain and the micturition diary chart. Post-injection, 9 of the 13 patients reported symptomatic improvements. The ICSI and ICPI scores were significantly improved by 71% and 69%, respectively. The pain score, daytime frequency and nocturia were significantly improved by 79%, 44% and 45%, respectively. These effects were short-term and lasted for an average of 3 months, and 5 patients underwent another BOTX A injection after 6 months.

Surgical interventions include bladder hydro distension, surgical transurethral resection or diathermy of Hunner's lesions, BOTX A injection and sacral neuromodulation. Severe resistant cases with Hunner's lesions, which are refractory to the previous medications, can be surgically treated through urinary diversion with or without cystectomy or partial cystectomy with augmentation cystoplasty (Hanno *et al.*, 2011).

5.1.8.1 Oral Therapeutic agents

Most of the medical treatments for BPS/IC show variable, short-term and often unsatisfactory improvements in the symptom scores of the patients, with symptom-improvement rates ranging from 40 to 60% of patients (Vella, Robinson and Cardozo, 2015; Cox *et al.*, 2016). It is believed that the efficiency of the current available treatment lines is negatively impacted by many factors. These may include the failure of identification of a definite etiological factor, a proper diagnostic tool and the multifactorial nature of the disease, which highlight the urge to develop a patient-directed treatment (Belknap, Blalock and Erickson, 2015).

In a randomised double-blinded clinical trial, oral amitriptyline, which is an anti-cholinergic anti-histaminic agent, induced statistically significant symptomatic improvements in 63% of the treated patients compared to 4% less symptoms in those treated with a placebo (van Ophoven *et al.*, 2004). However, anticholinergic side effects were reported in 92% of the treatment group. A study including naïve BPS/IC patients, who received standardized education and behavioral modification programs, oral amitriptyline induced improvements of symptoms only at a daily dose of 50 mg (Foster *et al.*, 2010). Statistically significant improvement was observed in 66% vs 47%, in the treatment group compared to the control groups. Such dose was only tolerated by 50% of patients.

In a non-controlled study, the therapeutic effect of an anti-histaminic agent, cimetidine, in BPS/IC patients refractory to conservative treatment was investigated (Seshadri, Emerson and Morales, 1994). 300 mg of cimetidine were given orally twice daily for one month to 9 BPS/IC patients refractory to conservative treatments. 6 out of 9 patients (66%) reported variable improvement, which was very satisfactory in 4 patients (44%).

Another RCT using another oral anti-histaminic (Hydroxyzine) and oral Pentosane Polysulphate Sodium (PPS), which is thought to replenish the glycosaminoglycan (GAG) layer lining the bladder wall (SANT *et al.*, 2003). Hydroxyzine showed a 31% improvements in the symptoms of the patients, while PPS showed 34% improvement. Both of which were not statistically significant from the placebo group 20%. Combination

of both drugs induced 40% improvement. Side effects were very common in all treatment groups.

Regarding oral PPS, in several RCT around 50% of patients reported 50% symptoms improvement (Hwang *et al.*, 1997; Nickel *et al.*, 2005a). An observational study reported that 50% of patients displayed 50% symptoms improvement after 22 months of treatment (Al-Zahrani and Gajewski, 2011), while, on the contrary, another study reported that only 6.8 to 17% of patient maintained therapeutic effect after 18 months of oral PPS treatment (Jepsen *et al.*, 1998). In a very recent RCT, 24-week treatment with oral PPS 100 mg (once or thrice daily) did not induce any statistically significant improvements in the patients' symptoms compared to the placebo group (Nickel *et al.*, 2015).

Cyclosporine A is an anti-inflammatory agent used orally for treatment of BPS/IC patients. Observational studies showed symptomatic improvements, which are more obvious in ulcerative patients compared to their non-ulcerative counterparts (68% vs 30%, respectively) (Forrest, Payne and Erickson, 2012). RCT showed 59% symptom improvements with cyclosporine A treatment. Such improvement was statistically significantly higher than in the PPS treated group (13% improvement) (SAIRANEN *et al.*, 2005).

5.1.8.2 Intravesical Therapeutic agents

Bacillus Calmette-Guerin (BCG) has been proposed to be used as an anti-inflammatory intravesical therapy for BPS/IC patient, which is thought to downregulate the pro-inflammatory cytokine IL-6 (Peeker *et al.*, 2000b). A randomized clinical trial showed no advantage of intravesical BCG therapy compared to the control group (intravesical saline) (Mayer *et al.*, 2005). Such was associated with development of side effects including arthritis and septicaemia (Paterson and Patel, 1998; Mayer *et al.*, 2005).

Intravesical dimethyl sulphoxide (DMSO) is currently used for treatment of BPS/IC. The rationale for its use is that it is an anti-inflammatory and analgesic agent that desensitizes the bladder sensory afferents (Birder, Kanai and de Groat, 1997). It can cause temporary urothelial injury, increasing the bladder uptake of the other therapeutic drugs. A randomised control trial by Perez-Marrero et al reported objective improvements in 93% of patients compared to 35% of the placebo group. However, from the patients

prospective, only 53% of patients reported symptom improvements compared to 13% of placebo group (Perez-Marrero, Emerson and Feltis, 1988). Another double-blinded randomised clinical trial showed improvements in bladder pain and frequency in the DMSO group compared to BCG-treated group in both subtypes of BPS/IC patients.

However, the functional urine capacity was not improved by both medications (Peeker *et al.*, 2000b). Like the other BPS/IC medications, the evidence supporting the DMSO use is not complete nor conclusive, while only proportion of the patients responds with mostly unsatisfactory results (Ha and Xu, 2017).

Botulinum toxin A (BTX A) is a neurotoxin produced by clostridium botulinum. Acting through prevention of neurotransmission at the presynaptic nerve terminals, it can be a detrusor muscle relaxant and analgesic (Gao and Liao, 2015). A retrograde observational study analysed the randomised clinical trials using BOTX A, which has been performed from 2003 to 2013. Through the O'Leary Sant Questionnaire, they observed favourable 90% subjective symptomatic improvements in a number of patients after hydro distension + BOTX A intravesical instillation. Such effect was short-term and symptoms returned back to baseline within 12 months (Gao and Liao, 2015). On the contrary, a more recent double-blinded RCT by Manning et al, in which patients were randomly allocated between hydro distension plus+ intravesical BOTX A group (Distension group) and hydro distension plus intravesical saline (Placebo group). The BOTX A induced around 50% improvements in the patients' symptoms only in 5 patients of the treatment group. They concluded that in most of the patients, BOTX A therapy does not yield favourable therapeutic effect compared to controls in the treatment of severe refractory BPS/IC (Manning *et al.*, 2014). From the above evidence, we can conclude that the evidence for using BOTX A is not consistent, and its effects can be partial and temporary.
Lidocaine is a well-established local anaesthetic. Alkalinised Lidocaine (AL) has been shown to induce immediate symptoms improvement in 30% of patients compared to 9.6% in the control group. Such effect was temporary and lasted only for 10 days after stopping of therapeutic course.

GAG layer is the mucous layer covering the urothelial lining of the bladder wall. Hence, it is the first bladder permeability barrier against the harmful urine constituents (Parsons,

Greenspan and Mulholland, 1975). The replenishment of the defective bladder permeability barrier, including the GAG layer, has been a top priority halt the progression of this chronic inflammatory disease (Madersbacher, van Ophoven and van Kerrebroeck, 2013). Accordingly, the intravesical instillation of several GAG layer components have been presented as potential treatment lines. These components include chondroitin sulphate, Hyaluronic acid (HA), heparin sulphate and keratine sulphate.

RCTs using variable HA treatment regimens, HA induced significant improvements in the patients' symptoms on the O'Leary Sant questionnaire and visual analogue score (VAS) for bladder pain (Lai, Kuo and Kuo, 2013). Another RCT over a duration of 48 weeks, HA induced partial improvements in the patients' symptoms, and such effect lasted till the end of study (Lv *et al.*, 2012).

As an essential component of the human bladder GAG layer Chondroitin Sulphate (CS), several RCTs have been performed to investigate a potential therapeutic effect of intravesical sodium chondroitin sulphate on the BPS/IC patients' symptoms. Nickel et al, highlighted symptomatic improvements in 63% of the patients (Nickel *et al.*, 2009). On the contrary, more recent RCTs by the same research group showed no statistically significant improvement in the patients' symptoms compared to the control groups (Nickel *et al.*, 2010; Nickel *et al.*, 2012).

Despite the failure of the CS monotherapy, the combined HA and CS (currently available as iAluRil) has shown significant success in improving the patients' clinical picture in several RCTs (Cervigni *et al.*, 2008; Porru *et al.*, 2012; Gulpinar *et al.*, 2018).

As a component of the GAG layer, intravesical Heparin instillation has been hypothesized to replenish the GAG layer and restore the normal function of the urothelium in BPS/IC bladders. In a non-controlled clinical trial by Parsons et al., intravesical heparin induced symptom improvement in 63% of the patients (Parsons *et al.*, 1994). Currently, combination of heparin with AL proved to fasten the onset of the symptomatic improvements. A more recent controlled RCT showed an immediate symptomatic relief in 40% of the patient group following combined heparin and Alkalinised Lidocaine therapy (Parsons *et al.*, 2012). However, no evidence is available to support the sustainability of this treatment line (Ha and Xu, 2017).

Due to its structural similarity to the bladder GAG layer, PPS has been hypothesized to replenish the GAG layer and, consequently, restore a healthy urothelium in the bladders of BPS/IC patients. Oral PPS is the only FDA approved drug in the United States (Hanno *et al.*, 2011). Intravesical PPS has been proposed to augment the therapeutic effect induced by oral PPS. Non-controlled clinical trial showed significant symptomatic improvements with biweekly intravesical PPS (Daha *et al.*, 2008). In a RCT were patients were randomly allocated into a treatment group (Oral PPS + Intravesical PPS) and control group (Oral PPS + Intravesical normal saline). The symptomatic improvements were statistically significantly higher (46%) in the treatment group compared to the control group 27% (Davis *et al.*, 2008).

These results show the great benefit and advantage of the combined therapy over monotherapy, especially in the treatment of a complicated multifactorial conditions like BPS/IC with partial and temporary responses to the current treatment lines.

5.1.8.3 Surgical Treatment

As previously described, bladder cystoscopy with hydrodistension is a routine measure for diagnosis and phenotyping of BPS/IC. Additionally, several studies highlighted the positive therapeutic role of hydrodistension (distension of the bladder via intravesical instillation of physiological saline under a pressure of 100 cm^2) in BPS/IC patients (El-Hefnawy *et al.*, 2015). In a retrospective study of 84 BPS/IC patients, who went through detailed history, physical examination, urine analysis, urine culture, and filled voiding diary and pelvic pain and urgency frequency (PPUF) questionnaire for the lower urinary symptoms (Ottem and Teichman, 2005). Forty-seven of these patients underwent hydrodistension were compared to 37 patients did not receive hydrodistension. About 54% of those who received hydrodistension reported symptomatic improvements, which were temporary and lasted for a mean of 2 months.

Another study included 65 BPS/IC patients, which included a retrospective group of 33 patients, followed by a prospective group of 32 patients (Glemain *et al.*, 2002). Both patients displayed pain on bladder filling relieved by voiding and nocturia more than 2 times per night. On cystoscopy with short-term hydrodistension, all patients were non-ulcerative and showed only urothelial glomerulations. Therapeutic hydrodistension was performed under epidural anaesthesia, and the bladders were hydrodistended under a

pressure equal to the patient's mean arterial pressure for a period of 3 hours without rest. The symptomatic improvement was assessed by the disappearance or the relieving of pain perceived on bladder filling with no demand for treatment, in addition to the reduction in the frequency of nocturia (0-2 times/ night). Both groups showed symptomatic improvement following hydrodistension, which rated between 37.5% after 6 months, which dropped to 21.9% after another 6 months (one year post hydrodistension) in the retrospective group. A higher symptom improvement rate was observed in the prospective group, which was 60% after 6 months and dropped to 43.3% one year post hydrodistension. The higher response in the second group was explained by the better optimisation and increased hydrodistension volume in the prospective group.
Another study was conducted to evaluate the value of hydrodistension for the treatment of BPS/IC (Aihara *et al.*, 2009). The study included 36 patients with bladder pain, urgency and frequency for more than 6 months, and the daily voided volume was less than 200 ml on a 48-h frequency volume chart. Of the 36 patients, six were excluded duet to urinary tract infections, bladder neck obstruction, post voiding residual volume more than 50 ml or bladder cancer. Hydrodistension was performed 10 minutes after intravesical instillation of 10 ml lidocaine 4% in 40 ml physiological saline. For hydrodistension, physiological saline was instilled into the intravesically, while the patients' vital signs and blood pressure are regularly checked every 10 minutes. Instillation was stopped when the bladder pain was intolerable by the patient. Hydrodistension was safe, and no patient reported any adverse effects requiring hospital admission after the session. One moth post treatment, 70% of the patient reported significant improvements in the lower urinary symptoms using the O'Leary-Sant interstitial symptom index (ICSI) and interstitial cystitis problem index (ICPI), with significant increases in the minimal, maximal and average voiding volumes, in addition to the 24-hour frequency on the 48-h frequency volume chart. Using the Kaplan–Meier method, these symptomatic improvements lasted for a median efficacy period of around 20 weeks.

Despite the technical research differences, we can conclude from the above data that bladder hydrodistension provide a significant but temporary relief of the patients symptoms.

Ulcerative-type BPS/IC patients have been treated with fulguration (Cauterisation) or surgical resection of the ulcers under cystoscopy guidance (Peeker, Aldenborg and Fall, 2000; Ko *et al.*, 2020). A retrospective study of ulcerative-type BPS/IC patients who underwent fulguration of the ulcers on top of persistent severe symptoms refractory to conservative, oral and hydrodistension therapy during the period between 1993 and 2011 in a pelvic pain referral centre (Hillelsohn *et al.*, 2012). Among the 59 patients included, 27 patients (45.8%) needed repeated fulguration with mean time intervals of 20 months in between the sessions. Among these 27 patients, 13% had repeated fulguration after 12 months, while 57.8% had repeated fulguration after 48 months. For the 32 patients who only a single fulguration session, 78% reported significant improvement of the lower urinary symptoms on the 10-point Likert scale for pain and irritative voiding symptoms. In another retrospective study, 27 patients, who had refractory symptoms and underwent ulcer fulguration, were included (Ryu *et al.*, 2013). The patients reported significant symptomatic improvements in their symptoms 2 months post fulguration, as revealed by the significant drop of the symptom score using different symptom assessment questionnaires such as pelvic pain urgency and frequency questionnaire (from 15.1 pre-fulguration to 7.0 post-fulguration), interstitial cystitis symptom index (from 15.1 pre-fulguration to 7.2), interstitial cystitis problem index (from 13.8 pre-fulguration to 6). 2 months after fulguration, the symptom improvement rates were 94%. Unlike the previous study, these improvements dropped to 70% after 6 months, and 33% 10 months post fulguration.

Regarding transurethral resection (TUR) of the Hunner's ulcer, many studies have been conducted to investigate its efficacy and side effects. A retrospective study of 103 ulcerative BPS/IC patients who underwent TUR of Hunner's ulcers, 92 patients experienced symptomatic relief, while 11 patients did not report any improvement in bladder pain and frequency (Peeker, Aldenborg and Fall, 2000). Among the 92 symptomatically improved patients, the symptom relief lasted for more than 3 years, which is much better when compared to the symptomatic relief obtained after the initial cystoscopy and hydrodistension that lasted only for 4 months.

In a prospective randomized clinical trial including 127 ulcerative BPS/IC patients, patients were randomly allocated into 2 groups, one was treated with TUR, while the second was

treated with transurethral coagulation (TUC) (Ko *et al.*, 2020). In the two groups, patients reported significant improvement in the lower urinary tract symptoms, which lasted for an average of 12 months before symptom recurrence. Such symptomatic relief was much shorter compared to that reported in the previous study. Despite the high response rate achieved by TUR, bladder injury occurred in 7.9% of patients during TUR, which was significantly higher than in TUC (3.4%).

Many trials have been conducted to investigate a favorable increase the efficacy and potency of TUR through combination with hydrodistension, which yielded controversial results. For example, in a retrospective study of 44 females newly diagnosed ulcerative BPS/IC patients, patients were allocated into 2 groups, the first included those who had received only TUR, while the second group included those who had received TUR with bladder hydrodistension (Lee *et al.*, 2017c). Through a 10-point visual analogue scale for pain and 3-day micturition diary, patients in both groups reported significant improvements in the bladder pain, urgency, frequency and nocturia with significant increase in the functional bladder volume. There was no statistical difference in the augmented improvement rates between both groups denoting no advantage of hydrodistension with TUR on the TUR alone. However, the post-operative mean functional bladder capacity was significantly higher among the hydrodistension group, despite it was nearly the same in both groups preoperatively. In both groups, symptomatic improvements lasted for 12 months, but no follow up was done beyond this period.

In another study, 87 female ulcerative BPS/IC patients, who underwent TUR with hydrodistension, were studied retrospectively (Lee *et al.*, 2013). Using a 3-day micturition chart, a 10-point visual analogue scale (VAS) for pain and the global response assessment (GRA) for patient satisfaction, the lower urinary symptoms and the patient satisfaction were assessed pre and post-operatively, respectively. There was significant improvements in the patients' symptoms, which lasted for an average of 12 months before the symptoms started to recur. Similarly, the maximal functional bladder capacity increased significantly post-operatively before starting to decrease again after 12 months. Based on the GRA, symptom improvement was appreciated by 95% of patients after 1 month, while

only 63% of the patients reported symptom improvement and satisfaction after 12 months.

From the above evidence, we can conclude that transurethral resection and transurethral Fulguration are effective therapeutic procedures, which can effectively and temporarily control and improve the patients' symptoms for an average period of 12 months. However, this might carry the risk of bladder injury and perforation.

Intravesical submucosal injection of BOTX A is well hypothesized to exert positive effect in the control of the refractory BPS/IC symptoms (Chiu *et al.*, 2016; Torkamand *et al.*, 2019). BOTX A is injected in the submucosa of the bladder trigone and floor based on the fact that most of the bladder nociceptive sensory endings are located in the bladder trigone (Avelino *et al.*, 2002). In a recent single center prospective randomized double-blinded placebo-controlled clinical trial, patients were randomly allocated into two groups; the treatment group patients received trigonal submucosal BOTX A injections, while the control group patients received saline injections (Pinto *et al.*, 2018). After 12 months, the patients' symptoms were significantly improved in the treatment group compared to controls, with more than 50% reduction in the pain intensity on the visual analogue scale in more than 60% in the treatment group compared to 22% in the placebo group.

In a multicenter, randomized, double-blinded, placebo-controlled trial in patients with refractory BPS/IC, patients were randomly allocated into a treatment (40 patients) and placebo groups (20) patients (Kuo *et al.*, 2016). The treatment group patient were treated with hydrodistension with injection of 100 IU BOTX A in the posterior and lateral walls of the bladder excluding the trigone. The control group were hydrodistended and injected with physiological saline. 8 weeks later, the decrease in the pain intensity on the VAS was significantly higher in the BOTX A group with an average decrease of 2.6 points on the VAS compared to 0.9 point decrease in the control group. Similarly, the cystometric bladder capacity was significantly higher in the BOTX A group. However, there was no decrease in the other valuables including urgency, daytime frequency and nocturia between the two groups. On the GRA, 26 out of 40 patients in the BOTX A group reported symptom satisfaction with overall success rate of 63%, while only 3 out of 20 patients in the control group reported symptom satisfaction with overall success rate of 15%.

In a comparative randomized clinical trial, 39 patients were randomly allocated into two treatment groups, 20 patient received BOTX A in the bladder body, while 19 patients received trigonal BOTX A injection (Jiang *et al.*, 2018). Both patients experienced similar symptomatic improvements, where 65% and 52% of the patients reported decrease in the pain intensity of 2 points or more on the VAS, in the body and trigone groups, respectively. 45% and 52% of the patients reported symptom satisfaction on the global response assessment (GRA≥ 2) in the body and trigone group, respectively. Interestingly, 45% and 52% of the patients reported undesirable dysuria after treatment in the body and trigone group, respectively.

Another recent randomized clinical trial, 34 patients with refractory BPS/IC symptoms were allocated into two groups, A and B (Akiyama *et al.*, 2015). Both groups were injected with BOTX A, but group A patients were immediately injected, while group B patients were injected with BOTX A one month later. After 1 month, when only group A patients were injected, using the GRA and the symptom scales, the symptom satisfaction were significantly higher on the group A patient with 72% response rate, compared to 25% in the group B, which has not been injected yet. One month later, after injection of the group B patients, the success rate on the GRA for all patients in the 2 groups as a single cohort was as high as 73%. However, the monthly follow up of the 2 groups as a single cohort revealed gradual recurrence of the patients' symptoms and decrease in the augmented success rates, which dropped to 58% after 3 months, 38% at 6 months and 20% at 12 months. Using the Kaplan–Meier method, the duration of response averaged at 5.4 months, which was similar in ulcerative and non-ulcerative BPS/IC patients.

From the above evidence, we concluded that BOTX A injection, in either the body or the trigone of the bladder, yielded significant but short-term improvements in the patients' symptoms.

Sacral neuromodulation (SNM) is an FDA-approved less-invasive surgical treatment line, in par with BOTX A in the AUA guidelines, with good efficacy in the control of bladder pain in refractory BPS/IC (Srivastava, 2012; Derisavifard and Moldwin, 2020). Peters et al., investigated the therapeutic effect of sacral neuromodulation, using an InterStim® (Medtronic Inc., Minneapolis, MN) implantable neuroprosthetic device, on the patients refractory systems using the VAS, voiding diaries and GRA (Peters, Carey and Konstandt,

2003). At a mean follow up period of 15 months, 20 of the 21 included patients reported moderate to strong symptomatic improvements. Such was associated by a 35% decrease in the morphine doses, while 22% of the patients stopped taking morphine.

Another study included 27 refractory BPS/IC patients performed monthly follow up of patients who underwent SNM up to 2 years(Vaze *et al.*, 2005). In an initial stage I procedure, the efficacy of SNM was tested through the stage I InterStim using the tined lead approach. In such procedure, neuromodulation was initially tested using a traditional technique, through insertion of a needle connected to a neuro generator into the third sacral foramen of the prone-positioned patient after local anaesthesia of the skin of the buttock and the periosteum, followed by stimulation of the S3 nerve. Nearly 80% of these patients showed successive response with more than 50% improvement in the bladder pain and frequency, which qualified them to proceed to the second stage, which is the implantation of the Interstim permanent device. At 3 months follow up, 5 patients had their devices explanted (2 for infection and 3 for poor efficacy). Among the 17 patients who maintained the devices, 4 patients reported loss of efficacy by the end of the follow up period. They estimated a success rate of 48%.

In another report by Peters et al., the outcome of the traditional SNM technique (Initial traditional single stage test followed by permanent implant, in case of success) was compared to a more recent multi-stage technique, in which the traditional single stage test was replaced by multi-stage test followed by permanent implant (Peters, 2002). In accordance with the previous study, only 67% of the patients who underwent the traditional SNM technique were positive responders (14 patients), while only 11 of these patients approved to have the permanent device installed, giving a test to implantation rate of 52%. On the contrary, among the 12 patients who underwent the new SNM technique, 11 have responded positively and chose to proceed to the permenant device level, giving a test to implant rate of 92%. The final 22 patients who had their permanent devices installed were followed up over an average period of 5 months. There was an overall symptom improvement of 56%, with reported 50% improvement in frequency, urgency, pelvic pain, pelvic pressure, quality of life, incontinence, and vaginal pain, in 71%, 62%, 65%, 80%, 72%, 69% and 54% of the patients, respectively.

Another study compared the sacral nerve stimulation (SNM) compared to pudendal nerve stimulation (PNM) for treatment of a total of 22 refractory BPS/IC patients (Peters, Feber and Bennett, 2007). At 6 months follow up, SNM yielded 44% overall symptom improvement, compared to 59% by PNM.

In a retrospective meta-analysis, 10 PubMed articles reporting clinical trials of sacral neuromodulation therapy of BPS/IC patients from 1990 to 2010 were included (Marcelissen *et al.*, 2011). Among the included patients 55 to 70% responded positively to the initial testing and had the device inserted. Over a follow up period ranged between 5 and 87 months, the improvement in the bladder pain on the VAS ranged between 40 and 77%, with a re operation rate of 27 to 50% after long term follow.

From the previous evidence, we conclude that despite around 20 to 30% of the patients fail the initial testing; SNM induced significant improvement in the bladder pain, which can be associated with improvements in the other urinary symptoms. The evidence supporting long-term improvement is not consistent, and in many studies, it only lasted for an average of 5 months.

5.1.8.3.1 Invasive surgical treatment (Bladder reconstruction)

In case of refractory BPS/IC patients with severe symptoms refractory to the previous treatment lines and small, unresponsive fibrotic bladders, the last line of intervention will be the more invasive surgical reconstruction surgeries (Hanno *et al.*, 2011). The most common radical surgery performed for these patients is total cystectomy with cutaneous urinary diversion (Homma *et al.*, 2009), which can be either incontinent urostomy through an ileal conduit, or the continent pouch (Indiana Pouch) (Derisavifard and Moldwin, 2020). Studies report that cutaneous urinary diversion is more effective than supra trigonal cystectomy with augmentation cystoplasty in the control of the bladder and pelvic pain in the refractory non ulcerative BPS/IC patients (Peeker, Aldenborg and Fall, 1998).

The incontinent ileal conduit requires a small segment of the ileum with minimal maintenance required by the patient (Derisavifard and Moldwin, 2020). Although the bladder can be retained, total cystectomy improves the surgical outcome of cutaneous urinary diversion with more improvements reported in the patients' symptoms, and general satisfaction, with the advantage of avoidance of any complications of retained bladders (Peters *et al.*, 2013). One of the major disadvantages of the incontinent ileal

conduit is the presence of the urine collection reservoir, which has poor effect on the cosmetic body image, while the continent requires careful maintenance and clean self-catheterization (Homma *et al.*, 2009; Derisavifard and Moldwin, 2020).

On the other hand, the continent ileal urine pouch requires the patient to be willing to carefully self-catheterize and drain the pouch to avoid chronic urine retention or urinary tract infection. Additionally, the use of a large bowel segment for the urine pouch can result in bowel habit abnormalities including poor absorption and diarrhea (Hohenfellner *et al.*, 2000).

Supra trigonal cystectomy with augmentation cystoplasty is the treatment of choice in sever refractory ulcerative BPS/IC patients, who failed to respond the non-surgical and the less invasive surgical treatments (Peeker, Aldenborg and Fall, 1998). Refractory BPS/IC patients prefer this technique to the cutaneous urinary diversion as there is no need for a urine reservoir or catheterized channel (Derisavifard and Moldwin, 2020). In a retrospective study of 10 women with ulcerative IC, patients underwent supra trigonal cystectomy with augmentation after 3 previous failed electrocautery of Hunner's lesions. Postoperatively, all BPS/IC were effectively controlled in 9 of the 10 patients, with marked improvements in the patients' quality of life and sexual function (Peters *et al.*, 2013).

Another study included 22 women and 1 man with refractory BPS/IC with average age of 51 years old (Linn *et al.*, 1998). Patients were divided into two groups, group 1 underwent sub trigonal cystectomy with re implantation of the ureters, while group 2 underwent supra trigonal cystectomy sparing the ureteric orifices. Both groups underwent augmentation of the bladder with an ileocecal pouch. All patients in both groups experienced significant increases in the functional bladder capacity (P value<0.001) associated with strong decrease in the daytime frequency and nocturia (P value<0.001).

In a retrospective study, 12 women with refractory BPS/IC, whose age averaged at 55.9 years, underwent therapeutic supra trigonal cystectomy with ileocecal augmentation cystoplasty, and all were performed by the same surgeon (OPHOVEN, OBERPENNING and HERTLE, 2002). Sixteen out of the 18 patients had significant improvements in bladder pain, frequency, and nocturia on an average of 50 months follow up. 3 out of the

respondents required repeated self-catheterization for residual urine drainage. Interestingly, two patients reported failure of symptom control after the operation.

Despite the high success rates denoted by the previous reports, another study reported symptomatic improvements in only 25% of patients (Nielsen *et al.*, 1990). Such was associated with small preoperative cystometric bladder capacities of 200 cc or less (2 patients), while the remaining 6 patients whose cystometric capacities averaged at 534 cc reported no symptom improvement.

5.1.8.3.2 Complications of the invasive surgical treatment lines

Despite the high success rate of this technique, it has a number of disadvantage, which can develop into serious life threatening complications. For example, malignancies such as adenocarcinoma and transitional cell carcinoma can develop at the site of the uro-intestinal anastomosis (Ali-El-Dein *et al.*, 2002). In case of patients with dysuria and urethral pain, supratrigonal cystectomy is contraindicated, as the bladder will be removed but the pain will remain as urine will continue to pass by the urethra (Derisavifard and Moldwin, 2020). In such case, cutaneous urinary diversion will be the treatment of choice.

Additionally, many of the patients who underwent augmentation cystoplasty may not be able to push urine efficiently, resulting in chronic residual urine (Derisavifard and Moldwin, 2020), which requires careful follow up to avoid possible hydronephrosis (Homma *et al.*, 2009). Accordingly, these patients will require regular catheterization, which can be very irritant in patients with urethral pain (Derisavifard and Moldwin, 2020).

Moreover, in both cystectomy with augmentation cystoplasty or cutaneous diversion, unlike the urothelium, the direct contact between the bowel segment and urine can result in massive uptake of the urine ammonium chloride by the intestinal mucosa, which can result in chronic hyperchloremic metabolic acidosis (Ueda *et al.*, 2018). In patients whose glomerular filtration rate less than 40 cc/min/1.73 m^2 body surface, sever electrolyte disturbances can occur (Ueda *et al.*, 2018).

5.1.8.4 Conclusions on the efficacy of current BPS/IC treatment lines

Based on the above evidence, we can conclude that the current conservative therapeutic agents are mostly unsuccessful in terms of restoration of the normal quality of life of the patients, a situation made more complex by the diverse entities among BPS/IC

subpopulations, and which adds to current treatment inefficacy (Held, Hanno and McCormick, 2018). Most of these treatments show variable, short-term improvements, which can vary from one patient to another (Belknap, Blalock and Erickson, 2015).

In addition, we can conclude that the invasive surgical treatment lines can provide an effective end line of treatment for patients with severe refractory symptoms. However, this comes on the price of a high risk of development of severe complications, which can be life threatening. Even in elderly patients, who represent a large percentage of BPS/IC patients, the idea of a major surgery and general anaesthesia can be very serious and life threatening. Accordingly, in our opinion, the best way to improve the overall treatment outcomes is to further invest in the search for a more potent conservative or less invasive treatment lines, which can keep the patients symptoms under control while maintaining the highest levels of patient's satisfaction. Such will help to keep the patient in a favourable track avoiding case deterioration that might necessitate the end line surgical treatments. Given the recently seen increasing prevalence and incidence rates of the disease, BPS/IC has become an important health problem that needs more focused study of its elusive pathophysiology and the identification of a reliable medication with minimal side effects. All of the previous factors make it compelling to search for a natural therapeutic agent that can be used uniformly as an adjuvant to other medications for the treatment of all of the patient subgroups with the express aim of improving treatment outcomes.

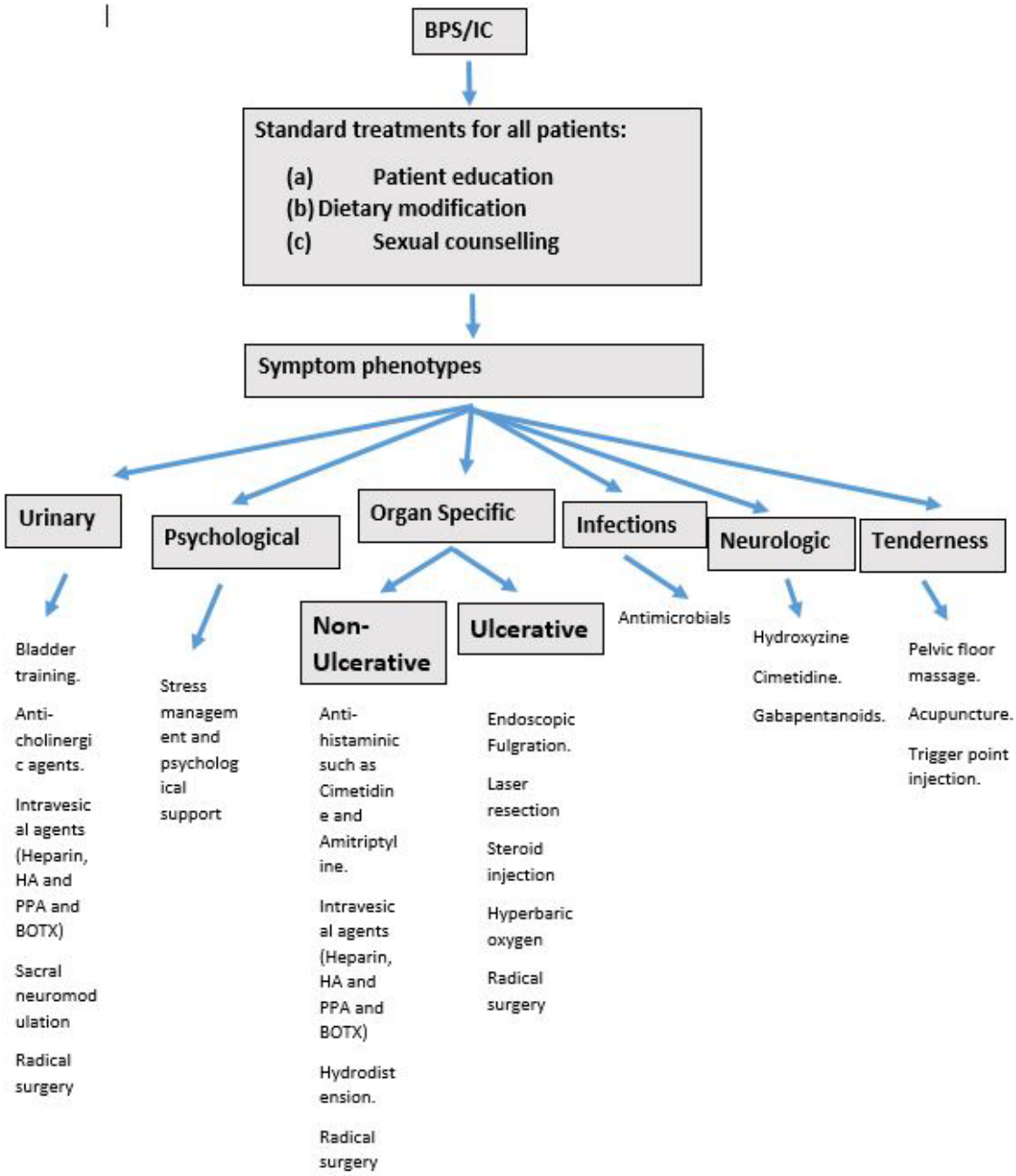

Figure 3: Treatment of BPS/IC, adopted from (Cox *et al.*, 2016).

5.2 Staining of mast cells in bladder biopsies

In BPS/IC bladders, the damaged urothelial cells and smooth muscle cells produce stem cell factor (SCF) (Theoharides, Kempuraj and Sant, 2001; Sant *et al.*, 2007b). The latter is responsible for the extravasation and migration of mast cells progenitors, originally circulating in the blood stream, which then localize in the perivascular spaces in the bladder wall. Additionally, SCF induces mast cell differentiation and maturation and increases mast cell responsiveness to degranulation factors such as SP (Tsai *et al.*, 1991;

Pang *et al.*, 1996a; Da Silva, Reber and Frossard, 2006). Such is the scientific basis for using stem cell factor for inducing growth and maturation of in vitro cultured mast cells. Several dyes have been used to stain and detect mast cells localized in tissues. These dyes include Giemsa, Toluidine blue and Alcain blue, which depend on the metachromatic staining of the poly anionic molecular contents of the mast cell granules such as heparin and GAG.

Tryptase immunostaining was found to be the most accurate method for identification of mast cells through the detection of the tryptase enzyme (a serine protease) exclusively produced by mast cells (Yamada *et al.*, 2000; Larsen *et al.*, 2008; Ribatti, 2018). Tryptase immunostaining detected higher numbers of mast cells in paraffin embedded urinary bladder biopsies than those detected by metachromatic staining using Toluidine Blue or Giemsa stain (Aldenborg, Fall and Enerback, 1986; Peeker *et al.*, 2000a).

5.3 Urinary bladder mastocystosis in BPS/IC patients

Generally, microscopic examination of bladder biopsies from BPS/IC patients reveals mast cell infiltration of the bladder wall with a significantly increased number of infiltrating mast cells (mastocystosis) in all of the layers of the bladder wall compared to non-IC controls. (Larsen *et al.*, 1982; Kastrup *et al.*, 1983; Sant and Theoharides, 1994; Yamada *et al.*, 2000; Theoharides, Kempuraj and Sant, 2001; Liu *et al.*, 2012a; Malik *et al.*, 2018b).

It is generally accepted that mast cell counts in ulcerative BPS/IC are higher than those in non-ulcerative BPS/IC, while in both BPS/IC subtypes mast cell counts are significantly higher compared to controls. Peeker et al. demonstrated a 6 to 10-fold increase in mast cell count in the bladders of ulcerative BPS/IC patients, and 2 to 3 fold increase in mast cell count in the bladders of non- ulcerative BPS/IC patients, compared to healthy controls using Tryptase immunostaining (Peeker *et al.*, 2000a). Malik et al. demonstrated 10-fold increase in the mast cell density in ulcerative BPS/IC patients compared with histologically normal controls, both in the detrusor and in the lamina propria (Malik *et al.*, 2018a). Another study by Yamada et al. reported a 2 to 3 fold increase in the mast cell counts in both the mucosa and detrusor layers (more prominent in the detrusor layer) in non-ulcerative BPS/IC patients above controls, which supports the previous results (Yamada *et al.*, 2000).

However, other research studies demonstrated different patterns of mast cell infiltration in BPS/IC bladders compared to controls. In 2015, Gamper et al. demonstrated 4.5 and 3 fold increase in mast cell density in detrusor layer of ulcerative and non-ulcerative BPS/IC patients, respectively, compared to controls. In the submucosa, they demonstrated 2-fold increase in mast cell density in ulcerative BPS/IC above controls, while there was no significant difference between non-ulcerative IC and controls (Gamper *et al.*, 2015).

Additionally, in 2018, Akiyama et al. demonstrated a 2-fold increase in mast cell density (in both the detrusor and lamina propria) in the ulcerative IC bladder above controls, while there was no significant difference in mast cell density between non-ulcerative IC and controls in both the detrusor and lamina propria (Akiyama *et al.*, 2018). Interestingly, recent studies have demonstrated a significantly higher mast cell counts in non-ulcerative BPS/IC compared to ulcerative BPS/IC, while both were significantly higher than controls (Jhang *et al.*, 2016; Kim *et al.*, 2017).

From the above evidence, we concluded that despite the large number of studies focused on mast cell counts in BPS/IC bladder biopsies, the results are inconsistent and unreliable making mast cell distribution and activation pattern a reasonable subject for further study.

5.4 Mast cell subtypes

Mast cells produce two main serine proteases, which are tryptase and chymase. Tryptase is present in large quantities in the two mast cell subtypes, the immune type ($MC_{T)}$ and non-immune type (MC_{TC}). Meanwhile, Chymase is present only in MC_{TC} (Krishnaswamy, Ajitawi and Chi, 2006). Consequently, mast cell chymase is less prominent than Tryptase. The increase in the level of MC Chymase is thought to be related to the development of fibrotic diseases and smooth muscle degradation, probably through the activation of $TGF\beta_{1,}$ which is a key regulating factor for fibrosis (Lindstedt *et al.*, 2001; Chen *et al.*, 2017).

MC_T are also termed mucosal mast cells as they are found facing the external environment in mucosal layers covering or lining the body surfaces (immune-type mast cells), while MC_{TC} are located in the deeper connective tissues (connective tissue mast

cells) (Krishnaswamy G. and Chi, 2006). The numbers of both subtypes can be abnormally elevated in many pathological conditions in patterns that can vary from one condition to another. In BPS/IC the numbers of the two subtypes were elevated compared to controls, while MC_T cells were predominant (Malik *et al.*, 2018a). In mild asthma, the numbers of the two subtypes were elevated. However, in severe asthma the total mast cell count was less elevated than in the mild form with MC_{TC} predominance (MC_{TC} accounts for more than 50% of the total mast cell count) (Balzar *et al.*, 2011).

These data support the importance of studying the numbers and the activation status of each mast cell subtype rather than just mast cells numbers as a whole, which might add valuable data for better clarification and phenotyping of BPS/IC.

5.5 Surface markers of mast cell activation

Despite the large number of studies highlighting the increased number of mast cells in the inflamed bladder tissue of BPS/IC patients, the actual activation status of these tissue resident mast cells and their actual involvement in the production of the pro-inflammatory mediators is yet to be clarified. This makes it very compelling to identify a specific activation pattern of mast cells in BPS/IC. Mast cells circulate in the peripheral blood in an immature form. The injured urothelial, smooth muscle and interstitial cells in the urinary bladder secrete stem cell factor (SCF) (Theoharides, Kempuraj and Sant, 2001; Sant *et al.*, 2007b). SCF is essential for mast cell extravasation and recruitment to the perivascular spaces in the body tissues, in addition to promoting mast cell proliferation, survival, differentiation and maturation in the body tissues. Consequently, SCF is essential for the in vitro culture of all mast cell lines, and its receptors (C-KIT) are consistently expressed on mast cell surfaces. (Galli, Tsai and Wershil, 1993; Iemura *et al.*, 1994; de Paulis *et al.*, 1999; Huang *et al.*, 2008; Gevaert *et al.*, 2017).

5.5.1 CD63

CD63 is a member of the tetraspanin family. It is a transmembrane type 4 protein expressed on the surface of activated mast cells in response to the formation of antigen-IgE-FcεR1 complex (Koberle *et al.*, 2012). By using monoclonal antibodies against activated human cord blood-derived mast cells, CD63 was exclusively expressed on

degranulated (active) but not quiescent mast cells. Interestingly, mast cells that had undergone degranulation as a result of Ag/IgE-FcεR1 interaction could undergo more degranulation cycles. Such repeated degranulation cycles were prevented by antibodies against CD63 (Schafer *et al.*, 2010). CD63 is essential for mast cell adherence to the extracellular matrix through binding of the integrins α4β1, α5β1 and α5β3 to the extracellular matrix proteins vimentin and fibronectin. A process that is augmented by activation of C-KIT or $Fc_{\varepsilon}RI$ receptors on the surface of mast cells, and results in intensifying mast cell degranulation (Kraft *et al.*, 2005).

In CD63 knockout mouse model, they found that mast cell numbers and distribution in tissues were not affected by the tetraspanin molecule deficiency. However, β-hexosaminidase assay revealed marked decrease in mast cell degranulation induced by FcεR1-IgE/Ag complex formation in CD63 knockout mice. Such an effect was associated with a significant decrease in the production of the cytokine TNF-α (granule stored) (Kraft *et al.*, 2013).

5.5.2 CD203c

CD203c (ENPP3) is a type 2 transmembrane protein and the third member of the family of enzymes called ecto-nucleotide pyrophosphates/phosphodiesterase. Members of this group catalyse the cleavage of phosphodiester and phosphosulfate bonds in molecules such as deoxynucleotides and NAD (Ono *et al.*, 2010). CD203c is responsible for the hydrolysis of extracellular nucleotides. It possesses ATPase and ATP pyro phosphatase activities, thus negatively regulating the chronic allergic and inflammatory responses produced by mast cells and basophils (Krishnaswamy, Ajitawi and Chi, 2006; Hauswirth *et al.*, 2008; Tsai *et al.*, 2015; Metcalfe *et al.*, 2016). CD203c is significantly overexpressed on the surface of the basophils during exacerbations of asthma, which decreases significantly on asthma remission (Ono *et al.*, 2010). It was found that CD203c is also overexpressed on the surface of neoplastic mast cells in the Systemic Mastocystosis (SM) patients but not in normal mast cells. It is also upregulated on the surface of normal mast cells in response to antigen-IgE/FcεRI reaction (Hauswirth *et al.*, 2008). In addition, It is also markedly expressed on immature in vitro-cultured mast cell progenitors in their early stages, while these levels decrease with further maturation (Schernthaner *et al.*, 2005).

Recently, an article by Bahri et al, published in 2018 proposed a new Mast cell Activation Test (MAT). In that study, peripheral blood-derived CD^{34+}, CD^{117+} myeloblast precursors were cultured in cytokine-supplemented growth medium to grow into mature mast cells. Then the serum of peanut or pollen allergic patients, which contains anti peanut or anti pollen IgE molecules, was used to passively sensitize the mast cells. They assessed mast cell activation and degranulation using several approaches including β-hexosaminidase release assay, prostaglandin D_2 release assay and the expression of activation markers including CD63 (LAMP-3), CD203c, and the lysosomal-associated membrane protein-1(CD107a) by flow cytometry. They found that mast cell activation, through incubation with peanut or grass allergen extract resulted in a dose dependant increase in the membrane expression of CD63 and CD107a, in addition to increase in the release of β-hexosaminidase from the mast cell granules (Bahri *et al.*, 2018).

Unlike CD63, which is upregulated in association with mast cell degranulation, CD 203c expression was more associated with the de-novo synthesis of cytokines and chemokines induced by IL-33, a cytokine known to induce cytokine and chemokine release by mast cells without degranulation (Cop *et al.*, 2018).

From the above evidence, it is evident that the above mentioned activation markers, CD 63, CD 107a and CD203c, are markers of allergic mast cell activation and degranulation. In the current study, we aim to study the expression of these markers in response to non-allergic mast cell activation using SP and IL-33.

5.6 Pro inflammatory biomarkers in the bladders of BPS/IC patients

Mast cell numbers and activity increase significantly in the bladders of patients of BPS, where they have a close spatial relationship with the bladder nerves in both the lamina propria and the detrusor muscle (Pang *et al.*, 1995). Mast cells have been considered to play a pivotal role in the pathogenesis of bladder pain syndrome/Interstitial cystitis (BPS/IC). Mast cells perform this role through the production of a range of serine proteases (tryptase and chymase), biogenic amines (histamine and serotonin), proteoglycans (heparin), cytokines (IL-6, IL-1 beta, TNF-alpha and interferon-gamma) and chemokines such as IL8, MCP-1, RANTES and CXCL-1. These mediators promote and maintain the inflammatory response underlying the pathology of BPS/IC (Gillenwater and

Wein, 1988; Letourneau *et al.*, 1996; Theoharides *et al.*, 1998; Theoharides, Kempuraj and Sant, 2001; Rashid *et al.*, 2004; Theoharides *et al.*, 2007; Patnaik *et al.*, 2017a).

BPS/IC is a chronic inflammatory bladder condition characterised by the upregulation of many pro-inflammatory cytokines and chemokines in the serum, urine and bladder tissue of affected patients. Such inflammatory mediators are considered as major contributors to the long-term tissue injury and debilitating symptoms experienced by patients. These cytokines and chemokines include IL-6, IL-8, IL-17A, NGF and TNF-alpha (Patnaik *et al.*, 2017a) .

5.6.1 Cytokines

Cytokines are small, secreted proteins produced by cells, which exert specific effects on the intercellular communications and interactions. They can be classified into Monokines (Released by Monocytes), Chemokines (Cytokines with chemotactic properties), Interleukins (Cytokines released by leukocytes, which interact with other leukocytes) and Lymphokines (Released by lymphocytes). Cytokines have autocrine, paracrine and endocrine effects (Zhang and An, 2007). In BPS/IC, both tissue resident and recruited Immune cells, including mast cells, lie in juxta-position to sensory nerve endings (Pang *et al.*, 1996b). Pro-inflammatory cytokines such as IL-1β, IL-6, IL-17a, TNF-α and IFN-γ are produced by mast cells (Theoharides *et al.*, 1998; Theoharides, Kempuraj and Sant, 2001; Theoharides *et al.*, 2007; Patnaik *et al.*, 2017a). Such pro-inflammatory cytokines are responsible for the activation and sensitization of bladder nociceptive sensory afferents, creating a chronic state of allodynia experienced by patients (Zhang and An, 2007). TNF-alpha induces bladder inflammation, increased urothelial apoptosis, bladder allodynia, increased voiding frequency and decreased voiding volume (Yang *et al.*, 2018). The cytokines TNF-alpha and IL-6 are expressed by damaged urothelial and smooth muscle cells, and induce activation of the JNK pathway, resulting in increased expression of pro-inflammatory genes, which maintain the bladder inflammatory response (Zhao *et al.*, 2016b).

These cytokines exert pro-inflammatory endocrine effects. TNF-α, IL-6 and IL-1β travel through the blood to the hypothalamus, where they induce fever. IL-6 migrates to the liver, where it targets hepatocytes to produce acute phase reactants such as the C-reactive protein and mannose-binding lectin. C-reactive protein acts as a primer for the

complement system, while mannose-binding lectin is essential for opsonisation of pathogens for phagocytosis. TNF-α and IL-6 also promote fat and protein catabolism, which in the long term can result in cachexia as observed in cancer patients.

Th2 cytokines, including IL-4, IL-6, IL-5, IL-10 and IL-13, are considered less inflammatory or anti-inflammatory when compared to their Th1/Th17 counterparts (Opal and DePalo, 2000). IL-4 is a highly pleiotropic cytokine. It induces the maturation of the naïve Th-cells into Th-2 phenotype, which produce IL-4 and IL-2 leading to augmentation of the Th-2 immune response. IL-4 inhibit the production of IL-12 by the activated macrophages, which suppresses the Th-1 lymphocyte development from naïve T lymphocytes. It also inhibits the expression of the pro-inflammatory cytokines IL-1, TNF-α, IL-8, IL-6 and MIP-1α. Additionally, it weakens the macrophage cytotoxic, anti-parasite and nitric oxide production activities, and induces the synthesis of the anti-inflammatory IL-1ra blocking the pro-inflammatory effect of IL-1.

Despite its prominent pro-inflammatory effects, IL-6 is considered an anti-inflammatory cytokine. It inhibits the production of the pro-inflammatory cytokines IL-1 and TNF-α, GM-CSF and IFN-γ, while it does not affect the synthesis of the anti-inflammatory cytokines e.g. IL-10 and TGF-beta. IL-10 is considered to be the most potent anti-inflammatory cytokine as it strongly inhibits the Th1/Th17 immune response. IL-10 is produced by $CD4^+$ Th-2 cells, monocytes and B-lymphocytes. It inhibits the production of the cytokines IL-1, IL-8, TNF-α, MIP-1α, MIP-2α and IL-12 by macrophages, supressing the Th-1 immune response. Generally, the Th2 cytokines IL-4, IL-5 and IL-13 contribute to the Th2-mediated immune response through the stimulation of T-cell growth and maturation into $CD34^+$ Th2 cells. In addition, they promote the growth of B-lymphocytes and the antibody class switch into IgG, IgA and IgE, which in turn contributes to the development of allergic immune responses e.g. bronchial asthma. However, they also possess anti-inflammatory properties, including inhibition of the development of the Th1/Th17 immune responses. In the bronchial walls of asthmatic lungs, IL-13 induces the production of the matrix metalo proteinases (MMP), which degrade pro-inflammatory cytokines in the bronchial walls pushing them into the airway lumen, so they can be easily cleared. It also inhibits the monocyte production of TNF, IL-1 and IL-8.

Table 4: The pro-inflammatory effects of cytokines and chemokines elevated in BPS/IC bladders

The Cytokine or Chemokine	Pro-inflammatory effects	Reference
TNF-α	Promotes vasodilatation and endothelial-leucocyte adhesion. Promotes vascular leakage and extravasation of pro-inflammatory cells, promoting tissue inflammation. Sensitizes nociceptive sensory afferents contributing to allodynia.	(Bradley, 2008; Cheng and Ji, 2008; Gonzalez, Arms and Vizzard, 2014)
CCL-2	Chemoattractant for macrophages, and mast cells. Enhances mast cell survival and maturation and enhances pro-inflammatory mediator release. Sensitizes bladder sensory afferent nerve fibres, contributing to allodynia.	(Bouchelouche *et al.*, 2004; Zhang and An, 2007; Dansereaux *et al.*, 2008; Jung *et al.*, 2008; Jainwei *et al.*, 2012; Gonzalez, Arms and Vizzard, 2014; Xu *et al.*, 2017)
CCL-5	Chemoattractant for lymphocytes, macrophages, eosinophils and mast cells. Sensitizes bladder sensory afferent nerve fibres, contributing to allodynia.	(Bouchelouche *et al.*, 2004; Zhang and An, 2007; Tyagi *et al.*, 2012; Gonzalez, Arms and Vizzard, 2014)

(CCL-9/CCL-10/CCL-11)	Chemoattractants for Th1 Lymphocytes. Promotes autoimmune tissue inflammation and damage.	(Ogawa *et al.*, 2010)
Interferon-γ	Enhances the Th1/Th17-mediated immune response, contributing to the development of chronic inflammation.	(Oxenkrug, 2011; Lee *et al.*, 2017b)
CXCL-8 (IL-8)	Neutrophil Chemoattractant and contributes to acute inflammation. Enhances the production of adhesion molecules by the vascular endothelial cells and increases vascular permeability.	(Mortier, Van Damme and Proost, 2008).
IL-6	Activates endothelial cells to secrete adhesion molecules and the chemoattractant chemokines MCP-1 and CCL-8. It enhances the production of the acute phase proteins such as C-reactive protein and serum amyloid A, promoting acute inflammation.	(Gabay, 2006; Tanaka, Narazaki and Kishimoto, 2014)

	Induces megakaryocyte maturation, which increase the platelet count. Promotes differentiation of the naïve $CD4^+$ T-cells, thus bridging the innate and acquired immune response. Enhances the maturation of the B-cells into antibody-producing plasma cells.	

Table 5 Potential Pro-inflammatory Biomarkers of Interstitial Cystitis

Pro-inflammatory Biomarker	Ulcerative BPS/IC	Non-Ulcerative BPS/IC	Reference
IL-6	+	+	(Peters, Diokno and Steinet, 1999; Erickson *et al.*, 2008; Jiang *et al.*, 2013; Logadottir *et al.*, 2014a)
IL-8 (CXCL-8)	+	+	(Peters, Diokno and Steinet, 1999; Erickson *et al.*, 2008; Jiang *et al.*, 2013)
IL-2	+	+	(Peters, Diokno and Steinet, 1999)

IL-17A	+		(Logadottir *et al.*, 2014a)
CCL-21	+		(Offiah *et al.*, 2016)
CXCL-10	+	+	(Tyagi *et al.*, 2012)
TNF-α	+	+	(Ogawa *et al.*, 2010; Jiang *et al.*, 2013)
NGF	+		(Tyagi *et al.*, 2012)
CXCL-1	+	+	(Tyagi *et al.*, 2012)
RANTES	Not elevated	+	(Tyagi *et al.*, 2012)
MCP-1	Not elevated	+	(Tyagi *et al.*, 2012)
IL-1β	Not elevated	+	(Jiang *et al.*, 2013)
CXCL-9	+	Has not been measured	(Ogawa *et al.*, 2010)
CXCL-11	+	Has not been measured	(Ogawa *et al.*, 2010)

5.6.2 Chemokines

Chemokines are a large family of secreted glycoproteins with small molecular weights ranging between 7 to 10 kDa. They are cytokines possessing chemoattractant properties, which can recruit inflammatory cells circulating in the blood to the peripheral tissues. They include more than 50 ligands exerting their effects through their interaction with seven transmembrane-spanning G protein-coupled receptors present on glycosaminoglycan (GAG) linked to endothelial cell layers (Mortier, Van Damme and Proost, 2008; Tyagi *et al.*, 2012; Kuo, Liu and Shie, 2013; Gonzalez, Arms and Vizzard, 2014). According to the positions of the first two conserved cysteine residues,

chemokines are classified into two major families, the CC and the CXC families. The CC family members are characterised by the adjacent position of the first two Cysteine residues and they include Monocyte Chemoattractant Protein-1 (CCL-2) and RANTES (CCL-5). CCL-2 is chemoattractant for monocytes that differentiate into macrophages in the tissue sites. It also chemo attracts and activates mast cells, enhancing their inflammatory cytokine production. It is produced by detrusor smooth muscle cells in inflamed bladders (Bouchelouche *et al.*, 2004; Jainwei *et al.*, 2012; Xu *et al.*, 2016a). CCL-5 is another chemokine with chemotactic properties for monocytes, lymphocytes, macrophages, eosinophils and mast cells but not neutrophils (Bouchelouche *et al.*, 2004; Tyagi *et al.*, 2012). Such chemokines are also implicated in the sensitization of bladder primary afferent nociceptive type-c sensory nerve fibres resulting in the development of allodynia (abnormal pain perception on non-painful stimuli e.g. minimal bladder filling), which is a major symptom experienced by BPS/IC patients (Zhang and An, 2007; Dansereaux *et al.*, 2008; Jung *et al.*, 2008; Gonzalez, Arms and Vizzard, 2014).

The CXC family of chemokines (chemotactic cytokines) are classified into two main families depending on the presence or absence of an ELR motif opposite to their terminal NH2-terminal Cysteine residue (Mortier, Van Damme and Proost, 2008). The chemokines CXCL-9, CXCL-10 and CXCL-11 belong to the ELR-negative chemokines, and they mediate their effects through interaction with the receptor CXCR3. These CXCR3-binding chemokines are involved in the perpetuation of chronic inflammatory tissue damage in autoimmune disorders such as Crohn's disease. In such autoimmune disease, injury of the tissue epithelial, endothelial and neuronal cells induces the release of the CXC-R3 binding chemokines. These chemokines chemo attract Th-1 lymphocytes expressing CXCR3 receptors. Acting on the CXCR3 receptors, these chemokines induce the production of IFN-γ by Th1 lymphocytes. Consequently, IFN-γ induces the production of the CXC-R3 binding chemokines by these epithelial, endothelial or neuronal cells attracting more Th-1 lymphocytes thereby creating a vicious cycle feeding the chronic inflammatory process in autoimmune disorders (Ogawa *et al.*, 2010; Tyagi *et al.*, 2012).

In the case of infection, macrophages phagocytose and digest the engulfed pathogens using lysosomal enzymes, and, finally, present the pathogen epitopes on their membranes bound to MHC class II molecules. Such complexes attach to T-cell receptors

on the surfaces of the $CD4^+$ Th1 lymphocytes, previously activated by this specific antigen (epitope), inducing the production of the cytokine IFN-γ, which is also produced by natural killer cells (NKs) on encountering pathogens. In turn, IFN-γ increases the expression of MHC molecules and the production of the cytokine IL-12 by macrophages. IL-12 enhances the differentiation of naïve $CD4^+$ T cells into activated Th1 cells, and enhances the activity of NK cells thereby promoting the Th1-mediated immune response.

$CD4^+$ Th17 cells are subsets of T helper cells, which produce the cytokine IL-17 that recruits neutrophils to the site of infection or tissue damage. It is a way by which the adaptive immune system maintains an innate immune response as long as the infection is present. Upon engulfment of pathogens, the activated macrophages and dendritic cells can produce IL-23, which induces the maturation of the $CD4^+$ naïve T cells into Th17 cells.

IL-2 is a cytokine produced by the $CD4^+$ Th0 and TH1 lymphocytes. It has an autocrine mode of action. When naïve T cells (Th0) encounter an antigen presented by antigen presenting cells (APCs), they start to express α chain of the IL-2 receptor (CD25) on their surfaces under the effect of the macrophage-produced IL-12. This allows these cells to respond to the autocrine effect of the IL-2, which induces proliferation of these lymphocytes promoting the inflammatory response.

The ELR-positive chemokines include CXCL-8 (IL-8) and CXCL-1, which are potent pro-inflammatory chemokines with strong neutrophil chemoattractant properties. In humans, CXCL-8 is well known as the chemokine with the highest potency as a neutrophil chemoattractant (Mortier, Van Damme and Proost, 2008). Interestingly, Tseng-Rogenski and Liebert demonstrated that exogenous recombinant IL-8 enhanced in vitro human urothelial cell growth through the Akt pathway, an effect mediated by CXC-R1 receptors expressed on the surface of the human urothelial cells. They also demonstrated that reduced m RNA levels of IL-8 could be responsible for urothelial cell death in BPS/IC (Tseng-Rogenski and Liebert, 2009).

5.6.3 Pro-inflammatory Cytokines and Chemokines in BPS/IC

The 24-hour urinary levels of the pro inflammatory cytokine IL-6 were found to be markedly elevated in BPS/IC patients compared to controls (Erickson *et al.*, 2002; Lamale

et al., 2006). In BPS/IC patients, Urinary levels of IL-6 correlated positively with the severity of bladder tissue inflammation (Erickson, Brelchis and Dabbs, 1997). Urinary levels of IL-2, IL-6 and IL-8 were significantly higher in active IC patients compared to controls or IC patients who had been in remission for 2.6 years after Intravesical BCG (Peters, Diokno and Steinet, 1999). Moreover, immunohistochemistry of the bladder biopsies from ulcerative IC patients revealed moderate to strong IL-6 immuno reactivity in both the urothelium and lamina propria (Erickson *et al.*, 2008).

In the bladder tissues from seven ulcerative IC patients, messenger RNA levels of the pro-inflammatory cytokines IL-6 and IL-17A, the anti-inflammatory cytokine IL-10, and the inducible nitric oxide synthase enzyme were significantly higher compared to bladder tissues from six healthy individuals (Logadottir *et al.*, 2014a).

In 2012, a research group quantified the urinary levels of a range of pro-inflammatory cytokines and chemokines from both ulcerative and non-ulcerative IC patients compared to healthy controls using specific Cytokine/Chemokine assays. They found that the levels of the cytokines IL-6 and NGF, in addition to the chemokines CXCL-1 and CXC-L10 were five to twenty-fold higher in ulcerative IC compared to the two other groups. CXCL-1 and RANTES were significantly higher in non-ulcerative IC patients compared to controls, while CXCL-10 and MCP-1 levels were close to significance. Interestingly, in ulcerative BPS/IC patients, the urine levels of CXCL-10 were found to be several fold higher than the serum levels indicating that the bladder tissue is the source of that chemokine (Tyagi *et al.*, 2012).

In a study that involved 30 non-ulcerative BPS/IC patients compared to 26 healthy controls, the serum levels of C-reactive protein, IL-1β, IL-6, IL-8 and TNF-alpha were significantly higher in BPS/IC patients (Jiang *et al.*, 2013). Positive correlation between the urinary levels of IL-8 and the mast cell counts in the bladder biopsies existed in both treated and untreated patients (Erickson *et al.*, 2008).

Using quantitative real time PCR, the m RNA levels of the CXCR-3 receptors and their binding chemokines, which include CXCL-9, CXCL-10 and CXCL-11, were found to be markedly elevated in the urothelium of ulcerative BPS/IC patients compared to controls.

These changes were accompanied by elevated m RNA levels of the cytokines interferon-gamma (IFN-γ) and TNF-α, which provides supporting evidence for the autoimmune theory as being responsible, at least in part, for the pathophysiology of BPS/IC (Ogawa *et al.*, 2010).

In the serum of ulcerative IC patients, the levels of the CXCR-3 binding chemokines were also elevated compared to controls (Sakthivel *et al.*, 2008). The blockage of CXCL-10 signalling markedly decreased the intensity of the cyclophosphamide-induced bladder inflammation in a rat model (Sakthivel *et al.*, 2008).

The secondary lymphoid tissue chemokine (CCL21) is a pro-inflammatory chemokine involved in the migration and recruitment of dendritic cells to injured tissue. Recently, both m RNA levels of CCL21 and the Fibroblast Growth Factor 7 were markedly elevated in the bladder tissue of BPS/IC patients compared to controls. Interestingly, their m RNA levels positively correlated with the patients' clinical outcomes (Offiah *et al.*, 2016).

The previous evidence reflects the compelling need for a potent, reliable and safe anti-inflammatory agent that can reduce the production and release of these pro-inflammatory mediators by activated mast cells.

Table 6: Pro-inflammatory Cytokines and Chemokines produced LAD2 cells upon 24-hour Secretagogue challenge

Mediator	Secretagogue	Reference
TNF-alpha	Substance P	(Kulka *et al.*, 2008; Catalli *et al.*, 2014; Patel and Theoharides, 2017)
CXCL-8 (IL-8)	Substance P	(Kulka *et al.*, 2008; Patel and Theoharides, 2017)
MCP-1 (CCL-2)	Substance P C3a	(Kulka *et al.*, 2008; Castellani *et al.*,

	Antigen-IgE	2009b; Catalli *et al.*, 2014)
RANTES (CCL-5)	Substance P	(Kulka *et al.*, 2008)
GM-CSF	Substance P	(Kulka *et al.*, 2008; Green *et al.*, 2019)
IL-3	Substance P	(Kulka *et al.*, 2008)
IP-10 (CXCL-10)	VIP	(Kulka *et al.*, 2008)
CCL-3 (MIP-1α)	Substance P	(Green *et al.*, 2019)
CCL-4 (MIP-1β)	C3a Substance P	(Catalli *et al.*, 2014; Green *et al.*, 2019)
CCL-1	Antigen-IgE	(Catalli *et al.*, 2014)

5.6.4 Interleukin 33 and bladder inflammation

Interleukin 33 is a member of the IL-1 family of cytokines (Liew, Pitman and McInnes, 2010). Under normal conditions, it is expressed constitutively by fibroblasts, endothelial, smooth muscle and epithelial cells as a nuclear protein that regulates the expression of intercellular barrier proteins (Martin and Martin, 2016). IL-33 binds to DNA through chromatin-binding motif located in its N-terminal nuclear domain. Deletion of this domain resulted in constitutive IL-33 extracellular release associated with ST-2 dependant serum hyper eosinophilia and lethal multi-organ inflammation (Bessa *et al.*, 2014). In chronic pulmonary inflammatory conditions, such as asthma and chronic obstructive pulmonary disease, IL-33 promoter activity and protein expression have been reported to be significantly increased indicating an inflammation-induced expression (Tapiainen *et al.*, 2010; Byers *et al.*, 2013).

Unlike the other members of the IL-1 cytokine family such as IL-1β and IL-18, IL-33 is released in an inactive form apoptotic epithelial cells, where caspases (apoptotic enzymes) cleave it into an inactive protein lacking any cytokine activity (Cayrol and Girard, 2008; Luthi *et al.*, 2009). However, in the case of epithelial damage (necrosis) and/or epithelial barrier breach, induced by environmental agents, IL-33 is released by the necrotic epithelial cells in an uncleaved (active) form (Moussion, Ortega and Girard, 2008; Jang and Kim, 2015). Active IL-33 recruits immune cells to the site of tissue damage and

stimulates a Th2 type immune response and so it is considered an alarmin protein (Schmitz *et al.*, 2005; Martin and Martin, 2016). Such evidence suggests that IL-33 pro-inflammatory effects are seen where there is tissue damage (necrosis), but not in programmed cell death (apoptosis).

Abnormal dysregulated release of IL-33 has been implicated in the pathophysiology of allergic, inflammatory and autoimmune conditions such as atopic dermatitis, anaphylactic shock, asthma, rheumatoid arthritis, and atopic dermatitis (Liew, Pitman and McInnes, 2010; Kurowska-Stolarska *et al.*, 2011; Imai *et al.*, 2013; Cayrol and Girard, 2014). IL-33 signals through a specific receptor complex formed of the ST2 receptor and the IL-1 receptor accessory protein (IL-1RAcP) (Schmitz *et al.*, 2005). In allergic conditions such as asthma, allergic rhinitis, atopic dermatitis and parasitic infections, IL-33 activates ST2-expressing IL-C2 cells (type-2 innate lymphoid cells) to induce the production of IL-13 and IL-5 cytokines inducing chronic inflammation independent of adaptive immunity (Cayrol and Girard, 2014).

Currently, the involvement of IL-33 in the pathophysiology of BPS/IC has not been sufficiently studied. Recent evidence revealed a clue to the potential role of IL-33 in the pathogenesis of BPS/IC. The 24-hour urinary levels of IL-33, normalized to creatinine, in BPS/IC patients were nearly twice as high as those in healthy controls (Kochiashvili and Kochiashvili, 2014). The nature of the pathology in BPS/IC bladders makes a contribution by IL-33 a strong possibility. Histology of BPS/IC biopsies reveals bladder inflammation, urothelial thinning and denudation, in addition to the urothelial barrier breach. With bladder inflammation, necrotic urothelial and detrusor smooth muscle cells are potential sources of IL-33. Mast cell released pro-inflammatory proteases such as cathepsin-G, tryptase and chymase can cleave the released IL-33 in its central domain releasing the IL-1cytokine-like domain, which is several times more potent than IL-33 (Lefrançais *et al.*, 2012; Cayrol and Girard, 2014; Lefrancais *et al.*, 2014). IL-33 has been proposed to enhance mast cell responses in both allergic and pseudo-allergic reactions (Hsu, Neilsen and Bryce, 2010; Hueber *et al.*, 2011; Fux *et al.*, 2014; Jang and Kim, 2015). Furthermore, IL-33 enhanced the survival of Human cord blood-derived mast cells and their adhesion to fibronectin (Lunderius Andersson, Enoksson and Nilsson, 2012), and active IL-33 induces the production of Th2 cytokines from mast cells (Ho *et al.*, 2007).

In animal models, Injection of IL-33 increased the numbers of both skin and peritoneal mast cells, in addition to increasing in vitro mast cell proliferation (Saluja *et al.*, 2014). Cord or peripheral blood-derived primary cultured human mast cells constitutively express IL-33 receptors (ST2), where IL-33 significantly accelerates their maturation (Allakhverdi *et al.*, 2007). IL-33 does not induce mast cell degranulation, but it activates mast cells to release pro-inflammatory cytokines and chemokines such as IL-5, IL-13 (immune regulatory cytokine), GM-CSF, CCL-2 CXCL-8 and CCL-17 (Allakhverdi *et al.*, 2007; Ho *et al.*, 2007). In a mouse model of cigarette smoking-induced COPD, both the expression of IL-33 and its ST2 receptors was significantly increased in lung tissue. Such was associated with neutrophil and macrophage infiltration and increased expression of the pro-inflammatory cytokines IL-17, TNF-α and IL-1β and the chemokine MCP-1. These pathological effects were blocked with intranasal injection of anti-IL-33 antibodies or sST2 decoy receptors (Qiu *et al.*, 2013).

Co challenge of LAD2 cells with IL-33 and Substance P induces several fold increases in the amounts of TNF-alpha released by both LAD2 and human cord-blood derived mast cells than when challenged by Substance P alone. However, it does not induce or enhance substance P-induced mast cell degranulation (Taracanova *et al.*, 2017). IL-33 pre-incubation enhanced the expression of the substance P receptor NK-1 on the surfaces of LAD2 cells (Taracanova *et al.*, 2017). In human CD-34^{+} peripheral blood-derived mast cells, flow Cytometric analysis of the expression of the degranulation marker CD63 and the CD-203c, a marker of mast cell activation and late de novo synthesis, revealed that IL-33 treatment induces mast cell activation without degranulation, while it augments IgE-FcεRI-induced but not Substance P-induced degranulation (Cop *et al.*, 2018).

In animal studies, both mouse bone marrow-derived mast cells and the murine mast cell line mc9 released IL-33 upon Antigen IgE-FcεRI reaction (Hsu, Neilsen and Bryce, 2010), which might induce an autocrine effect augmenting and prolonging the inflammatory response.

Mast cell proteolytic enzymes such as chymase can induce urothelial cell death (Choi *et al.*, 2016), which further contributes to the urothelial barrier breach exposing by

urothelial cells, fibroblasts, the interstitial cells and the smooth muscle cells to harmful urine constituents, which can result in further release of active IL-33.

These data present IL-33 as a potential pro-inflammatory mediator in BPS/IC, and accordingly, the expression of this cytokine and its correlation with patients' symptoms (Pain, Frequency and Urgency) and the other histopathological findings e.g. mast cell counts. This could form the basis for future study (see later).

Recent studies have highlighted that the systemic blocking of IL-33 may not be the treatment of choice in several inflammatory conditions. IL-33 might have other beneficial functions such as improving anti-tumour immunity and functional recovery after contusion. Additionally, systemic blocking of IL-33 signalling can produce serious side effects such as atherosclerosis (Jang and Kim, 2015). These facts make it compelling to obtain a local therapeutic agent that can ameliorate the pro-inflammatory effects of IL-33 without producing systemic side effects.

5.7 Honey

Honey is the sweet and sticky substance produced by honeybees following nectar collection. For a long period, honey has been used as a remedy for wounds as it speeds up wound healing and combats wound infection. Honey is mentioned in the Qur'an and is recommended as a cure for diseases (Surah 16. Verse 68, 69 The Bee). Interestingly, an ancient Egyptian papyrus dating to 1550 BC (Ebers Papyrus) revealed that the ancient Egyptians regarded honey as sacred and used it as a wound dressing.

5.7.1 Antimicrobial effects of Honey

Recently, Manuka honey has been proven by a large number of scientific studies to possess strong antimicrobial properties against wide range of pathogenic strains of bacteria including those resistant to antibiotics (Carter *et al.*, 2016; Halstead *et al.*, 2016; Emineke *et al.*, 2017). It has also been shown to strongly inhibit bacterial biofilm formation in a dose dependant manner (Emineke *et al.*, 2017). Both antibiotic-sensitive and antibiotic-non sensitive bacterial strains showed sensitivity to medical honey preparations (Cooper and Jenkins, 2012a). These include variable staphylococcus aureus species such as methicillin-resistant staphylococci aureus (MRSA), methicillin-sensitive staphylococci (MSSA), and coagulase-negative staphylococci (Girma, Seo and She, 2019).

It also inhibit the growth of gram negative organisms such as pseudomonas aeruginosa and enterobacteriaceae (Girma, Seo and She, 2019).

The antimicrobial properties of honey can be easily demonstrated by its ability to remain unspoiled for long periods while stored at room temperature. The antimicrobial effects of Honey have been attributed to a number of properties. Firstly, the high sugar and low water content allows honey to be hygroscopic draining water from the surrounding environment. By the enzymatic production of glucose acids from glucose, honey creates a dry and acidic environment, which is unsuitable for the growth of microorganisms. Secondly, the oxidation of glucose by the glucose oxidase enzyme secreted by the honeybee produces hydrogen peroxide. The latter activates proteases through oxidation resulting in enhancement of autolytic debridement of necrotic tissue, cutaneous circulation and new tissue formation (Cooper, 2007; Evans and Mahoney, 2013). Interestingly, Manuka honey from New Zealand and Jelly bush honey from Australia possess antibacterial properties which are attributed to unidentified contents other than the high sugar content and hydrogen peroxide (Cooper, 2007). More recently, the strong antibacterial properties of Manuka honey, honey produced by bees foraging on Leptospermum scoparium (Manuka bushes) in New Zealand, have been attributed to its high methylglyoxal (MGO) content (Mavric *et al.*, 2008).

5.7.2 Honey and wound healing

Manuka honey-based dressings, including those using Manuka honey, have been demonstrated to yield satisfactory results significantly reducing the healing time for bed sores and partial thickness burns (Molan, 1998). Honey induced partial or total autolytic debridement of necrotic or infected tissue, promoting angiogenesis, tissue regeneration, in addition to combating tissue infection and inflammation (Molan, 2001;2002; Cooper and Jenkins, 2012b; Evans and Mahoney, 2013).

5.7.3 Anti-inflammatory effect of Honey

Much evidence supports a positive anti-inflammatory role for Manuka honey. The strong antibacterial properties of Manuka honey adds to its anti-inflammatory effects through prevention of the inflammation induced by infection (Molan, 1998; Yaghoobi, Kazerouni and Kazerouni, 2013). However, the anti-inflammatory properties of honey have been noted in aseptically induced wounds in animal models indicating an anti-inflammatory effect of honey independent of its anti-microbial properties (Molan, 2002).

In animal models of cut wounds and burns, Honey dressings not only induced the fastest wound healing rates, but also histological examination of wound biopsies revealed lower numbers of acute inflammatory cells in honey-dressed wounds compared to other dressings (Molan, 1998). The anti-inflammatory effect of Manuka honey is attributed to the inhibition of the production of the reactive oxygen species (ROS) by activated polymorphonuclear neutrophils (PMNs) through limitation of the complement system, which activates and recruits PMNs to the wound site. Honey also scavenges the superoxide anion produced by activated PMNs at the wound site (antioxidant effect) (van den Berg *et al.*, 2008).

Interestingly, Malaysian honey extracts, including honey methanol extract (HME) and honey methyl acetate extract (HMAE), displayed strong in vitro anti-inflammatory effects. Honey extracts exerted 40 to 80 % inhibition of nitric oxide (NO) production by both lipopolysaccharide (LPS) and IFNγ-activated macrophages. They also significantly inhibited cytotoxicity produced by TNF-α on L929 cells (Fibroblast cell line) (Kassim *et al.*, 2010).

Manuka Honey treatment successfully decreased the inflammation in atopic dermatitis patients compared to untreated patients. It also inhibited the production of IL-4 and CCL-26 from the keratinocyte cell line HaCaT and inhibited human LAD2 mast cell degranulation and histamine release (Alangari *et al.*, 2017a). Furthermore, various honey formulations inhibited Ca^{++} ionophore-induced mast cell degranulation and histamine release (Birch *et al.*, 2011; Lwaleed *et al.*, 2014). Moreover, Honey is well tolerated by urothelial cells and can protect them from harmful chemical injuries (Lwaleed *et al.*, 2014). Such evidence supports the use of Manuka Honey as an Intravesical anti-inflammatory agent in the treatment of BPS/IC.

5.8 Study Summary:

5.8.1 Search Strategy:

The research study was based on **PICO** model as follows:

P (Forni *et al.*) BPS/IC Patients.

(2) Human mast cell line (LAD2).

I The intervention is the challenge of LAD2 cells with substance P after Manuka Honey treatment.

C (Forni *et al.*) Healthy bladder tissues.

(2) The comparison group are LAD2 cells challenged with sub P but untreated with Medihoney.

O Decrease in both degranulation and the levels of the pro-inflammatory cytokines and Chemokines release

The literature search was conducted on the following databases using free text: Embase, Medline, EMBASE, Scopus and Web of Sciences as follows:

(Forni *et al.*) (bladder pain syndrome or Pain* bladder syndrome or Interstitial cystitis).mp.

(2) (Manuka or Honey).mp

(3) Mast cell*.mp.

(4) 1 and 2

(5) 1 and 3

(6) 2 and 3

(7) (Mast cell* AND (MRGPRX2 OR mrgprx2)) .mp.

(8) ((Manuka OR Honey) AND inflammation).mp

(Forni *et al.*) pathophysiology OR pathology OR aetiology OR cause OR mechanism

(10) 1 and 9

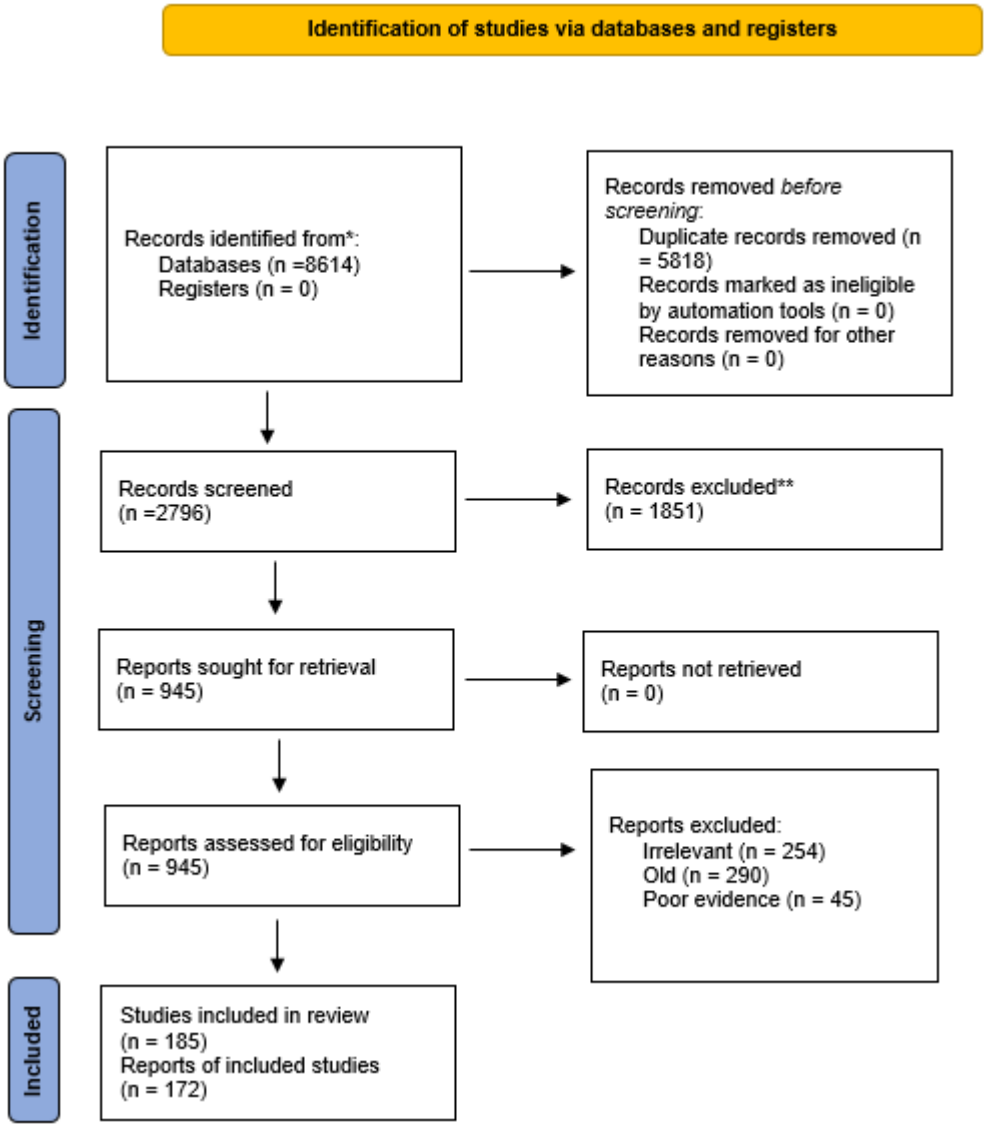

Figure 4: PRISMA flow diagram for the database research strategy

5.8.2 Research questions:

(A) Is neurogenic inflammation through the interaction between mast cells and the depolarized sensory nerve endings involved in the chronic bladder wall inflammation seen in BPS/IC patients?

(B) Can Manuka Honey treatment decrease mast cell-induced inflammation?

5.8.3 Research Aims:

(A) To investigate the actual involvement of the neurogenic inflammation theory in the pathophysiology of the bladder wall inflammation in BPS/IC patients, through studying the expression of the substance P receptors on the surfaces of the bladder mast cells, and consequently their responsiveness to substance P in the bladders of BPS/IC patients.

(B) Investigating a potential positive role of a medical-grade Manuka honey (Medihoney) as a mast cell stabilizer, which will consequently decrease the degree of bladder wall inflammation found in the disease.

5.8.4 Research Objectives

(A) To assess mast cell numbers and the substance P responsiveness in the bladders of BPS/IC patients using immunohistochemistry technique.

(B) To study the effect of Manuka Honey treatment on mast cell degranulation and the levels of cytokines and chemokines produced by these cells when treated with Substance P using specific protein analysis array.

(C) To study the effect Manuka Honey pre-treatment on the mast cell signalling pathways involved in mast cell activation.

6 Chapter two: The effect of Medihoney on the Pro-inflammatory responses in LAD2 mast cells

6.1 Abstract

Mast cells numbers and activity are significantly elevated in several inflammatory conditions including interstitial cystitis (BPS/IC). This condition is associated with the release of neuroactive substances, e.g. substance P, which induces mast cell degranulation and tissue inflammation. Medihoney® (MH) is a medical grade Manuka honey with strong anti-microbial properties. Recent reports also highlight its anti-inflammatory properties through inhibition of histamine release by mast cells. In the present study, we investigated the anti-inflammatory effect of MH against neurogenic inflammation by studying its effect on mast cell degranulation and pro-inflammatory cytokine release induced by the neuropeptide substance P (SP). LAD2 human mast cells were activated by SP (1µM) for 40 minutes (for degranulation assays) or 24 hours (for cytokine release assays) with or without 20-minute pre-incubation with MH. Degranulation was assessed by measuring release of the lysosomal enzyme β-hexosaminidase (β-hex) in the cell culture supernatant, while cytokine release was assessed using a highly specific electroluminescent ELISA assays (MSD, USA). MH (4%) significantly inhibited mast cell degranulation by approximately 90%. MH (4%) pre-treatment inhibited both the spontaneous and the SP-induced release of the cytokines GM-CSF, IL-8, while it enhanced the release of the cytokine IL-1β. Its effect on the release of MCP-1 was contradictory and inconclusive. The Akt inhibitor 10-DEBC HCL strongly inhibited mast cell degranulation in a dose dependent fashion, but it had no effect on cytokine release.

MH inhibits substance P-induced LAD2 cell degranulation possibly through downregulation of protein kinase B (Akt) activation, which is involved in mast cell activation pathways. Moreover, Medihoney has the potential to inhibit the release of various pro-inflammatory cytokines from mast cells, however, these results need further confirmation. Our findings suggest that MH (used intravesically) could potentially be useful as an anti-inflammatory agent directed against the neurogenic inflammation involved in the pathophysiology of BPS/IC.

6.2 Introduction

As previously described, mast cells are key effector cells in both the innate and acquired immune response. Their abnormal activation is involved in the pathophysiology of various chronic inflammatory conditions including BPS/IC. Their interaction with substance P^{+ve} nerve endings is believed to mediate the neurogenic inflammation involved in the pathophysiology of the chronic bladder wall inflammation seen in the disease. In this chapter, the potential anti-inflammatory properties of Medihoney as a candidate therapeutic agent in BPS/IC will be explored by studying its effect on mast cell degranulation and pro-inflammatory cytokine release induced by the neuropeptide substance P using LAD2 cells, a well-known in vitro mast cell model.

6.3 The effect of Medihoney on mast cell degranulation

6.3.1 Methodology

6.3.1.1 LAD2 cell culture

Human LAD2 mast cells (Mast cell line developed by the laboratory of allergic diseases, NIH, USA from the blood of systemic mastocystosis patients) have been obtained as a kind gift from Professor Kirshenbaum, NIH, USA, and different batches of these cells are currently being cultured and used in inflammation studies. LAD2 cells are cultured in Stem Pro-Serum free medium (Invitrogen) enriched with recombinant human Stem cell factor 100 ng /ml (Peprotech), 2mM L-Glutamine with penicillin/streptomycin (Sigma Aldrich), and growth supplement (Invitrogen) (Kirshenbaum *et al.*, 2003).
These cells have been tested for Substance P responsiveness using the β-hexosaminidase assay (Kuehn, Radinger and Gilfillan, 2010). The cells were strongly responsive when treated with 1 µM of substance for 40 minutes, with an average percentage release of 50%, which is an essential requirement for this work. Currently, these experiments are performed in the Health Sciences Laboratory (AA81), in collaboration with the Immuno-pharmacology Group at the Faculty of Medicine, University of Southampton.

6.3.1.2 Preparation of LAD2 cell suspension in Tyrode's buffer

LAD2 cells were washed twice in PBS 1X via centrifugation for 10 minutes at room temperature and 225 x g. Cells were resuspended in Tyrode's buffer and placed on roller

for 30 seconds before spreading the suspension in a 96-well plate at a density of 50,000 cells per 160 µl Tyrode's buffer/ well. Each condition was performed in three wells (Triplicates) to provide reliable results. Based on the experimental design and number of wells, the cell count and Tyrode's buffer volume can be easily calculated.

6.3.1.3 Drug Preparations

6.3.1.3.1 Sub P preparation (Sigma Aldrich)

Substance P was dissolved in 0.1 M acetic acid, and was used at 1µM concentration.

6.3.1.3.2 Substance P receptor blockers

6.3.1.3.2.1 QWF preparation (TOCRIS biosciences)

QWF is a non-selective blocker of the substance P receptors MRGPCRX2 and NK-1 receptor (Ali, 2016a). QWF is a powder that is dissolved in DMSO (Stock). QWF was used at 100, 10 and 1µM concentrations, where in each concentration, the final DMSO concentration was les that 0.1%.

6.3.1.3.2.2 L-733,060 preparation (TOCRIS biosciences)

L-733,060 is a selective blocker of the substance P receptor NK-1. It was prepared in ultrapure water and used at 100, 10 and 1µM concentrations, where in each concentration, the final DMSO concentration was les that 0.1%.

6.3.1.3.3 Inhibitors of ERK and Akt protein kinases

As per our results in chapter three, Honey pre-treatment inhibited substance P-induced phosphorylation of the protein kinases Akt and ERK. In order to find out if these effects underlie the inhibitory effect of Medihoney on mast cell degranulation following substance P challenge, we treated LAD2 cells with specific inhibitors of Akt and ERK, before treatment with substance P to see the effect of inhibition of these kinases on mast cell degranulation.

6.3.1.3.3.1 **The Akt Inhibitor 10-DEBC hydrochloride (TOCRIS Biosciences**)

10-DEBC hydrochloride was dissolved in DMSO (Stock). 10-DEBC hydrochloride was used at 30, 20, 10, 5 and 1 µM concentrations, where in each concentration, the final DMSO concentration was les that 0.1%.

6.3.1.3.3.2 The ERK1/2 Mitogen activated protein kinase inhibitor U0126 (TOCRIS biosciences)

U0126 was dissolved in DMSO (Stock). 10-DEBC hydrochloride was used at 30, 20, 10, 5 and 1 µM concentrations, where in each concentration, the final DMSO concentration was les that 0.1%.

6.3.1.3.4 Medihoney Preparations

0.2, 0.4 and 0.6 grams of each of Medihoney or the sugar free Manuka extract were dissolved in TB (to give a total volume of 1 ml), to prepare 20, 40 and 60% Medihoney concentrations (W/V), respectively. In addition, 200, 400 and 600 µL of the sugar free Manuka extract were dissolved in TB (to give a total volume of 1 ml), to prepare 20, 40 and 60% concentrations (V/V), respectively. As 20 µl of each honey preparation was added to a total volume of 200 µL in each well, the final working concentration in each well ranged from 1 and 6%.

The sugar-free Manuka honey extract was developed and kindly provided by COMVITA New Zealand Ltd. It has been provided to the research group near to the end of the study, hence it has not been used in the cytokine release assays. However, the inclusion of this extract is crucially important for the overall value of the message delivered by the study. For example, the fact that this extract is sugar free makes it more suitable than the raw honey for the treatment of diabetic patients. In addition, it allows the study of the therapeutic properties of Manuka-specific agents away, while excluding the effects of the sugar content of honey.

6.3.1.4 Loading the cell culture plate

Each well in the 96-well cell culture plate was loaded with a total volume of 200 µL, including 160 µL of the cell suspension in TB. In the spontaneous well (cell suspension alone), the rest of the volume (40 µL) was added as TB. Each treatment was added in a volume of 20 µL so that each drug will be further diluted 10 times. Before LAD2 cell activation, cell suspensions were treated with the potential mast cell stabilising agent, including Medihoney or the protein kinase inhibitors, for 20 minutes incubation at 37°C in 5% CO_2. Such is followed by LAD2 cell activation (degranulation) by adding substance P (1µM), compound 48/80 (10 µg/ml) or Ca^{++} ionophore (1µM) to the designated wells and the plate was further incubated for 40 minutes. The plate was then centrifuged at

225 x g for 10 minutes at 2-8°C (acceleration 2, brake 9) to stop the reaction and precipitate the cells.

6.3.1.5 Total preparation

480 uL of the cell suspension (equivalent for cell suspension in three wells) was placed in a 1.5 ml Eppendorf tube and centrifuged at 1500 xg for 5 minutes. After discarding the supernatant, the cell pellet was re-suspended in 600 uL of 1% v/v Triton-x 100 in 1x PBS, and vortexed vigorously for 2-3 minutes. The tube was centrifuged for 10 minutes in 3500 xg. Finally, the supernatant was pipetted out and placed on ice for the final step.

6.3.1.6 B-Hexosaminidase assay

30 µL of the supernatant in each well of the 96-well cell culture plate, in addition to 30µL of the total, were transferred into the corresponding well of a 96-well assay plate. 50 µL of the β-hexosaminidase substrate were added into each well of the assay plate, which already contained 30µL of the supernatant in each well, and then the plate was incubated for one hour. After incubation, 100 µL of the stop solution 0.2mM glycine were added into each well. Finally, each plate was read by a plate reader at Endpoint L1-L2, wave length from 410 to 595. Data were analysed using GraphPad Prism 9 and Excel 2016 software programmes.

Table 6: Constituents of each well of the cell culture plate in case of studying the effect of MH4% pre-treatment on the substance P- induced mast cell degranulation.

	Constituent per well			
CONDITIONS	Cell suspension (50,000 cells/160 µL)	MH 40%	TB	Substance P 10 µM
Spontaneous	160 µL	0	40 µL	0
MH4% alone	160 µL	20 µL	20 µL	0

Substance P 1µM alone	160 µL	0	20 µL	20 µL
Substance P 1 µM + MH4%	160 µL	20 µL	0	20 µL

- It is worth mentioning that 20 µL of each drug was added to a total volume of 200 µL in each well, which means that each drug was further 10-time diluted after addition to each well. To make up for this, each drug is prepared in 10 times of the desired final working concentration. For example, to activate LAD2 cells with substance P 1µM, 20 µL of substance P 10 µM was added into a total volume of 200 µL in each well.
- Nevertheless, substance P 1µM was replaced by other secretagogues such as Compound 48/80 10 µg/ml or Ca^{++}ionophore 1µM to study the effect of these substances on mast cell degranulation in LAD2 cells. Similarly, MH4% was replaced by other potential mast cell stabilizers including other Medihoney concentrations, Manuka honey extract, protein kinase inhibitors, or substance P blockers such as QWF protein. Such a protocol allowed for the study of the effect of various drugs on mast cell degranulation, in addition to characterisation of the mechanisms by which Medihoney inhibits mast cell degranulation.

6.3.2 Results

6.3.2.1 The effect of Medihoney on mast cell degranulation

- When LAD2 cells were incubated for one hour with variable concentrations of Medihoney, β-hexosaminidase levels remained very close to the levels seen with spontaneous release, indicating that honey incubation did not induce mast cell degranulation (See Figure 5).
- Similar to Medihoney (MH), all concentrations of Manuka extracts did not induce significant release of β-hexosaminidase when compared to the spontaneous untreated control (Figure 6).

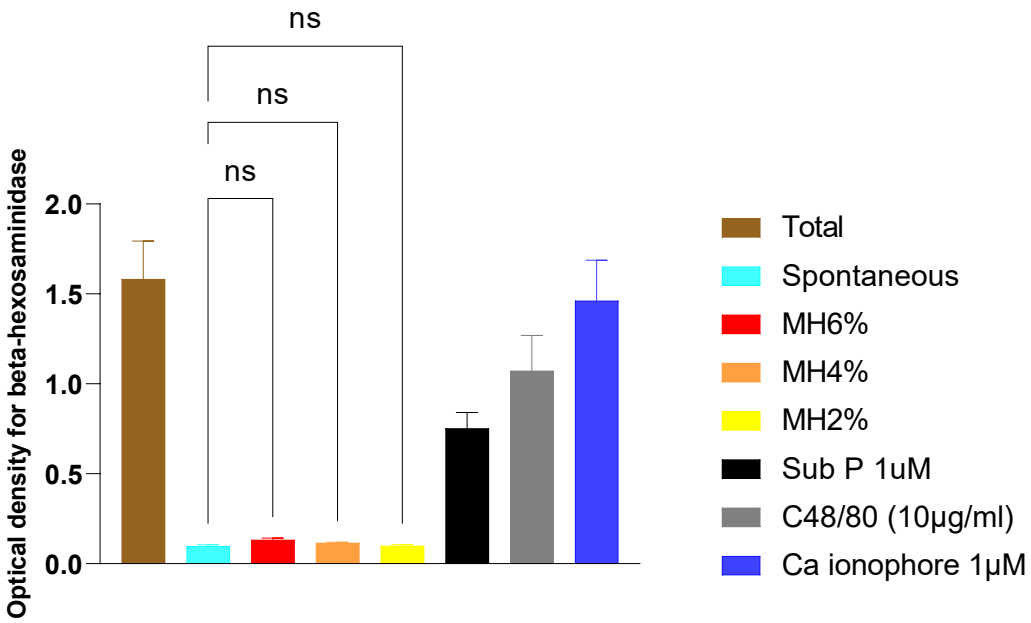

Figure 5: Optical density for β-hexosaminidase release by LAD2 cells upon one hour incubation with s Medihoney (MH), at 2, 4 and 6% concentrations, substance P 1µM, Compound 48/80 10 ng/ml and Ca ionophore 1 µM. The experiment was repeated twice and each assay was performed in triplicates (n=2). Statistical differences were analysed by Kruskal-Wallis test using GraphPad Prism 9 software.

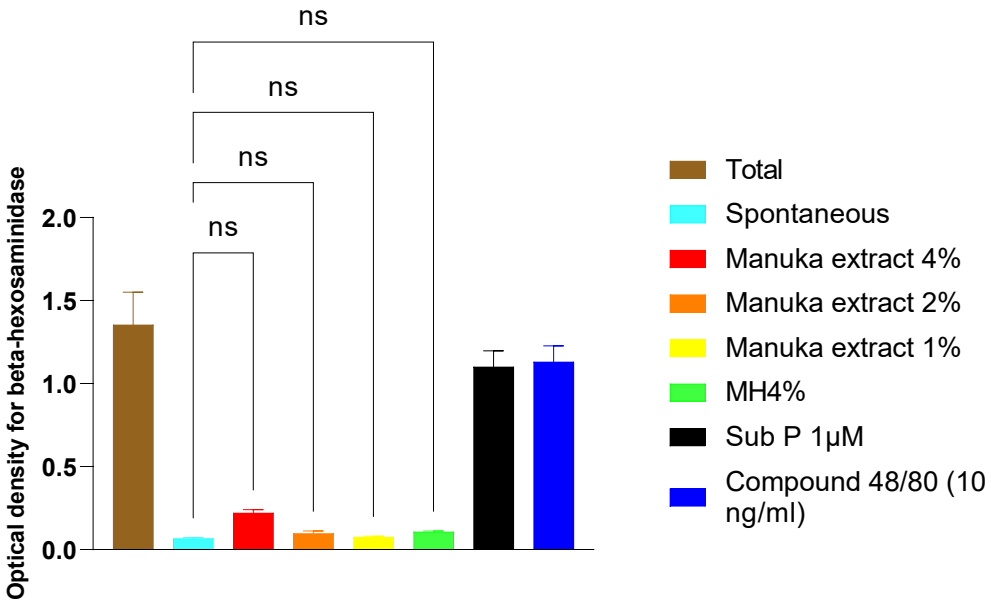

Figure 6: Optical density for β-hexosaminidase release by LAD2 cells upon one hour incubation with Manuka extract at 4, 2 and 1 % concentrations, Medihoney 4%, substance P 1µM, Compound 48/80 10 ng/ml and Ca ionophore 1 µM. The experiment

was repeated twice and each assay was performed in triplicates (n=2). Statistical differences were analysed by Kruskal-Wallis test using GraphPad Prism 9 software.

6.3.2.2 The effect of the neuropeptide substance P-induced, Compound 48/80 and Ca^{++} ionophore on mast cell degranulation in LAD2 cells

Through our experience, and after several repetitions of beta-hexosaminidase release (degranulation) assays, we were able to conclude that LAD2 cells respond strongly to a substance P concentration of 1µM (40% release above spontaneous rates) (see figures 5&6). It is also worth mentioning that 1 µM is the most widely used concentration for studying the effects of substance P, which is both potent and physiological, especially when dealing with cell lines (Kulka *et al.*, 2008; Castellani *et al.*, 2009b; Taracanova *et al.*, 2017). In addition, Compound 48/80 (10 ng/ml) and Ca^{++} ionophore (1µM) induced an average release of 61% and 86% above spontaneous release, respectively.

6.3.2.3 The effect of Medihoney pre-incubation on the substance P, Compound 48/80 and Ca^{++} ionophore-induced mast cell degranulation

- LAD2 cells were incubated with 2, 4 and 6% concentrations of Medihoney in TB for 20 minutes before 40-minute incubation with either substance P 1µM, Compound 48/80 10 ng/ml or Ca^{++} ionophore 1µM. Following tens of β-hexosaminidase assays, MH4% was observed to consistently induce around 90% inhibition of substance P-induced mast cell degranulation. This effect was reduced significantly to around 70% inhibition when the MH concentration was reduced to 2%, while increasing the concentration of MH to 6% did not yield any increase in the anti-inflammatory effect of MH (Figure 7 & 10).
- Similarly, regarding Compound 48/80, 4% MH pre-incubation induced maximal inhibition of degranulation averaging around 97%. Again, this effect dropped significantly to around 77% when the MH concentration was reduced to 2%, while increasing the concentration of MH to 6% did not yield any increase in the anti-inflammatory effect of MH (Figures 8 & 11).
- In the case of Ca^{++} ionophore, all of the three concentrations induced strong 97% inhibition of LAD2 cell degranulation (Figures 9 & 12).

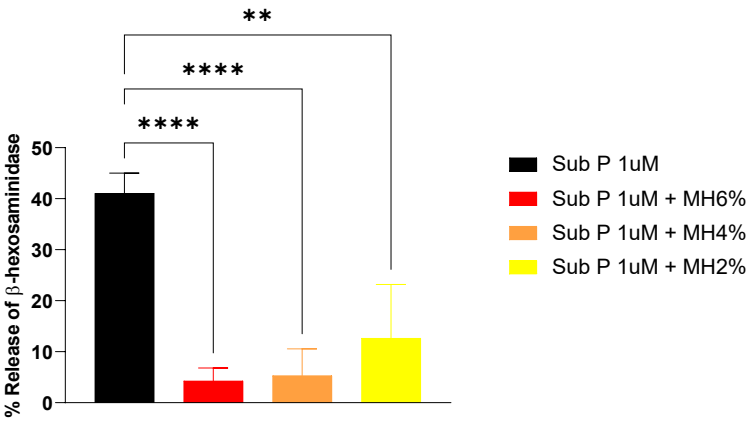

Figure 7: Percentage release of β-hexosaminidase by LAD2 cells upon 40-minute challenge with substance P 1µM alone or following 20-minute pre-incubation with Medihoney at 2, 4 and 6% concentrations. Statistical analysis of the differences was performed by Kruskal-Wallis test using GraphPad Prism 9 software. Each graph represent the mean ± standard deviation. The Graph represent the results of 5 independent assays each performed in triplicates (** P value < 0.005, **** P value<0.0001) (n=5).

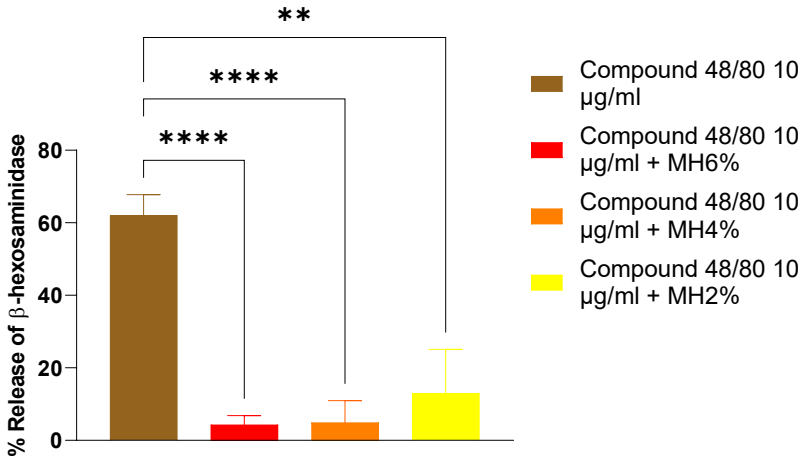

Figure 8: Percentage release of β-hexosaminidase by LAD2 cells upon 40-minute challenge with Compound 48/80 10 ng/ml alone or following 20-minute pre-incubation with Medihoney at 2, 4 and 6% concentrations. Statistical analysis of the differences was performed by Kruskal-Wallis test using GraphPad Prism 9 software. Each graph represent the mean ± standard deviation. The Graph represent the results of 3 independent assays each performed in triplicates (** P value < 0.01, **** P value<0.0001) (n=3).

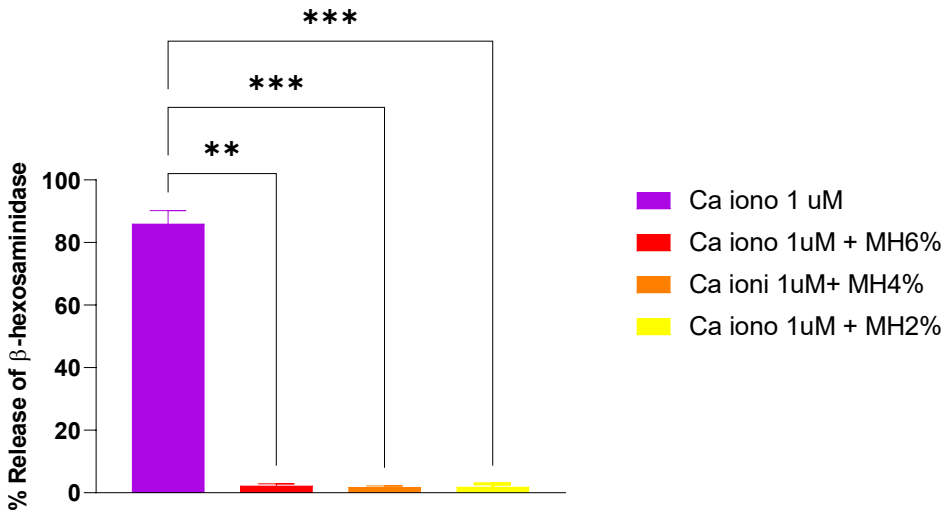

Figure 9: Percentage release of β-hexosaminidase by LAD2 cells upon 40-minute challenge with Ca^{++} ionophore alone or following 20-minute pre-incubation with Medihoney at 2, 4 and 6% concentrations. Statistical analysis of the differences was performed by Kruskal-Wallis test using GraphPad Prism 9 software. Each graph represent the mean ± standard deviation. The Graph represent the results of 3 independent assays each performed in triplicates (** P value < 0.01, *** P value <0.0005) (n=3).

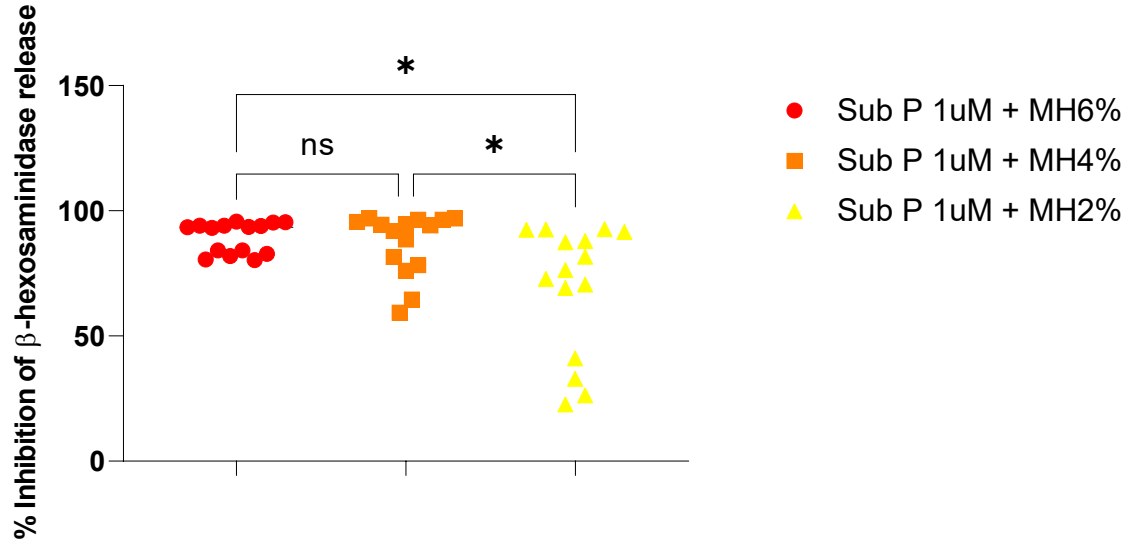

Figure 10: Scatter plot represents the percentage inhibition of substance P-induced LAD2 cell degranulation upon 20-minute pre-incubation with 2, 4 and 6% Medihoney. Statistical analysis of the differences was performed by Kruskal-Wallis test using GraphPad Prism 9 software. The Graph represent the results of 3 independent assays each performed in triplicates (* P value < 0.05) (n=3).

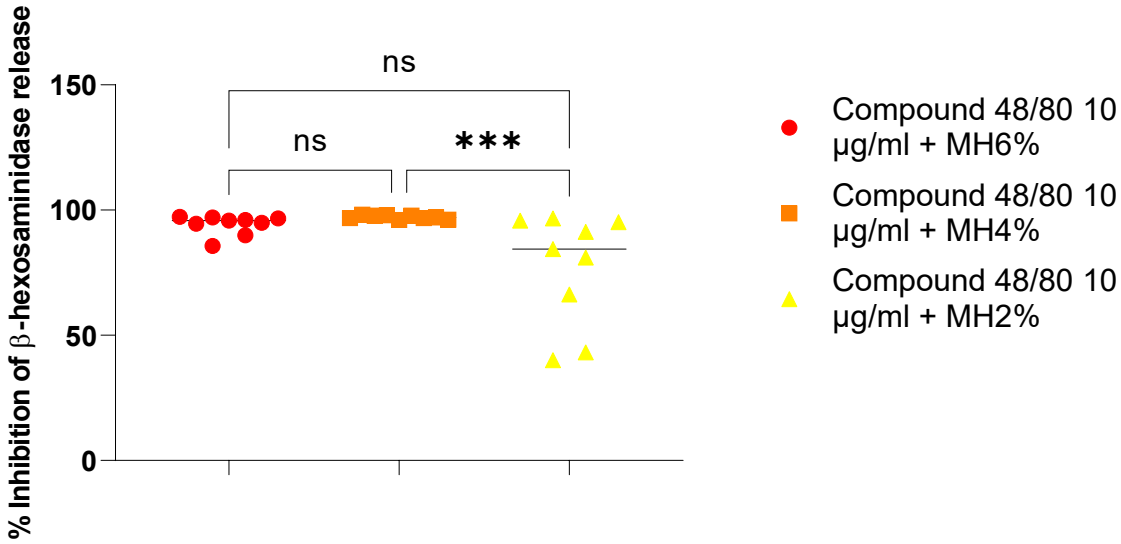

Figure 11: Scatter plot represents the percentage inhibition of the compound 48/80-induced LAD2 cell degranulation upon 20-minute pre-incubation with 2, 4 and 6% Medihoney. Statistical analysis of the differences was performed by Kruskal-Wallis test using GraphPad Prism 9 software. The Graph represent the results of 3 independent assays each performed in triplicates (* P value < 0.05) (n=3).

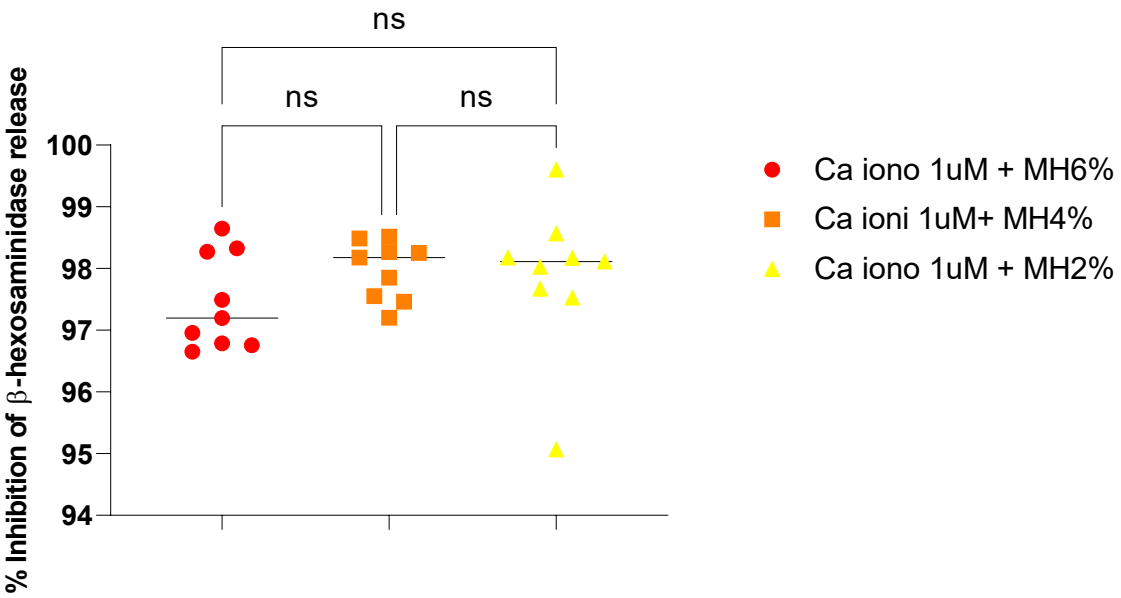

Figure 12: Scatter plot represents the percentage inhibition of the Ca^{++} ionophore-induced LAD2 cell degranulation upon 20-minute pre-incubation with 2, 4 and 6% Medihoney. Statistical analysis of the differences was performed by Kruskal-Wallis test using GraphPad Prism 9 software. The Graph represent the results of 3 independent assays each performed in triplicates (* P value < 0.05) (n=3).

6.3.2.4 The effects of the substance P blockers QWF and L-733,060 on the substance P-induced Mast cell degranulatio

Pre-incubation of LAD2 cells with the tripeptide QWF, a non-selective blocker of the substance P receptors NK-1 and MRGPRX2, resulted in a dose dependant inhibition of substance P-induced β-hexosaminidase release. QWF 100 µM induced 60-70% inhibition of mast cell degranulation following substance P 1µM challenge, while both 10 and 5 µM concentrations failed to produce the same effect (figure 13).

The neuokinin-1 receptor blocker L-733,060 inhibited mast cell degranulation by 70 % when used in 100 µM concentration, while 10 µM concentration only induced 13% inhibition (figure 14).

It is worth mentioning that both substance P blockers were weaker than Medihoney, which inhibited the substance P-induced mast cell degranulation by 89% and 87% using 4 and 6% concentrations, respectively.

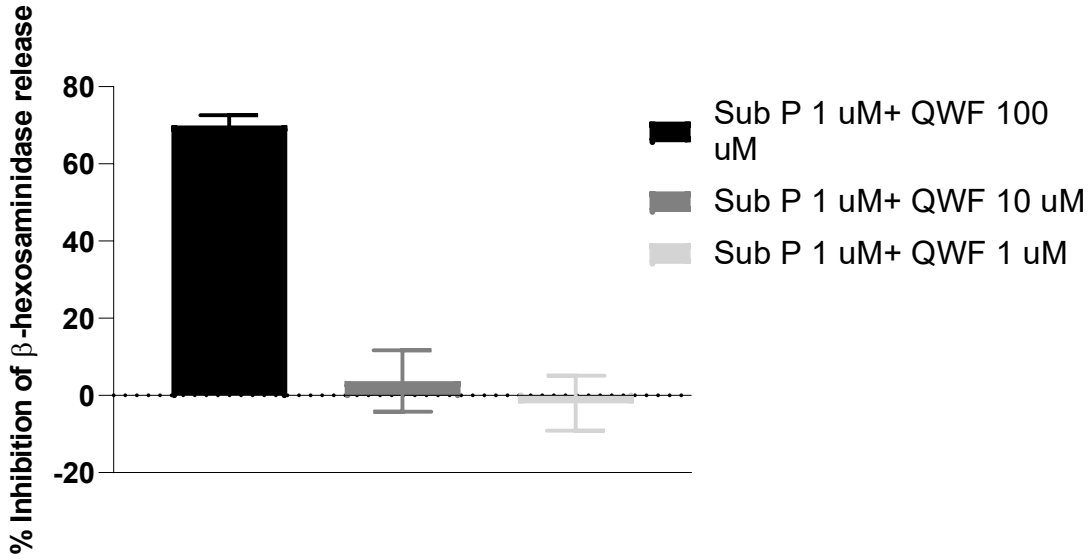

Figure 13: The effect of the substance P blocker QWF on the substance P-induced β-hexosaminidase release (n=3).

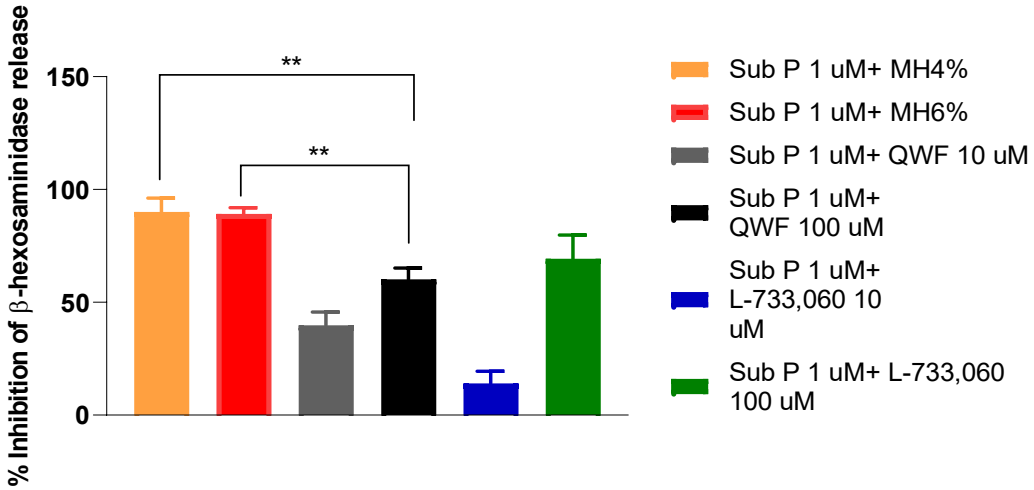

Figure 14: The Inhibition of mast cell degranulation induced by 40-minute challenge with substance P 1µM, through 30-minute pre incubation with MH (4 and 6%), and the substance P blockers (QWF and L-733,060) (**P 0.005) (n=3).

6.3.2.5 The effect of the selective Inhibition of Protein kinase B (Akt) on the substance P-induced mast cell degranulation

In this experiment, LAD2 cells were pre-incubated for 20 minutes with the selective Akt inhibitor, 10-DEBC hydrochloride, before a 40-minute challenge with substance P, Ca^{++} ionophore 1µM or Compound 48/80 10 µg/ml. The percentage (Net) release of β-hexosaminidase was maximally inhibited (P value> 0.0005) when the cells were pre-incubated with 30 and 20 µM of 10-DEBC hydrochloride, yielding 77% and 57% inhibition, respectively. 10 µM induced significant inhibition only in Ca^{++} ionophore-induced degranulation, while the lower 5 and 1µM failed to inhibit the degranulation (See figures 15, 16 & 17).

Given the previous findings, these results suggest that the inhibition of substance P-induced Akt activation and phosphorylation by the Medihoney pre-incubation (See chapter 3), is the mechanism by which Medihoney inhibits mast cell degranulation.

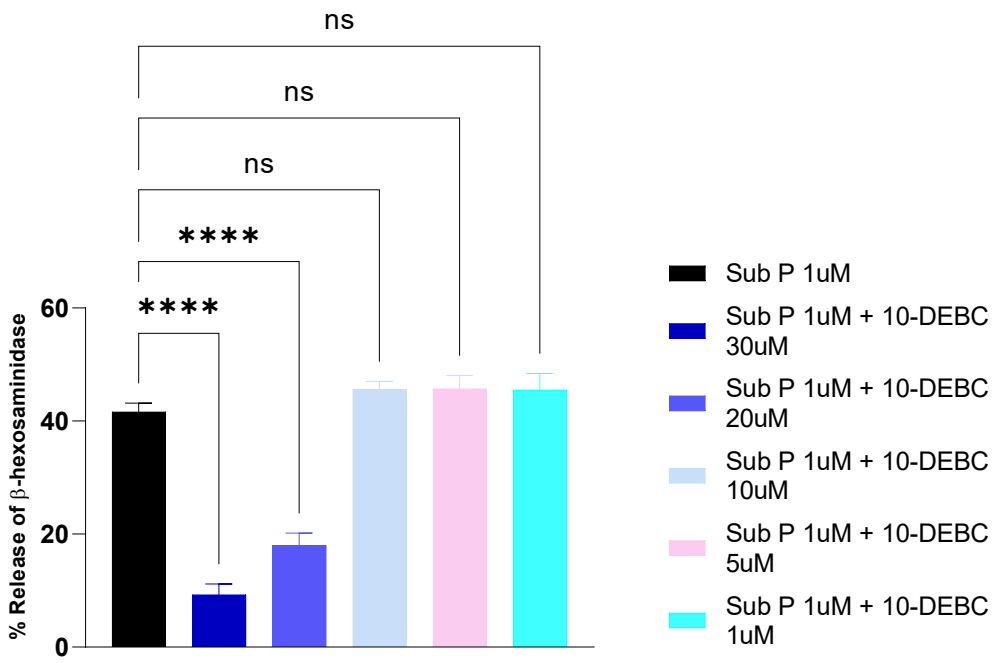

Figure 15: The effect of the Akt inhibitor 10-DEBC HCL on substance P-induced mast cell degranulation. Error bars represent means ± standard error means. The graph represent the results of three independent assays, each performed in triplicates. Statistical analysis was performed by one-way ANOVA using GraphPad Prism 9 software (**** P <0.0005, n=3).

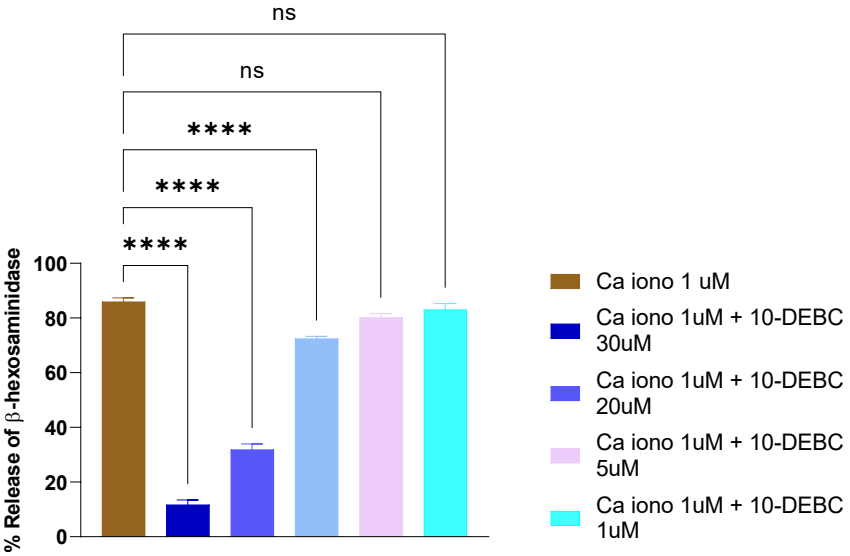

Figure 16: The effect of the Akt inhibitor 10-DEBC HCL on the Ca^{++} ionophore-induced mast cell degranulation. Error bars represent means ± standard error means. The graph represent the results of three independent assays, each performed in triplicates.

Statistical analysis was performed by one-way ANOVA using GraphPad Prism 9 software (**** P <0.0005, n=3).

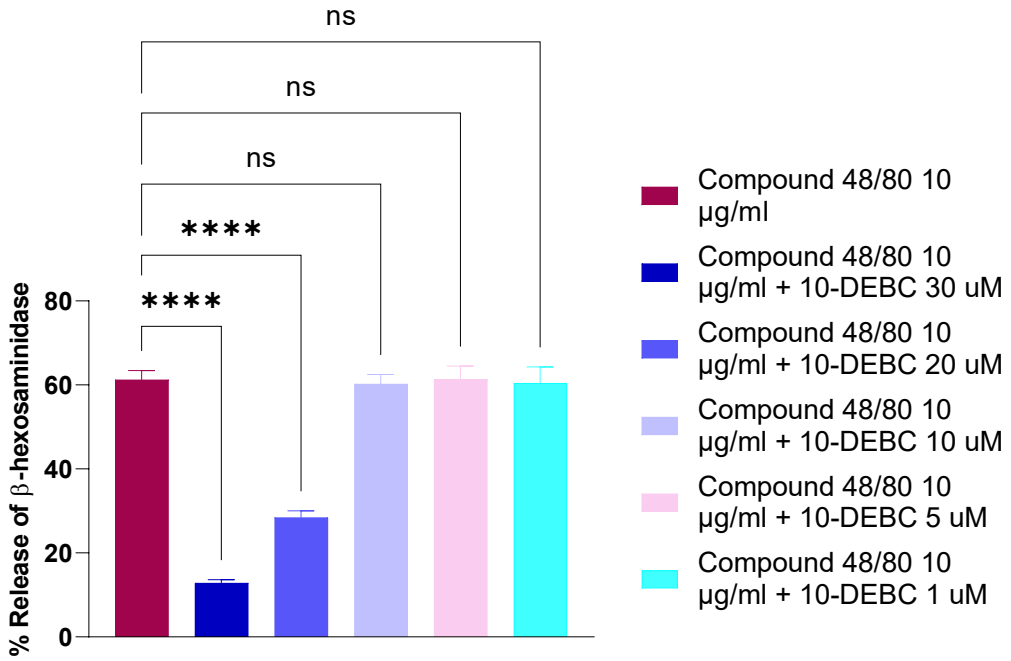

Figure 17: The effect of the Akt inhibitor 10-DEBC HCL on the Compound 48/80-induced mast cell degranulation. Error bars represent means ± standard error means. The graph represent the results of three independent assays, each performed in triplicates. Statistical analysis was performed by one-way ANOVA using GraphPad Prism 9 software (**** P <0.0005, n=3).

6.3.2.6 The effects of the selective ERK1/2 U0126 inhibitor on mast cell degranulation

In this experiment, LAD2 cells were pre-incubated for 20 minutes with the selective ERK1/2 inhibitor, U0126, before a 40-minute challenge with substance P 1µM, Ca^{++} ionophore 1Mm or Compound 48/80 10 µg/ml. With all secretagogues, β-hexosaminidase release was maximally inhibited (P value> 0.0005) when LAD2 cells were pre-incubated with 30 and 20 µM of U0126, producing 46% and 24% inhibition, respectively. The lower 10 and 5 µM only inhibited the Ca^{++} ionophore – induced degranulation, while 1µM failed to inhibit degranulation induced by all secretagogues (See figures 18, 19 & 20).

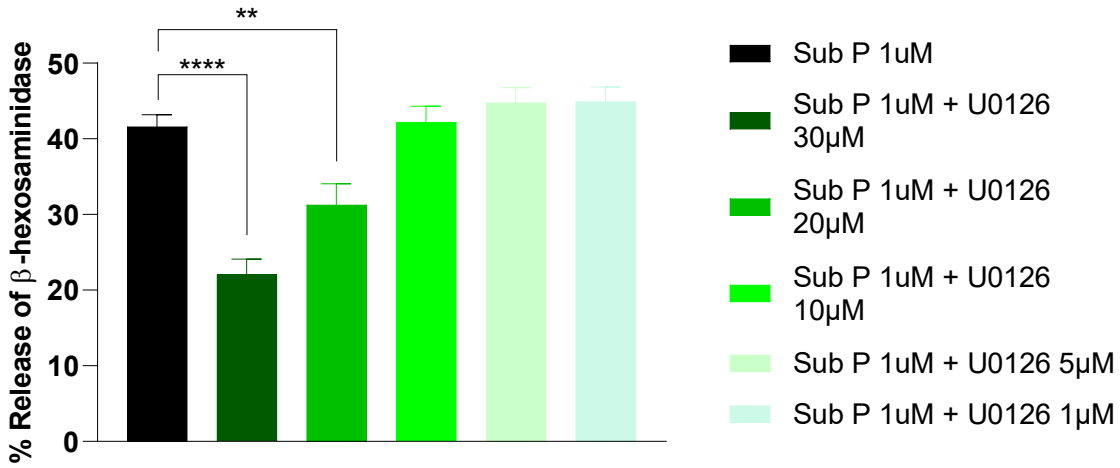

Figure 18: The effect of the ERK1/2 inhibitor U0126 on the substance P-induced mast cell degranulation. Error bars represent means ± standard error means. The graph represent the results of three independent assays, each performed in triplicates. Statistical analysis was performed by one-way ANOVA using GraphPad Prism 9 software (** P <0.005, **** P <0.0001, n=3).

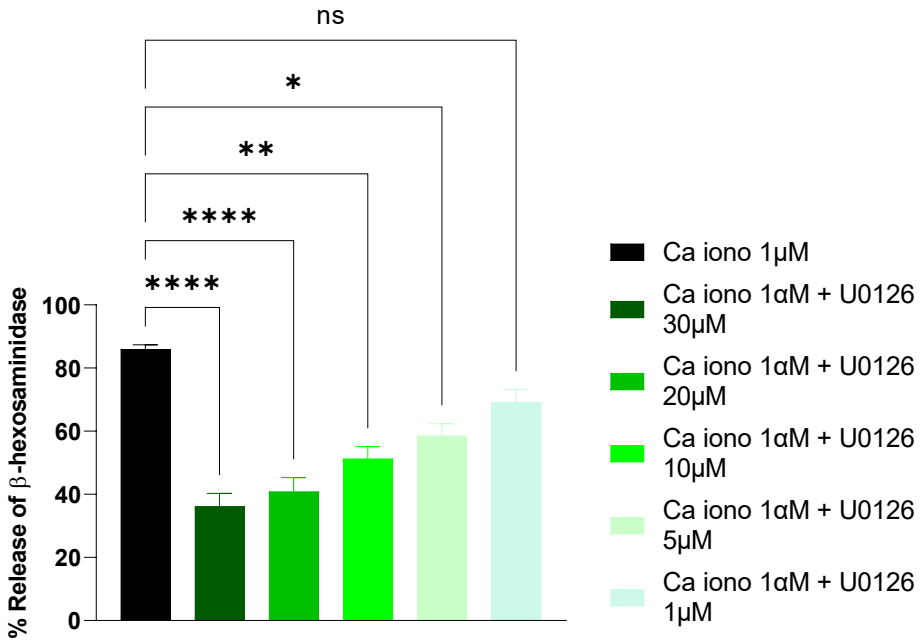

Figure 19: The effect of the ERK1/2 inhibitor U0126 on the Ca^{++} ionophore-induced mast cell degranulation. Error bars represent means ± standard error means. The graph represent the results of three independent assays, each performed in triplicates. Statistical analysis was performed by Kruskal-Wallis test using GraphPad Prism 9 software (* P <0.05, ** P <0.005, **** P <0.0001, n=3).

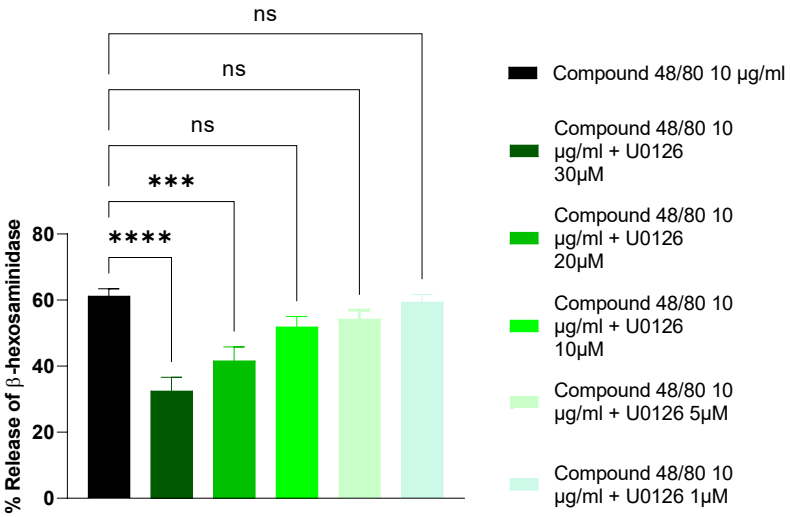

Figure 20: The effect of the ERK1/2 inhibitor U0126 on the Compound 48/80-induced mast cell degranulation. Error bars represent means ± standard error means. The graph represent the results of three independent assays, each performed in triplicates. Statistical analysis was performed by one-way ANOVA using GraphPad Prism 9 software (*** P <0.0005, **** P <0.0001, n=3).

6.3.2.7 The effect of the sugar-free Manuka extract on degranulation in LAD2 cells

Similar to Medihoney, pre-incubation of the LAD2 cells with the Manuka extract induced a dose-dependent significant inhibition of degranulation induced by substance P 1µM or Compound 48/80 10 µg/ml, culminating at 70% inhibition at 4% concentration.

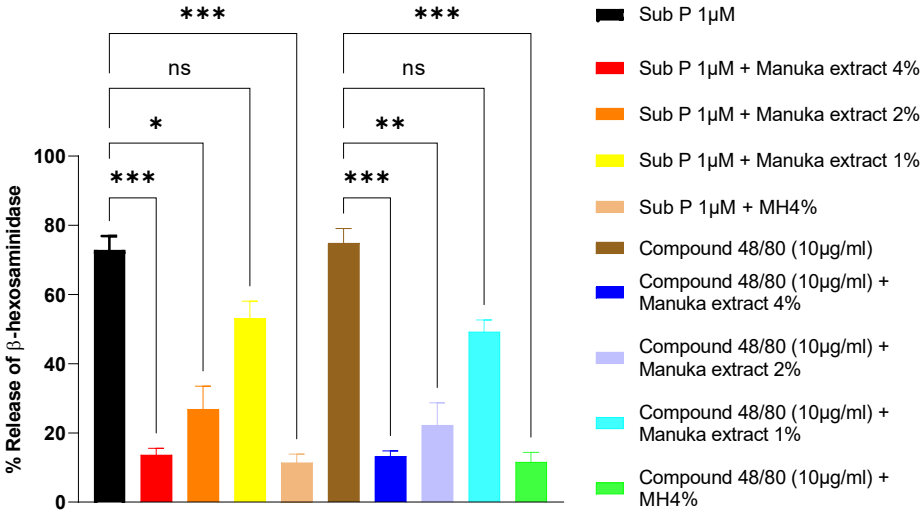

Figure 21: The effect of the Manuka honey extract on LAD2 cell degranulation induced by Substance P and Compound 48/80-induced mast cell degranulation. Error bars represent means ± standard error means. The graph represents the results of three

independent assays, each performed in triplicates. Statistical analysis was performed by Kruskal-Wallis test using GraphPad Prism 9 (*** P <0.0005, **** P <0.0001, n=3).

6.4 The effect of Medihoney pre-treatment on the substance P-induced release of pro-inflammatory cytokines and chemokines by LAD2 mast cells

6.4.1 Methodology

We studied the effect of Medihoney 20-minute pre-treatment on the pro-inflammatory cytokines and chemokines produced by LAD2 cells upon 24-hour challenge with the neuropeptide substance P or Ca^{++} ionophore (as a positive control) (Described in the next page). The rationale for this was that such an approach might provide further evidence supporting the validity of Medihoney as a therapeutic anti-inflammatory agent in BPS/IC patients.

The whole methodology is very similar to the previous experiment (LAD2 cell degranulation assay), except for the following: The number of LAD2 cells used was 4 million cells/well. This large number aims at obtaining the maximum possible levels of cytokines in the cell culture medium (Theoharides *et al.*, 2010), especially as LAD2 cells do not tend to produce generous amounts of cytokines and chemokines upon activation. The de novo synthesis of cytokines and chemokines requires a period of 24 hours from the activation process (adding substance P) until measurable levels of these proteins can be detected in the cell culture supernatant (Castellani *et al.*, 2009b).

Cells were incubated with substance P 1µM or Ca^{++} ionophore 1µM for 24-hours, with or without 20-minute with MH4% or the Akt inhibitor 10-DEBC HCL 30 µM at 5% CO_2 and 37°C). Following the 24-hour incubation period, the cell culture plate was centrifuged for 10 minutes at 4°C and 225x g To quantify the pro-inflammatory cytokines and chemokines in the cell culture supernatant, two highly sensitive and specific cytokine and chemokine quantification electroluminescent kit from MSD, USA were used. The first kit is an electroluminescent Human V-PLEX MCP-1 kit that quantifies the cytokine CCL2 (MCP-1). The other kit is a multi-analyte U-PLEX electroluminescent kits (Human U-PLEX Group 1, MSD, USA). Such kit simultaneously quantifies the cytokines IL-1β, TNF-α, IL-6, IL-17A, and the chemokines IL-8, MCP-1, GM-CSF, MIP-1α, IP-10, I-TAC and CXCL-1.

Statistical analysis of the results was performed by one-way ANOVA using GraphPad Prism 9. It is worth mentioning that the 2 assays, the V-PLEX and U-PLEX, were performed using different batches of LAD2 cells, which might provide an explanation of the differences in the results between the 2 assays.

Table 7: Constituent of each well of the cell culture plate in case of studying the effect of MH4 % pre-treatment on the substance P-induced cytokine and chemokine release. MH4 % can be replaced by any other drug, for example QWF protein, so we can study the effect of any proposed drug on the substance P-induced cytokine release.

	Constituent per well			
CONDITIONS	Cell suspension (4 million cells/160 µL)	MH 4%	TB	Substance P 1 µM
Spontaneous	160 µL	0	40 µL	0
MH4% alone	160 µL	20 µL	20 µL	0
Substance P 1µM alone	160 µL	0	20 µL	20 µL
Substance P 1 µM + MH4%	160 µL	20 µL	0	20 µL

6.4.2 Results

6.4.2.1 The single analyte human MCP-1 V-PLEX assay

- In this assay, Medihoney alone induced a significant decrease in the levels of the cytokine MCP-1 in the cell culture supernatant below the spontaneous levels ($P < 0.05$).

- Both substance P 1µM and Ca^{++}ionophore 1µM 24-hour challenge induced statistically significant increase in the release of MCP-1 in the LAD2 cell culture supernatant when compared to the untreated control levels ($P < 0.05$).
- MH4% pre-incubation significantly dropped the substance P and Ca^{++}ionophore-induced release of MCP-1 ($P< 0.0005$ and 0.005, respectively) (n=2) (figure 22).

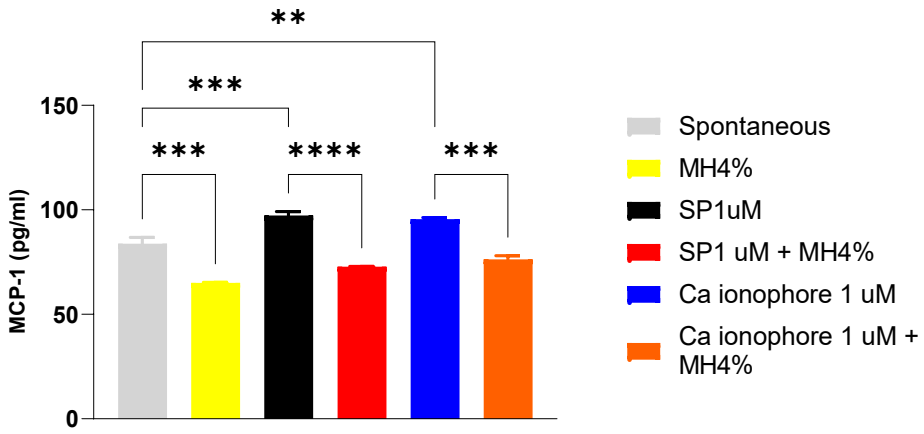

Figure 22: The levels of MCP-1 released by LAD2 cells following 24-hour challenge with Substance P and Ca ionophore with or without 20-minute pre-incubation with MH4%. Experiment was repeated twice. Statistical analysis was performed by one-way ANOVA using GraphPad Prism 9 software (** $p<0.005$, *** $P< 0.0005$, **** $p< 0.0001$, n=3).

6.4.2.2 The multi-analyte Human U-PLEX assay

- Despite using 4 million cells per well, out of the previously mentioned 10 pro-inflammatory cytokines and chemokines, only 4 analytes were above the limit of detection. These were the chemokines IL-8, GM-CSF and MCP-1, in addition to the cytokine IL-1 β.
- It is worth mentioning that the by the time we started to do this assay, we ran out of the raw medical-grade Manuka honey (Medihoney batch# 405), which has been used for both degranulation and intracellular signalling assays. Accordingly, we had to use another Medihoney batch (#395), which is specifically processed and wax added to give it a creamy consistency.
- Medihoney 4% induced a statistically significant drop in both the spontaneous and the induced release of the cytokines GM-CSF and IL-8 with both SP and Ca^{++} ionophore challenge, while 10-DEBC induced significant reduction of the Ca^{++} ionophore-induced release of IL-8 and GM-CSF.

- On the contrary, MH4% pre-incubation induced statistically significant increase in both the spontaneous and the induced release of the cytokines MCP-1 and IL-1β with both SP and Ca^{++} ionophore challenge (see figure 23 & 24).

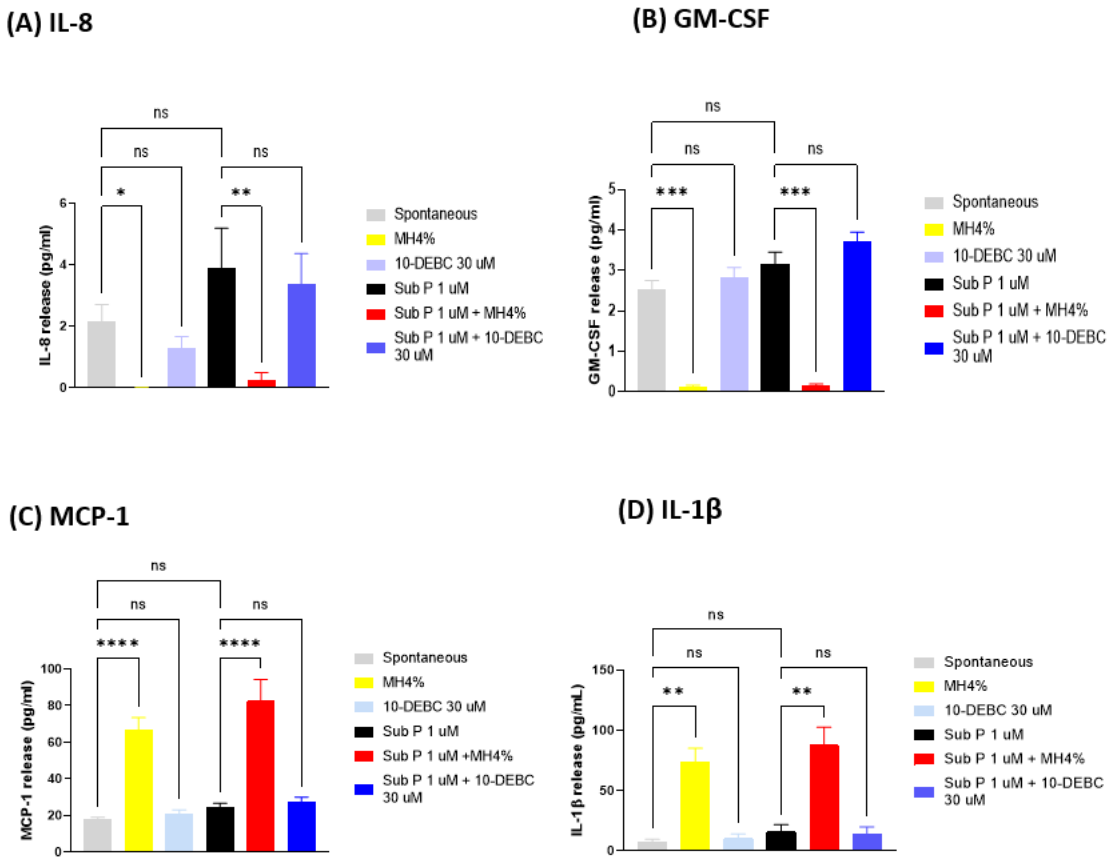

Figure 23: A, B, C and D represent the LAD2 cell expression of the cytokines IL-8, GM-CSF, MCP-1 and IL-1β, respectively, following 24-hour substance P 1µM challenge with or without Medihoney or 10-DEBC HCL pre-incubation. Each experiment was repeated three times where each condition was done in duplicates. Statistical analysis was performed by one way ANOVA and Kruskal-Wallis tests using GraphPad Prism 9 software (* P value <0.05, ** P value <0.005, *** P value <0.0005, **** P value <0.0001 n=3). The kit lower detection limit is 0.01 pg/ml for all the 4 analytes.

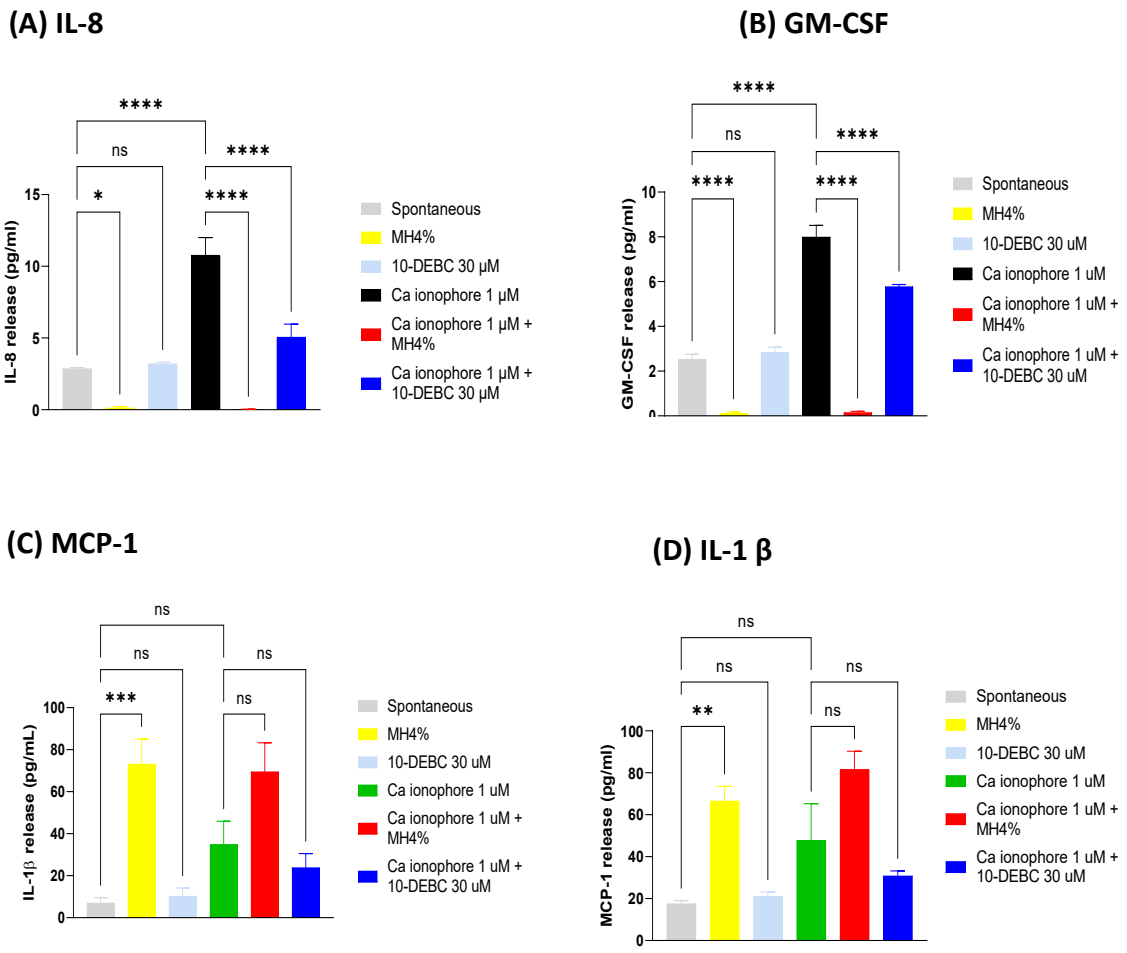

Figure 24: A, B, C and D represent the LAD2 cell expression of the cytokines IL-8, GM-CSF, MCP-1 and IL-1β, respectively, following 24-hour Ca^{++} ionophore 1µM challenge with or without Medihoney or 10-DEBC HCL pre-incubation. Experiment was repeated three times where each condition was done in duplicates. Statistical analysis was performed by one way ANOVA and Kruskal-Wallis tests using GraphPad Prism 9 software (* P value <0.05, ** P value <0.005, *** P value <0.0005, **** P value <0.0001 n=3). The kit lower detection limit is 0.01 pg/ml for all the 4 analytes.

6.5 The effect of Medihoney or the sugar-free Manuka extract on the viability of the LAD2

6.5.1 Methodology

Lactate dehydrogenase (Schmitz *et al.*) is an oxidoreductase enzyme that catalyses the interconversion of pyruvate and lactate. Cells release LDH into the bloodstream after tissue damage or red blood cell haemolysis. Since LDH is a fairly stable enzyme, it has

been widely used to evaluate the presence of damage and toxicity of tissue and cells. The effect of the various concentrations of Medihoney and sugar free Manuka extract on the viability of the LAD2 cells was assessed by quantification of the lactate dehydrogenase assay in the cell culture supernatant using a sensitive lactate dehydrogenase (Schmitz *et al.*) activity assay (MAK066, Sigma) as follows:

6.5.1.1 Sample preparation

LAD2 cells were suspended in Tyrode's buffer and placed in 96-well cell culture plate at a density of 200,000 cells per well. Similar to β-hexosaminidase assay, LAD2 cells were challenged with substance P 1µM for 40 minutes at 5% CO2 and 37°C with or without 20-minute pre-incubation with various concentrations of Medihoney and Manuka honey extract, and 30µM 10-DEBC HCL as previously described. After challenge, the plates were centrifuged for 10 minutes at 2000 rpm and 4°C, and 50 µL of each supernatant were transferred to the corresponding well of a 96-well assay plate.

6.5.1.2 Standard preparation

0, 2, 4, 6, 8, and 10 µL of the 1.25 mM NADH Standard were added in duplicates into the 96 well plate, then, the volume in each well was topped up to 50 µL using the LDH assay buffer, generating 0 (blank), 2.5, 5, 7.5, 10, and 12.5 nmole/well of the standards, respectively.

6.5.1.3 Addition of the substrate (Master Mix reaction)

50 µL of the master reaction mix (2 µL of the LDH substrate + 48 µL of LDH assay buffer) were added to each well.

6.5.1.4 Plate reading

Finally, plates were read at 450 nm wave length after 2 minutes ($T_{Initial}$), then the incubated at 37°C for 12 minutes, followed by final reading at 450 nm (T_{Final}).

6.5.1.5 Calculation of the LDH activity (cytotoxicity)

The LDH activity of each sample may be determined by the following equation:

LDH Activity = B × Sample Dilution Factor /(Reaction Time × V)

B = Amount (nano mole) of NADH generated between the initial and final readings (T_{Final} - $T_{Initial}$).

Reaction Time = 12 – 2 = 10 minutes. **V** = sample volume added to well in ml (0.05 ml). LDH activity is reported as nano mole/min/ml = milliunit/ml.

6.5.2 Results

- A total of one-hour incubation of the LAD2 cells with the variable concentrations of Medihoney did not induce any significant rise in the LDH activity, indicating no cytotoxic effect on LAD2 cells (Figure 23).
- Similarly, substance P 1µM with or without pre-incubation with all the MH and Manuka extract concentrations did not induce any cytotoxic effect on the LAD2 cells (Figures 25 & 26).
- Interestingly, the Akt inhibitor 10-DEBC HCL 30 µM induced strong cytotoxic effect on LAD2 cells (Figures 23&24). This finding indicates that 10-DEBC may inhibit LAD2 cell degranulation via a cytotoxic effect rather than inhibition of Akt phosphorylation.

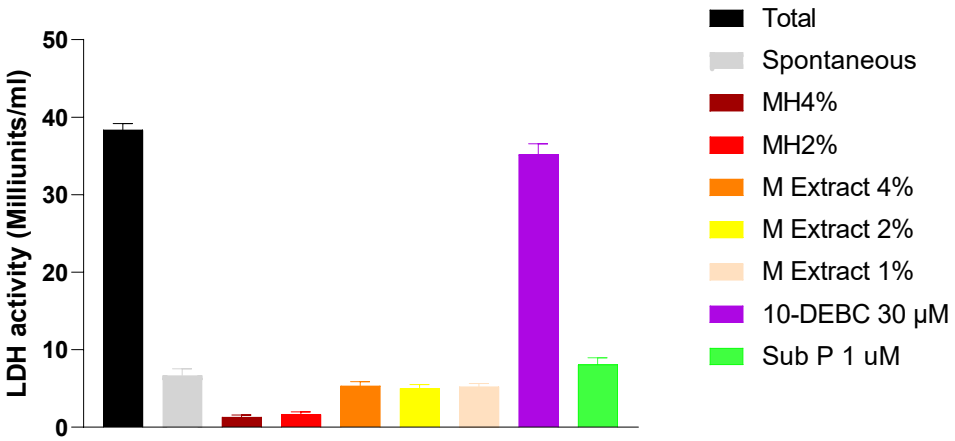

Figure 25: The lactate dehydrogenase activity of LAD2 cells upon one-hour challenge with various concentrations of Medihoney (2 and 4%), Manuka extract (1, 2 and 4%), in addition to 10-DEBC 30 µM or substance P 1 µM.

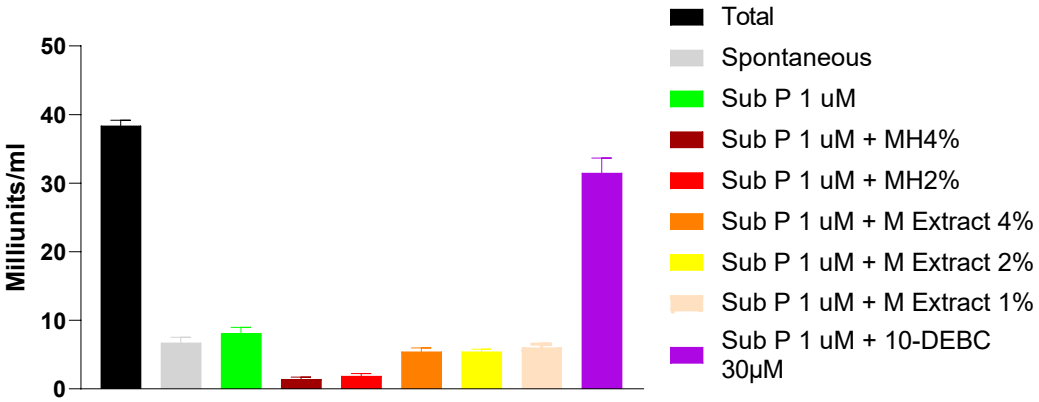

Figure 26: The lactate dehydrogenase activity of LAD2 cells upon one-hour challenge with substance P 1 µM with or without pre-incubation with various concentrations of Medihoney (2 and 4%), Manuka extract (1, 2 and 4%), in addition to 10-DEBC 30 µM.

6.6 Discussion

Medihoney displayed strong anti-inflammatory properties through inhibition of mast cell degranulation. Medihoney at various concentrations did not induce significant mast cell degranulation above spontaneous levels and MH at 4 % concentration strongly inhibited substance P, Ca^{++} ionophore and Compound 48/80-induced mast cell degranulation, while the 6% concentration did not display any advantage above 4%, and the 2% concentration did not induce consistent effect.

The inhibition of the substance P and Compound 48/80-induced mast cell degranulation by Medihoney indicates that Medihoney antagonizes the MRGPRX2 signalling pathway underlying mast cell degranulation. However, the fact that Medihoney inhibits Ca^{++} ionophore-induced degranulation might indicate that Medihoney also inhibits degranulation through the inhibition of the cell signalling pathways commonly utilised by secretagogues which induce mast cell degranulation. This might provide an advantage for Medihoney as a multimodal mast cell stabilizer valid for the treatment of various mast cell related chronic inflammatory conditions.

Nevertheless, Medihoney pre-incubation strongly and significantly inhibited both the substance P and Ca^{++} ionophore-induced expression of the chemokines IL-8 and GM-CSF, however, Medihoney increased the expression of the cytokine IL-1β.
Strangely, the effect of Medihoney on the expression of MCP-1 was contradictory between the two assays. Such may be explained by the fact that the 2 assays were performed using two different batches of Medihoney. In more detail, in the first cytokine assay testing MCP-1, the honey used was a raw gamma-irradiated Medical grade Manuka honey (Medihoney batch #405), which is the same batch as used in degranulation and signalling studies. In this assay, Medihoney strongly inhibited MCP-1 release. In the second U-PLEX cytokine assay, due to supply shortages, the honey used is Medihoney batch#395, which is a specifically processed Medihoney with wax addition

to obtain a different creamy consistency, which was associated with enhanced MCP-1 release. Such difference might give an explanation to the controversial results.

In addition, Medihoney has unstable constituents, which might gradually transform into other compounds with time, a factor that might affect its anti-inflammatory properties. These compounds include dihydroxyacetone (DHA), which gradually transforms into methylglyoxal (MGO), a major anti-bacterial constituent of Manuka honey (Girma, Seo and She, 2019).

Generally speaking, the LAD2 cell line is a very good model for studying mast cell degranulation, but less so for the expression of de novo synthesized cytokines and chemokines, as they do not produce generous amounts of these cytokines (Gaudenzio *et al.*, 2016). Moreover, a report from our lab shows that when the same batch of LAD2 cells were activated with compound 48/80 in an in-vitro urinary bladder co-culture model (LAD2 + primary human urothelial and fibroblast cells), the Medihoney batch #405 significantly inhibited IL-6 and IL-8 production induced by the LAD2 cell degranulation (Yusuh *et al.*, 2021). Nonetheless, the inhibition of substance P-induced phosphorylation of Akt (serine 473), ERK1/2 and STAT3 (serine 727) (See chapter 3) are strong indicators in favour of a reduction rather than an increase in the pro-inflammatory cytokine release (Gao *et al.*, 2001; Kim, Rådinger and Gilfillan, 2008; Blatt *et al.*, 2012; Siegel *et al.*, 2013; Qin *et al.*, 2016; Bao *et al.*, 2018).

The Akt inhibitor 10-DEBC HCL (Akt inhibitor X) inhibited mast cell degranulation in a dose dependant fashion culminating in a strong 90% inhibition at 30 µM concentration, while it had no effect on the cytokine release. Interestingly, such effect was associated was strong cytotoxic effect on the LAD2 cells as evident by enhanced lactate dehydrogenase release, which may indicate that 10-DEBC inhibits degranulation by via its cytotoxic effect rather than via a modulatory effect on Akt phosphorylation.

On the contrary, the ERK inhibitor FR18084 failed to inhibit mast cell degranulation, indicating that mast cell degranulation is dependent on Akt phosphorylation. 10-DEBC HCL at 30 µM inhibited the Ca^{++} ionophore, but not the Substance P, induced release of

GM-CSF and IL-8, indicating differences in the signalling pathways underlying the cytokine release in LAD2 cells between substance P and Ca^{++} ionophore challenge. This is consistent with recent reports highlighting the differences in both the cytokine release pattern (Gaudenzio *et al.*, 2016) and the underlying signalling pathways (Gilfillan and Tkaczyk, 2006; Gilfillan, Austin and Metcalfe, 2011) upon activation of mast cells with different secretagogues.

From the studies in this chapter, it was concluded that Medihoney at 4% concentration strongly and consistently inhibited mast cell degranulation. However, the effect of Medihoney on LAD2 cell pro-inflammatory cytokine release is variable with inhibition of some mediators and increase of others. However, these data needs to be confirmed using primary explanted human mast cells, which can produce much larger amounts of cytokines compared to LAD2 cells. Both mast cell models will be treated by Medihoney (batch#405 and the sugar-free Manuka extract).

6.6.1 LAD2 cell in vitro model as representative of the in vivo mast cell activity (Pros & cons)

LAD2 cells are very well established as a representative and reliable model for studying mast cell responses in vitro (Kirshenbaum *et al.*, 2003; Kirshenbaum *et al.*, 2014; Kirshenbaum *et al.*, 2019; Lazki-Hagenbach, Ali and Sagi-Eisenberg, 2021). However, this famous model does not come with limitations both in their lab use and their informative outcomes. For example, in degranulation assays, LAD2 are very sensitive and can be easily activated during the various steps of the assay starting from dislodging the cell clumps in the cell culture flask, repeated washing in Pbs 1X, and the transfer into the assay 96-well plates. Moreover, with time, LAD2 cells tend to increase their clumping, which is accompanied by weakened or even lost responsiveness to various secretagogues (Radinger *et al.*, 2010). We were able to overcome these issues by always maintaining the cell count between 2.5-5 * 10^5 cells /ml and repeated dislodging of the LAD2 cells by repeated pipetting against the bottom of the flask at least twice a week. In addition, at the beginning of the study, we had to repeat degranulation assays for several times as LAD2 cells were easily activated during the cell preparation before adding the mast cell secretagogues. This strongly undermined the obtained results and makes it hard to analyse the mast cell stabilising potentials of the studies Manuka derivatives. However, the repeated practice allowed us to refine the procedures, which

resulted in an average spontaneous release of 6.7 % ± 0.09 % of the total amount of β-hexosaminidase enzyme in LAD2 cells.

Moreover, despite that in vitro study of mast cell degranulation using LAD2 cells is a valid and well-established model to study the potential anti-inflammatory properties of various agents, such mono cellular model may not necessarily reflect the full in vivo picture. For example, as described earlier in chapter one, mast cells are not the only factor involved in tissue inflammation in BPS/IC. For example, the injured urothelial cells release the cytokine stem cell factor (SCF), which is responsible for chemoattraction and maturation of the bladder mast cells in BPS/IC (Ke and Kuo, 2015). At the same time, the activated mast cells release the proteases tryptase and chymase, which lyse the urothelial cells leading to more SCF release (Choi *et al.*, 2016). In addition, tryptase desensitises the sensory nerve endings resulting in upregulation of the bladder sensory nerve endings and substance P release (Patnaik *et al.*, 2017a). Furthermore, substance P is not the only secretagogue inducing mast cell activation in IC/BPS, and several other factors might affect and potentially induce mast cell degranulation. These include intrinsic factors such as human β-defensins, eosinophil derived Major basic protein and eosinophil peroxidase, and IL-33 (cytokine release) (Patnaik *et al.*, 2017a), or extrinsic factors such as bacterial toxins and allergen-IgE complex (Gregory and Brown, 2006; Galli and Tsai, 2012; Jhang and Kuo, 2015).

Importantly, other cellular elements are involved in the pathophysiology of IC/BPS. These elements include structural cells of the urinary bladder, such as the urothelial cells, interstitial cells of Cajal and the detrusor smooth muscle cells (Keay *et al.*, 2004; Graham E. and C., 2006; Hurst, Moldwin and Mulholland, 2007; Parsons, 2007; Shie and Kuo, 2009; Parsons, 2011; Janssen *et al.*, 2013; Lee and Lee, 2013; Marentette *et al.*, 2013; Lee and Lee, 2014; Hurst *et al.*, 2015; Choi *et al.*, 2016; Chavda *et al.*, 2017). Nevertheless, immune cells other than mast cells are involved in the pathophysiology of chronic inflammation in IC/BPS. These include eosinophils, macrophages and T-lymphocytes. Similar to mast cells, the numbers of these immune cells are significantly elevated in the bladder tissues of IC/BPS patients, indicating their involvement in the pathophysiology of the IC/BPS-related inflammation (Bouchelouche *et al.*, 2001; Dodd and Tello, 2011).

Based on the above, the monocellular in vitro mast cell model may be indicative of the potential anti-inflammatory properties of Medihoney and the Manuka honey extract, but the information can be further completed via involving more complex in vitro co-culture models involving more than one cell line. Moreover, our main focus was to investigate and analyse the effect of honey on the neurogenic substance P-induced mast cell activation, but we did not cover mast cell activation induced by other secretagogues, which was left for the next stage of the study. Such stage was immediately started where we have developed a co-culture model of primary urinary bladder urothelial cells, fibroblasts and LAD2 cells. In such model, mast cell activation was induced by both substance P and Compound 48/80 induced bladder urothelial cytotoxicity, pro-inflammatory cytokine release and loss of the urothelial integrity (intercellular junctions) as assessed by TEER measurements. Interestingly, these effects were significantly blocked upon pre-incubation with Medihoney at 4% concentration (Yusuh *et al.*, 2022).

In addition, we are in the process of obtaining the LADR cells, an in vitro mast cell line recently developed by the laboratory of allergic diseases, NIH, US. Such cell line has stronger expression of the IgE receptor FcεRI compared to LAD2 cells, allowing for properly studying the effect of honey on the allergic mast cell responses, knowing that the recent LAD2 cell batches are very deficient in FcεRI expression. In addition, we have obtained staph aureus endotoxin and bacterial lipopolysaccharide (LPS) and another study will be launched to assess their effects on mast cell degranulation.

Regarding IgE-mediated mast cell degranulation, despite being known to express the IgE receptor FcεRI, recent batches of LAD2 cells did not response to the anti-IgE/IgE mediated activation. Excitingly, LAD2 cells were sensitised to respond to the typical IgE mediated allergic response following 2 weeks of priming with a combination of the th2 cytokines IL-4 and IL-13 at 10 ng/ml each. This was accompanied by a 40% β-hexosaminidase release, which was efficiently inhibited upon pre-incubation with MH4% (See chapter 5). Similarly, in these primed cells, the staph aureus δ-toxin induced 30% β-hexosaminidase release, which was strongly inhibited upon Medihoney 4% pre-incubation. However, it is expected that the effect of such priming will be transient, which makes this process clinically impractical as large amounts of the expensive IL-4 and IL-13 will be needed to maintain this effect.

Excitingly, using HEK-293 cells stably transfected to express the MRGPRX2 receptor, we have just demonstrated that Medihoney, but not the artificial honey (35% glucose and 35% fructose) inhibits the intracellular Calcium signalling upon MRGPRX2 activation by substance P (See chapter 5).

Importantly, we are planning to obtain a commercially available cell line transfected to express the protease -activated receptor 2 (PAR2). Such cell line is well designed to study the PAR2 receptor activation via the assessment of the post-activation β-arrestin-mediated receptor internalisation and Calcium signalling. This will allow us to assess the potential pain and inflammation -relieving properties of Medihoney via a potential inhibitory effect on the PAR2 receptors expressed by the bladder sensory nerve endings.

6.6.2 Active agents in Manuka honey responsible to its anti-inflammatory properties

The therapeutic properties of Manuka honey are diverse, and they include bactericidal, anti-inflammatory, tissue regeneration and wound healing. This plethora of Manuka honey properties can be attributed to the fact that Manuka honey is a collection of a wide range of nutritive agents, each of which has the potential to contribute to the medicinal properties of Manuka Honey (El-Senduny *et al.*, 2021). Despite tha crucial contribution of the honeybees to the formulation of honey in its form, the plant-derived components of honey seems to carry the major responsibility for its physiological effects. This assumption is supported by several facts as follows. Among different types of honey, Manuka honey is characterised by its high content of the plant-derived flavonoid, mainly luteolin (>0.05 mg/100 g honey). Of these flavonoids, pinobanksin, pinocembrin, chrysin, and luteolin account for 61% of its total flavonoid content. Quercetin, 8-methoxykaempferol, isorhamnetin, kaempferol, and galangin are present at much lower levels.

Phenolic acids are the second most abundant metabolites in Manuka honey after Flavonoids. Gallic acid is the prevalent followed by protocatechuic acid, syringic acid, p-hydroxy benzoic acid, 2,3,4 trihydroxybenzoic acid, gentisic acid, caffeic acid, 4-methoxyphenyllactic acid, and chlorogenic acid. These acids contribute to the acidic nature and, consequently, the anti-inflammatory properties of Manuka honey.

Additionally, methyl syringate and its glycoside leptosperin (formerly known as leptosin) are regarded as markers for MH from other uni-floral honeys (El-Senduny *et al.*, 2021). 3,6,7-trimethyl-2,4(1H,3H)-pteridinedione; lepteridine is a terideinedione unique to Manuka honey and currently has been proposed as a unique fluorescence marker for Manuka honey derivatives.

The fourth metabolite class that characterizes Manuka honey is 1,2-dicarbonyl compounds represented by glyoxal, 3-deoxyglucosulose, and methylglyoxal (MGO) (Fig. 1), a low-molecular-weight aldehyde. MGO is a distinctive chemical for MH that arises from non-enzymatic dehydration of dihydroxyacetone (DHA), a component of Manuka flower nectar (Adams, Manley-Harris and Molan, 2009), thus leading to a continuous increase in MGO levels during honey maturation.

Importantly, several of these plant-derived agents have well established mast cell stabilising properties. For example, flavonoids are well to exert potent anti-inflammatory properties via the inhibition of both mast cell (Park *et al.*, 2008; Weng *et al.*, 2015; Noshita *et al.*, 2018; Shaik *et al.*, 2018; Cordaro, Cuzzocrea and Crupi, 2020) and basophil degranulation (Wu *et al.*, 2022). Importantly, luteolin is one of the most abundant flavonoids in Manuka honey, and it has been strongly linked with the suppression of mast cell degranulation and pro-inflammatory cytokine release (Kempuraj *et al.*, 2008; Kritas *et al.*, 2013; Weng *et al.*, 2015).

7 Chapter Three: The Effect of Manuka Honey on the Signalling Pathways underlying Mast Cell Activation

7.1 Abstract

Mast cell numbers and activity have been positively associated with increased severity of tissue inflammation in many diseases, including Bladder Pain Syndrome. In these patients, the involved bladder sensory nerves release neuroactive substances, such as substance P, which activate mast cells, promoting inflammation. Medihoney®, a medical grade Manuka honey, has proven to inhibit substance P-induced mast cell degranulation. In order to explore the mechanism by which honey possesses this mast cell stabilizing effect, we have studied the effect of pre-treatment with Manuka Honey on the phosphorylation of a wide group of intracellular protein kinases, including mitogen-activated protein kinases (MAPK), following LAD2 cell (mast cell line) challenge with substance P.

Interestingly, 4% Medihoney pre-treatment inhibited substance P-induced phosphorylation of Akt, ERK1/2, STAT3 and WNK1. These kinases are deeply involved in mast cell maturation, survival, degranulation and nuclear expression of a variety of pro-inflammatory mediators.

This is the first evidence to explain the mechanism by which Medihoney inhibits mast cell degranulation.

7.2 Introduction

Bladder Pain Syndrome/ Interstitial Cystitis (BPS/IC) is a chronic inflammatory disease of the urinary bladder, which is associated with poor responsiveness to antibiotic treatment and no evidence on infection. A large spectrum of BPS/IC cases have an associated mast cell infiltration of the bladder wall. These mast cells are localized around the bladder sensory nerve afferents in the lamina propria and in the detrusor muscle layer. Through our current research, we are implementing an immunohistochemistry technique to detect MRGPRX2, the G protein-coupled receptor for substance P, on the surface of mast cells in the bladder wall of BPS/IC patients. We were able to detect consistent expression of this receptor on the surfaces of mast cells in the bladder biopsies from 18 patients clinically diagnosed as BPS/IC patients (see Chapter 4). In addition to the increased density of the substance P $^{+ve}$ sensory nerve afferents in the bladders of BPS/IC patients, we can state that an interaction between substance P and mast cells could be responsible for the increased mast cell activity and degranulation seen in the bladders of these patients.

In our previous studies, Manuka honey showed high potency inhibition of histamine release by both substance P-induced degranulation and Ca^{+2} ionophore-induced mast cell degranulation (Birch *et al.*, 2011; Alangari *et al.*, 2017c). Such evidence adds to the value and importance of using a Manuka Honey bladder instillation to combat the bladder wall inflammation seen in BPS/IC patients.

In the current study, we aimed to investigate the mechanism by which honey inhibits substance P-induced mast cell degranulation. This was performed through studying the effects of Manuka Honey pre-treatment on the intracellular signalling pathways responsible for mediating the G protein-coupled receptor substance P signal which induces degranulation and pro-inflammatory mediator release.

7.2.1 The signalling pathway responsible for substance P-induced mast cell activation

Mast cells express two G protein-coupled receptors responsible for binding to substance P and mediating its effects on intra-cellular cell signalling pathways. As previously mentioned, these receptors are NK-1 and MRGPCRX2 (Douglas and Leeman, 2011; Wu

et al., 2015; Green *et al.*, 2019). Each receptor is a transmembrane spanning polypeptide chain that is bound to a heterotrimeric G protein. The G protein is comprised of 3 subunits (α,β and γ) (Vines and Prossnitz, 2004). The alpha subunit is of several isoforms including, α_s (Stimulates the enzyme adenylyl cyclase), α_i (Inhibits adenylyl cyclase), α_q (Stimulates the enzymes PLC_β and BTK), and $\alpha_{12/13}$ (Stimulates the guanine nucleotide exchange factor Rho) (Kuehn and Gilfillan, 2007). Upon binding of the ligand (In this case it is substance P) to its G protein-coupled receptor, the receptor undergoes a conformational change that allows the GDP nucleotide, which is bound to the α subunit in the resting condition, to be replaced by a GTP nucleotide. As a result, the GTP-bound α subunit can dissociate form the β and γ subunits and all work as secondary messengers promoting the substance P-induced pro-inflammatory response (Vines and Prossnitz, 2004).

Mast cells express a variable group of G protein-coupled receptors allowing them to respond to variable ligands (Vines and Prossnitz, 2004). These receptors differ from each other in the type of α subunit of the G protein, which in turn determines the resultant effect on the mast cell. For example, human mast cells express two isoforms of adenosine purinoreceptors $A2_A$ and $A2_B$. These receptors differ in their effects on mast cell functions according to their different G proteins. The $A2_A$ receptor deploys the G protein α_S subunit, which activates the enzyme adenylyl cyclase resulting in the production of cAMP and activation of Protein Kinase A and the nuclear factor CREB. Such signalling events are linked with inhibitory effects on mast cell functions, including degranulation and cytokine release. However, the other adenosine receptor $A2_B$ deploys the G protein α_q subunit, activating PLC_β enzyme with a resultant increase in cytoplasmic Ca^{++} levels thereby mediating mast cell activation (Kuehn and Gilfillan, 2007).

In the case of substance P receptors (MRGPCRX2 and NK-1), the G protein α subunit comprises the isoforms α_i (inhibitory to the enzyme adenylyl cyclase) and α_q (Activates PLC_β and BTK) (Burstein *et al.*, 2006; Kulka *et al.*, 2008). The signalling pathway underlying G-protein coupled receptor-mediated mast cell degranulation is dependent on the phosphorylation of the enzyme Phospholipase C-β (PLC-β) by the G-protein $\alpha_{i/q}$ and βγ subunits, and the resultant hydrolysis of the membrane associated

Phophoinositol 4,5 bisphosphate (PIP2) into Diacylglycerol (DAG) and Inositol triphosphate (IP3) (Gilfillan, Austin and Metcalfe, 2011). The latter two are responsible for increased intracytoplasmic Ca^{++} levels and the activation of the enzyme protein kinase C (PKC) resulting in mast cell degranulation (Kuehn and Gilfillan, 2007).

Additionally, the G protein α and/or βγ subunits phosphorylate and activate the enzyme Phosphoinositide-3 kinase (PI3K), which in turn phosphorylates the membrane bound PIP2 into Phophoinositol 3,4,5 trisphosphate (PIP3) (Kim, Rådinger and Gilfillan, 2008). PIP3 provides docking sites for phosphorylation and activation of the enzymes protein kinase B (AKT), PDK-1, BTK and PLC-β (Gilfillan and Tkaczyk, 2006; Gilfillan, Austin and Metcalfe, 2011). BTK augments the PLC-induced intracytoplasmic Ca^{++} mobilisation described earlier (Gilfillan and Tkaczyk, 2006). Activated AKT phosphorylation downstream signalling, including phosphorylation of the MAPK (ERK 1/2, P-38 and JNK) and the mammalian target of rapamycin (mTORC1 and mTORC2), is essential for gene expression leading to pro-inflammatory cytokine and chemokine release (Mócsai *et al.*, 2003; Blatt *et al.*, 2012; MacNeil *et al.*, 2014; Kanazawa *et al.*, 2016; Patel and Theoharides, 2017).

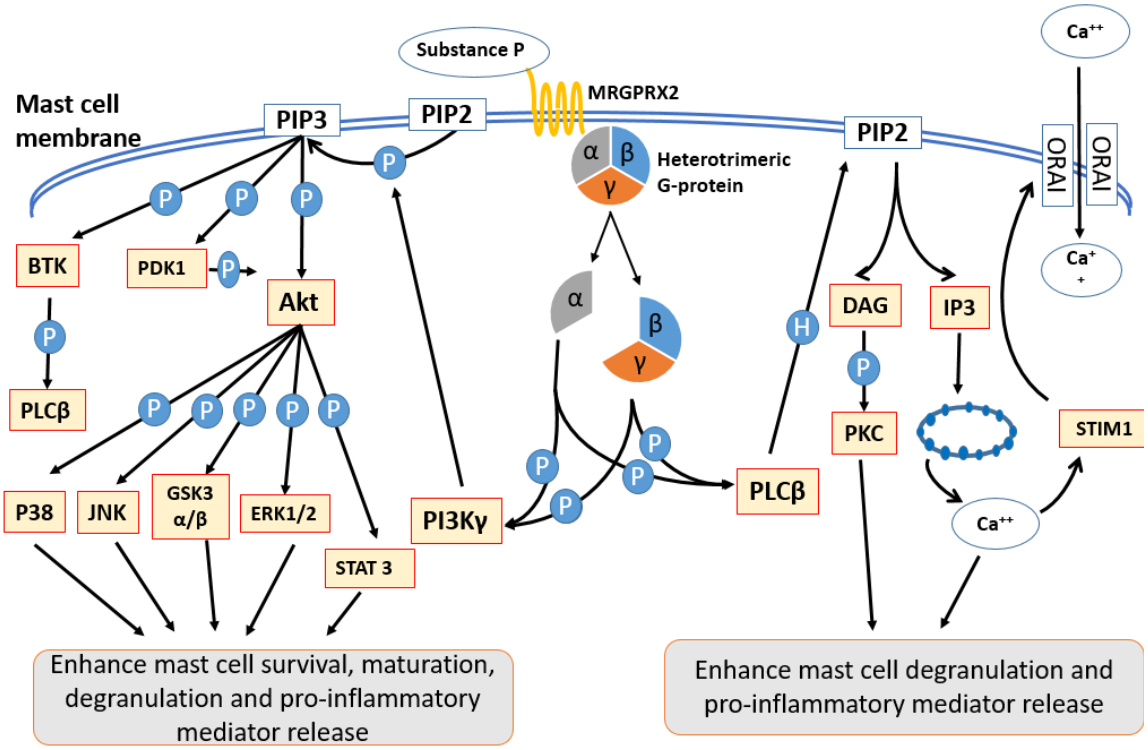

Figure 27: Schematic representation of the G-protein coupled receptor intracellular signalling pathway underlying the SP-induced mast cell degranulation. (P) stands for phosphorylation and (H) stands for hydrolysis.

7.3 Methods

In this experiment, we studied the effect of Manuka honey pre-treatment on the signalling pathways responsible for mast cell activation upon treatment with SP.

7.3.1 Human mast cells

The human mast cell line (LAD2), a well-known mast cell model for in vitro experiments, was used in the current study (Kirshenbaum *et al.*, 2014). LAD2 cells were cultured in Stem Pro-Serum free medium (Invitrogen) enriched with recombinant human Stem cell factor 100 ng /ml (Peprotech), 2mM L-Glutamine with penicillin/streptomycin (Sigma Aldrich), and growth supplement (Invitrogen) (Kirshenbaum *et al.*, 2003).

7.3.2 Honey and substance P challenge

Using the degranulation assay (beta-hexosaminidase), we were able to identify the most potent physiological dose of substance P (1µM), which induces mast cell activation, as indicated by 40 to 50% release. Based on our degranulation assays, the LAD2 cells were pre-treated with Medihoney 4% for 20 minutes before activation with 1 µM of substance P. As the substance P-induced signalling events (mediated via G-protein coupled receptor signalling), which underlie mast cell degranulation are much faster

compared to the degranulation itself, a literature search was performed to choose the optimal substance P activation incubation period. As expected, substance P-induced signalling events could be detected between 1 and 20 minutes from the beginning of the challenge (Lazki-Hagenbach, Ali and Sagi-Eisenberg, 2021; Wang *et al.*, 2022). Based on the fact that the utilised phosphorylation kit arrays a wide range of early and late signalling proteins, we opted to incubate LAD2 cells with substance P 1µM for 10 minutes as follows.

LAD2 cells were suspended in tyrode's buffer (TB) (contains Ca++ and Mg essential for mast cell activation and degranulation, in addition to gluscose as a source of cell energy). Ten million cells suspended in 790 µL TB were added to each of the four 1.5 ml Eppendorf tubes. The total volume in each of the four tubes was 1 ml as follows:

(1) In tube 1, only 210 µL of TB was added. (Only cell suspension and Tyrode's buffer, which is the spontaneous).
(2) In tube 2, 100 µL of Medihoney (MH) 40 % and 110 µL of TB were added to the cell suspension. (Cell suspension + Medihoney alone). In this tube 100 µL of MH 40% were diluted 10 times in a total volume of 1 ml to give a final concentration of 4%.
(3) In tube 3, 110 µL of TB was added to the 790 µL cell suspension.
(4) In tube 4, 100 µL of MH 40 % and 10 µL of TB were added to the cell suspension (Medihoney pre-incubation, then substance P will be added).

The four tubes were incubated for 20 minutes at 37˚C in 5% CO_2. After pre-incubation, 100 µL of substance P 10 µM was added to tubes 3 (substance P alone) and 4 (substance P after honey pre-incubation). In tubes 3 and 4, 100 µL of substance P 10 µM were diluted 10 times in a total volume of 1 ml to give a final concentration of 1 µM. The tubes were incubated again for 10 minutes after adding substance P.

Table 8: The contents of each tube in the Phospho-MAPK quantification experiment

	Tube 1 (Spontaneous)	Tube 2 (MH4%)	Tube 3 (SP 1µM)	Tube 4 (SP 1µM + MH4%)
Cell suspension	790 µl	790 µl	790 µl	790 µl

Tyrode's buffer	210	110 µl	110 µl	10 µl
MH4%	0	100 µl	0	100 µl
Substance P 1Mm	0	0	100 µl	100 µl

7.3.3 Cell lysis

After challenge, each tube was centrifuged for 5 minutes at 6000 G at 4°C and the supernatant discarded.Cells in each tube were washed with 1x PBS and centrifuged again at 5000 G at 4°C for 5 minutes, and then the PBS was discarded. Then, 500 µL of lysis buffer (R and D systems) was added to each tube, followed by short vortexing. Then, the tubes were placed in large glass bottle filled with ice and left on roller for 30 minutes. After adding the lysis buffer and proper mixing, each tube was centrifuged for 5 minutes at 4°C and 14,000 G to lyse the cells. The cell lysates were pipetted out and cell pellets were discarded. Finally, the total protein content in each tube was measured by Nanodrop, with the following results:

(a) For tube 1 (Spontaneous), the protein concentration was 18.8 mg/ml.
(b) For tube 2 (MH4%), the protein concentration was 19.073 mg/ml.
(c) For tube 3 (substance P), the protein concentration was 18.479 mg/ml.
(d) For tube 4 (Sub P + MH4%), the protein concentration was 18.779 mg/ml.
(e) Lysis buffer protein concentration was 16.898 mg/ml.

7.3.4 Measurement of the levels of the phosphorylated MAPKs in the cell lysates

For this purpose, we used a Human Phospho-MAPK Array KIT (Cat. Number ARY002B from R and D systems). This KIT is an economical and accurate tool to simultaneously measure the phosphorylated levels of 26 protein kinases.

This KIT contains 4 membranes, one for each sample, where detection and control antibodies for each protein kinase are plated on a pair of dots (duplicates). Each sample (cell lysate) was mixed with a cocktail of biotinylated detection antibodies (according to the manufacturer's instructions), and then incubated overnight with a nitrocellulose membrane. Streptavidin-biotin and chemo luminescent detection reagents were applied. Finally, membranes were membranes were visualised using AmershamTM imager 600. Signalling intensity in each of the four membranes were analysed by image

lab software and transferred into an excel sheet for the statistical analysis. Finally, the data were analysed using GraphPad Prism version 9. Differences between conditions were analysed using 2-way ANOVA.

7.4 Results

7.4.1 The effects of the neuropeptide substance P with or without Medihoney 4% on the phosphorylation of mitogen activated protein kinases in LAD2 cells using Phospho MAPK Array Kits (R&D Systems).

- When LAD2 cells were challenged with substance P (1 μM) for 10 minutes, this resulted in a statistically significant increase in the levels of the phosphorylated protein kinases Akt (Serine 473), ERK1/2, STAT3 (Serine 727), PRAS 40 (Threonine 246) and WNK1.
- Akt, ERK1/2, STAT3, PRAS40 and WNK1 phosphorylation was strongly and significantly inhibited upon 20-minute pre-incubation with 4% MH, while the inhibitory effect of MH4% on ERK1/2 phosphorylation was not statistically significant (Figures 28-39).
- In addition, despite the fact that substance P did not induce a significant rise in the phosphorylated levels of the Glycogen Synthase Kinase 3 (GSK3), MH pre-incubation induced strong and significant drops in the levels of phosphorylated GSK3 (α/β) at positions serine 21/ serine 9, respectively (Figure 38).
- Interestingly, substance P- enhanced the phosphorylation of the protein kinase RSK 1/2/3 (serine 380/386/377). Such phosphorylation was even enhanced upon Medihoney pre-incubation (Figure 52).

7.4.2 The effects of the Akt inhibitor 10-DEBC HCL on the neuropeptide substance P-induced signalling events in LAD2 mast cells

- Interestingly, on the contrary of the MH effect, 10-DEBC pre-incubation enhanced the Sub P-induced phosphorylation of Akt (Serine 473), WNK1 and STAT3 (serine 727) (Figures 31, 40, 41 & 42).

7.4.3 The effects of the sugar-free Manuka honey extract at 2 and 4% concentrations on the neuropeptide substance P-induced signalling events in LAD2 mast cells

- Similar to Medihoney, pre-incubation of LAD2 cells with the Manuka extract resulted in inhibition of the substance P-induced phosphorylation of the protein kinases Akt (Serine 473), WNK1, and STAT3 (serine727).
- The inhibitory effect of the Manuka extract on Akt phosphorylation was dose-dependent starting at partial inhibition at 2% concentration, which culminated in complete blocking of the substance P-induced Akt phosphorylation at 4% concentration (Figures 41 & 42).
- In case of WNK1 phosphorylation, the inhibitory effect of the Manuka extract was only seen with the 4% concentration, while 2% failed to produce any inhibitory effect (Figures 43 & 44).
- Both Manuka extract concentrations completely blocked the substance P induced STAT3 phosphorylation at position serine 727 (S727) (Figures 45 & 46).
- Similar to Medihoney, only at 4% concentration, the Manuka extract completely inhibited the substance P-induced phosphorylation of the protein kinase PRAS40 at Threonine 246 position (T246) (Figures 47& 48).
- Contrary to the effect of Medihoney, Manuka extract at both 2 and 4% concentrations completely inhibited substance P-induced phosphorylation of the protein kinases P90 S6 kinase (RSK1/2/3) at positions serine 380/366/377 (Figures 49 & 50), while 4% Medihoney significantly enhanced that phosphorylation (Figure 53).
- It is worth mentioning that the effect of the Manuka honey extract at 2 or 4% concentration on the substance P-induced signalling events was only assayed once (Pilot data), so repetition is needed to obtain a solid conclusion.

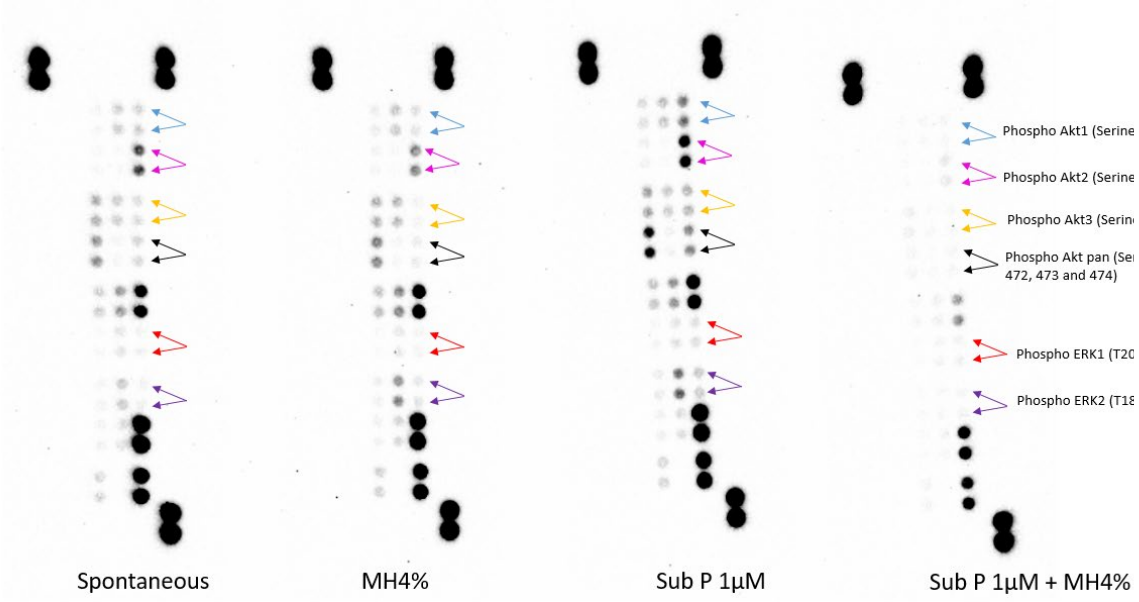

Figure 28: Image of 4 sets of nitrocellulose membranes plated with monoclonal antibodies against various phosphorylated intracellular signalling protein kinases (Phospho MAPK array, Cat# ary002b, R&D systems, USA). The blue, pink, yellow and black arrows point to duplicated dots plated with monoclonal antibodies against phosphorylated Akt at positions serine 473, 474, 472 and AKT pan (all positions), respectively. Red and violet arrows point to duplicated dots plated with monoclonal antibodies against phosphorylated ERK 1 and 2, respectively (First assay, July 2019).

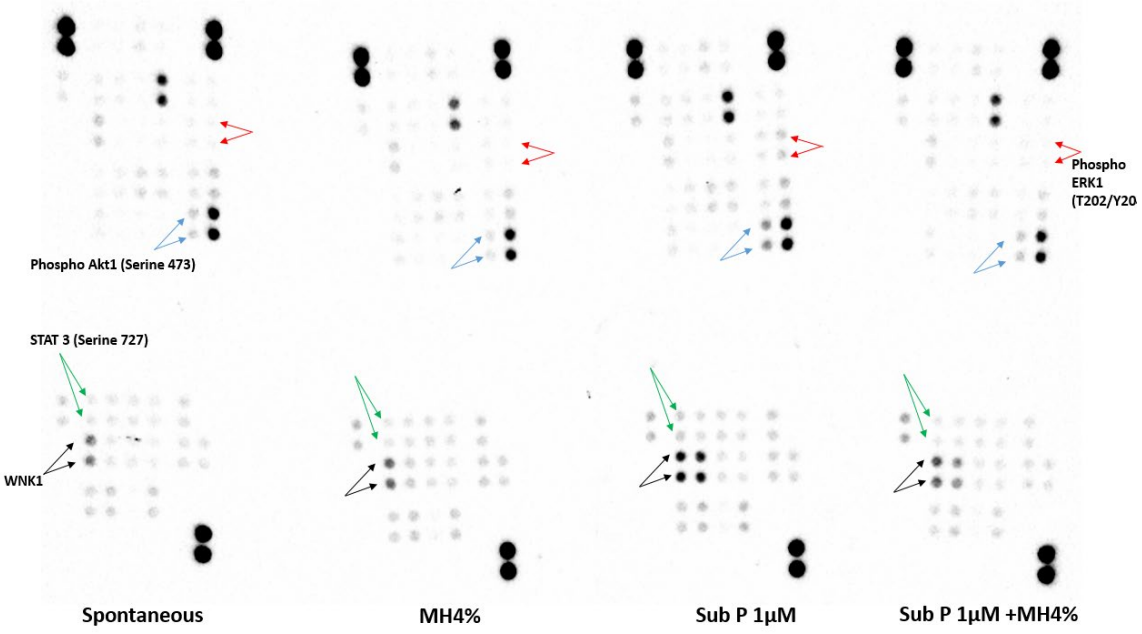

Figure 29: 4 sets of nitrocellulose membranes plated with monoclonal antibodies against various phosphorylated intracellular signalling protein kinases (Phospho MAPK array, Cat# ary003b, R&D systems, USA). Blue, red, green and black arrows point to

duplicated dots plated with monoclonal antibodies against phosphorylated Akt, ERK1/2, STAT3 (serine 727) and WNK1, respectively. (Second assay, January 2020).

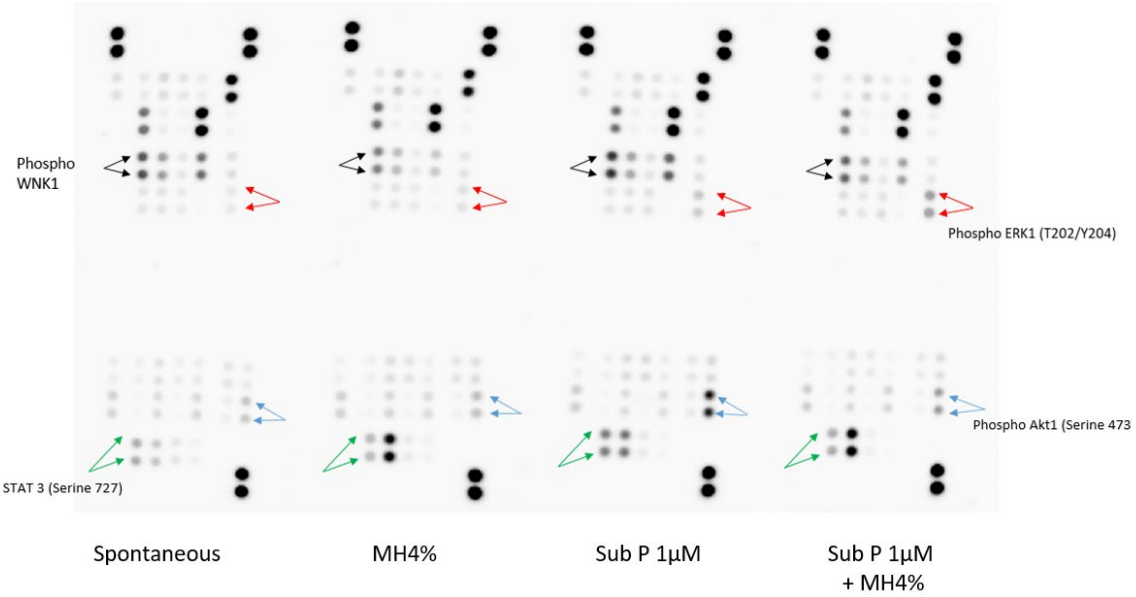

Figure 30: 4 sets of nitrocellulose membranes plated with monoclonal antibodies against various phosphorylated intracellular signalling protein kinases (Phospho MAPK array, Cat# ary003c, R&D systems, USA). The black, red, blue and green arrows point to duplicated dots plated with monoclonal antibodies against phospho WNK1, Akt (serine 473), ERK1/2 and STAT 3 (serine 473), respectively. (Third assay, April 2021).

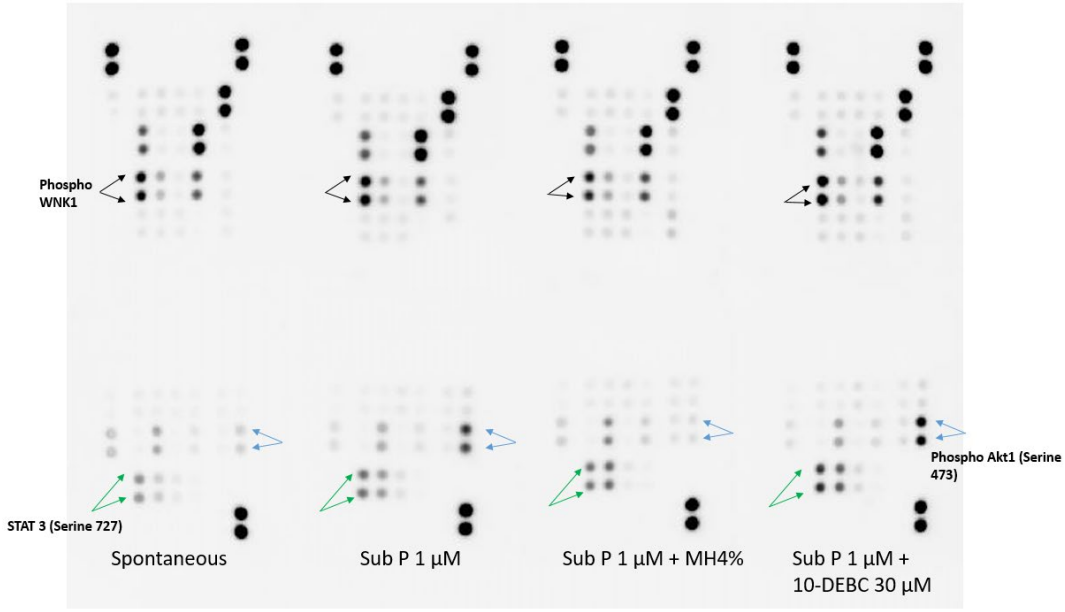

Figure 31: 4 sets of nitrocellulose membranes plated with monoclonal antibodies against various phosphorylated intracellular signalling protein kinases (Phospho MAPK array, Cat# ary003c, R&D systems, USA). The black, red, blue and green arrows point to

duplicated dots plated with monoclonal antibodies against phospho WNK1, ERK1/2, Akt (serine 473) and STAT 3 (serine 473), respectively (4TH assay, September 2021).

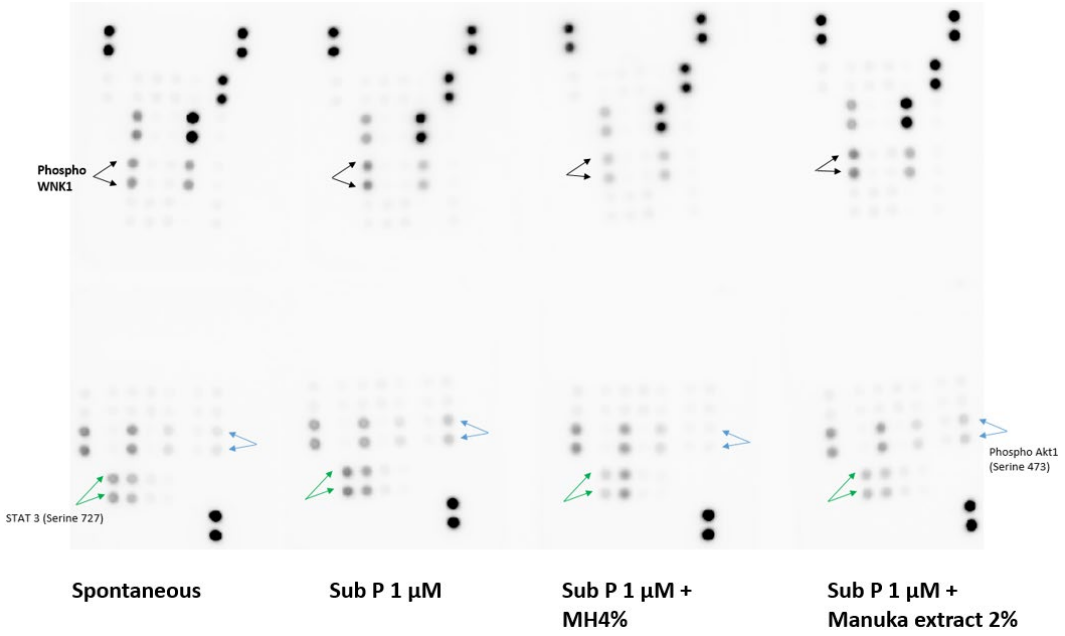

Figure 32: 4 sets of nitrocellulose membranes plated with monoclonal antibodies against various phosphorylated intracellular signalling protein kinases (Phospho MAPK array, Cat# ary003c, R&D systems, USA). The black, blue and green arrows point to duplicated dots plated with monoclonal antibodies against phospho WNK1, Akt (serine 473) and STAT 3 (serine 473), respectively (5th assay, October 2021).

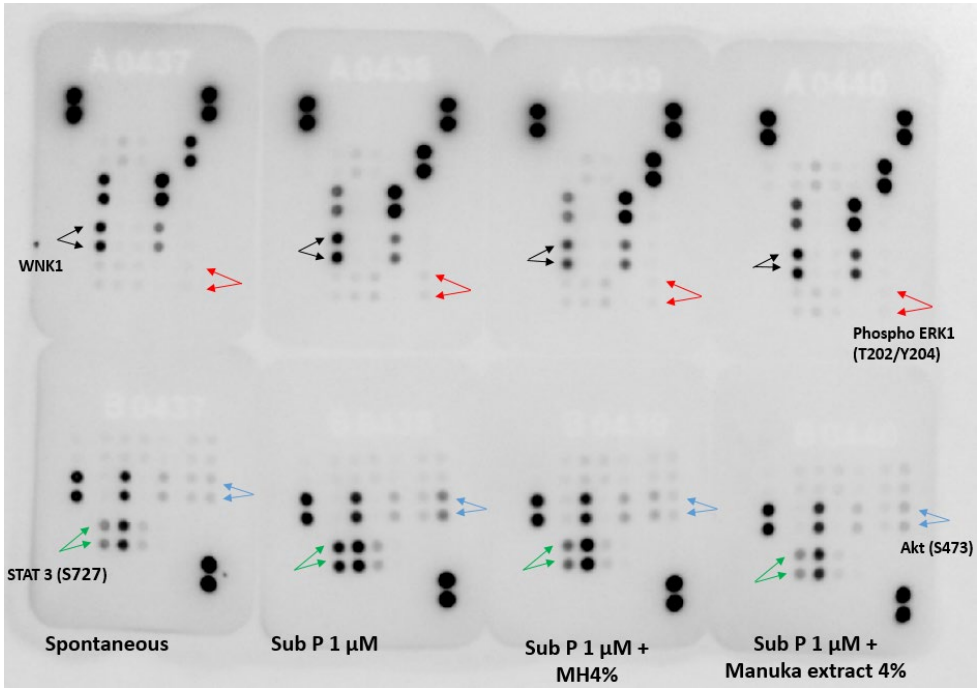

Figure 33: 4 sets of nitrocellulose membranes plated with monoclonal antibodies against various phosphorylated intracellular signalling protein kinases (Phospho MAPK

array, Cat# ary003c, R&D systems, USA). The red, black, blue and green arrows point to duplicated dots plated with monoclonal antibodies against phospho ERK1/2, WNK1, Akt (serine 473) and STAT 3 (serine 473), respectively. (6th assay, October 2021).

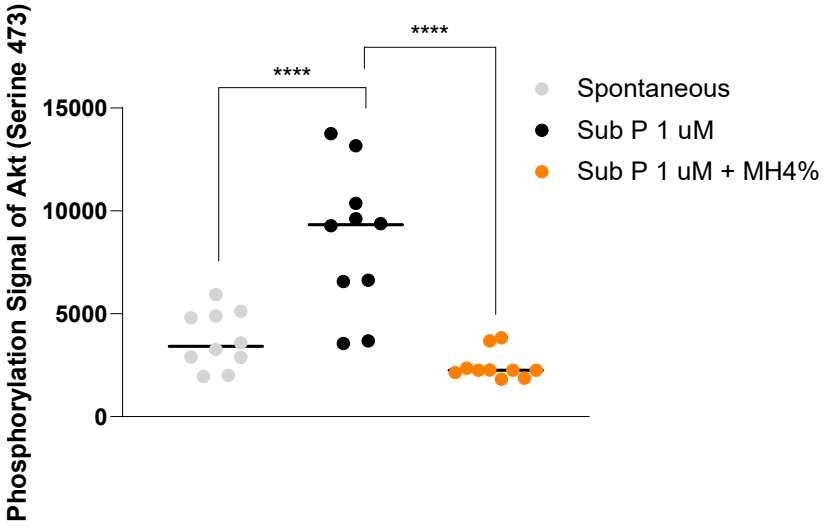

Figure 34: Scatter plot represents the levels of phosphorylation of AKT at positions serine 473 following 10-minute substance P treatment with or without 20-minute Medihoney (MH) 4% pre-incubation. Error bars represent the minimal and maximal values (*** P value <0.005,* n=10). Statistical differences were analysed by 2-way ANOVA using GraphPad Prism 9 software.

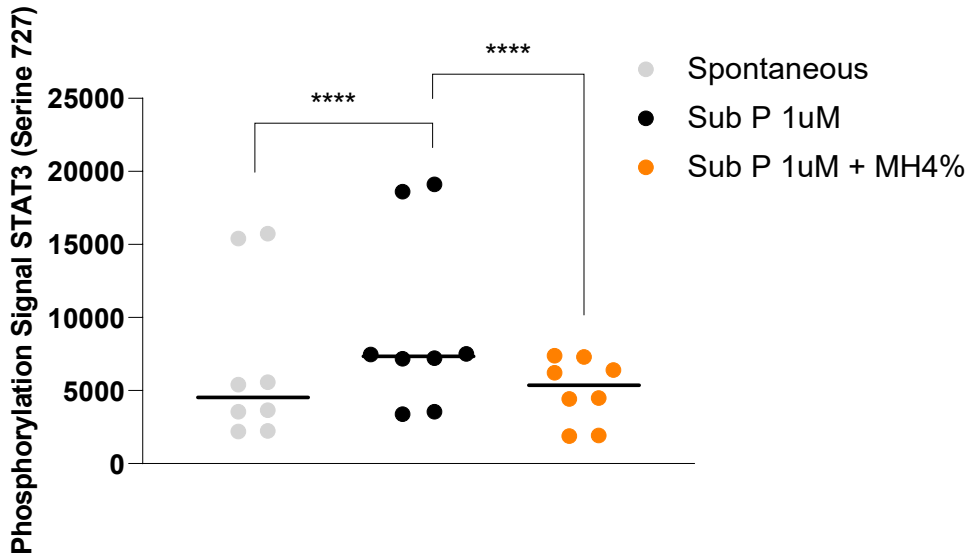

Figure 35: Scatter plot represents the levels of phosphorylation of STAT3 at position serine 727 following 10-minute substance P treatment with or without 20-minute

Medihoney (MH) 4% pre-incubation. Error bars represent the minimal and maximal values (*** P value <0.01,* n=8). Statistical differences were analysed by 2way-ANOVA using GraphPad Prism 9 software.

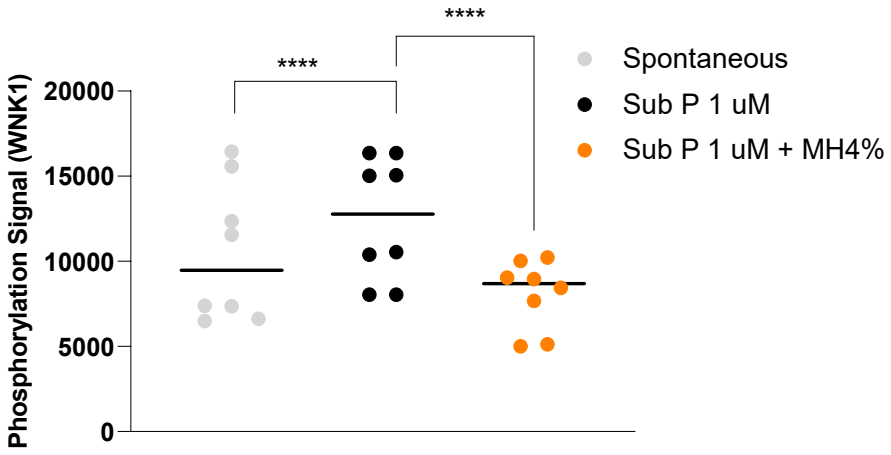

Figure 36: Scatter plot represents the levels of phosphorylation of the lysine deficient protein kinase (WNK1) at position serine 727 following 10-minute substance P treatment with or without 20-minute Medihoney (MH) 4% pre-incubation. Error bars represent the minimal and maximal values (** P value < 0.05, ** P value <0.005,* n=8). Statistical differences were analysed by 2-way ANOVA using GraphPad Prism 9 software.

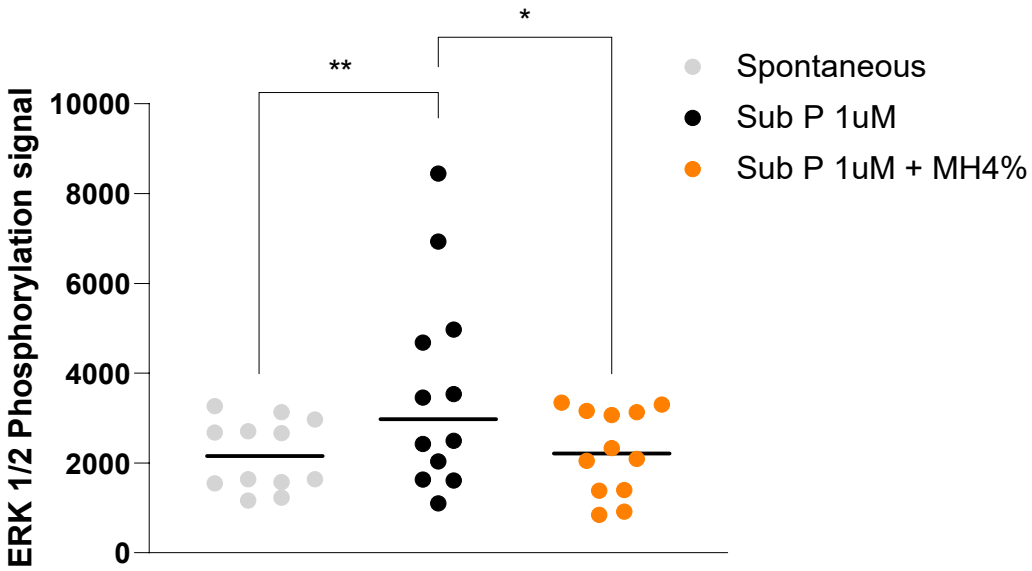

Figure 37: Scatter plot represents the levels of phosphorylation of ERK1/2 following 10-minute substance P treatment with or without 20-minute Medihoney (MH) 4% pre-incubation. (* P value <0.05, ** P value <0.005, n=12). Statistical differences were analysed by 2-way ANOVA using GraphPad Prism 9.

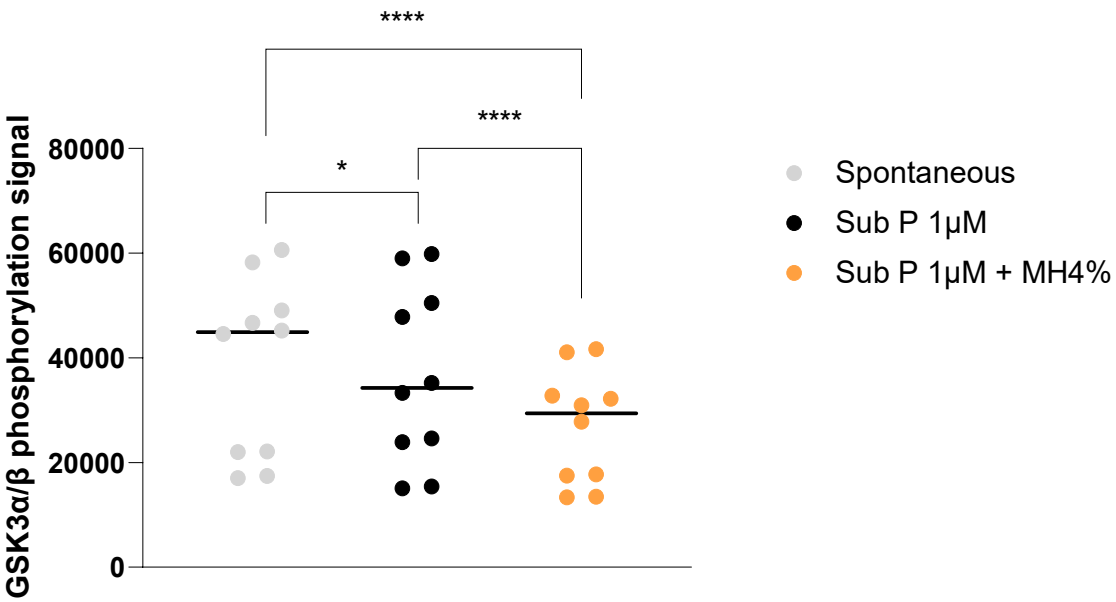

Figure 38: The levels of phosphorylation of GSK3 α/β following 10-minute substance P treatment with or without 20-minute Medihoney (MH) 4% pre-incubation. Error bars represent the minimal and maximal values (* P value <0.05, ** P value <0.005 n=10). Statistical differences were analysed by 2way-ANOVA using GraphPad Prism 9.

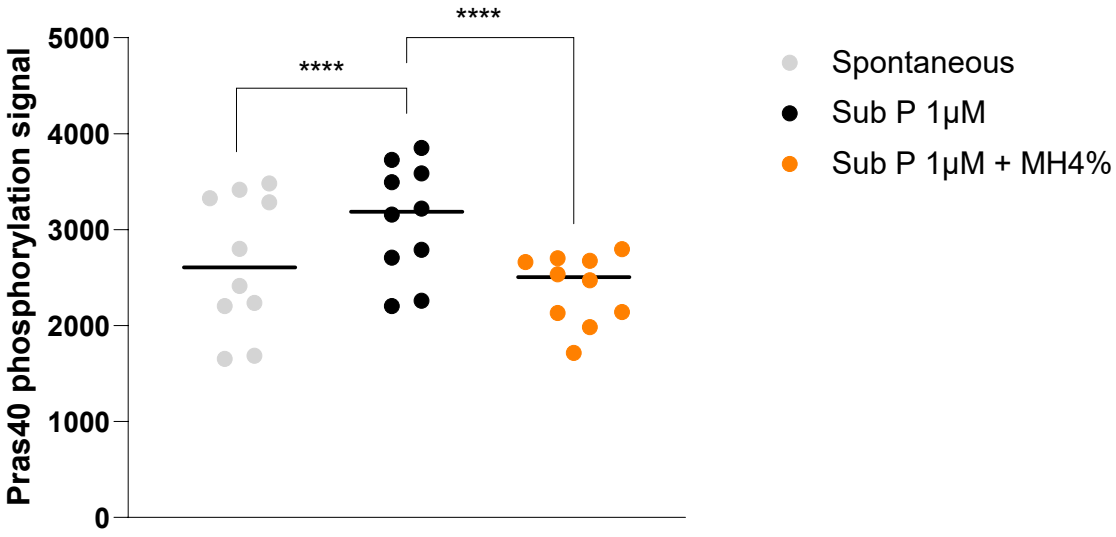

Figure 39: The levels of phosphorylation of Pras40 following 10-minute substance P treatment with or without 20-minute Medihoney (MH) 4% pre-incubation. Error bars represent the minimal and maximal values (* P value <0.05, ** P value <0.005 n=10). Statistical differences were analysed by 2way-ANOVA using GraphPad Prism 9.

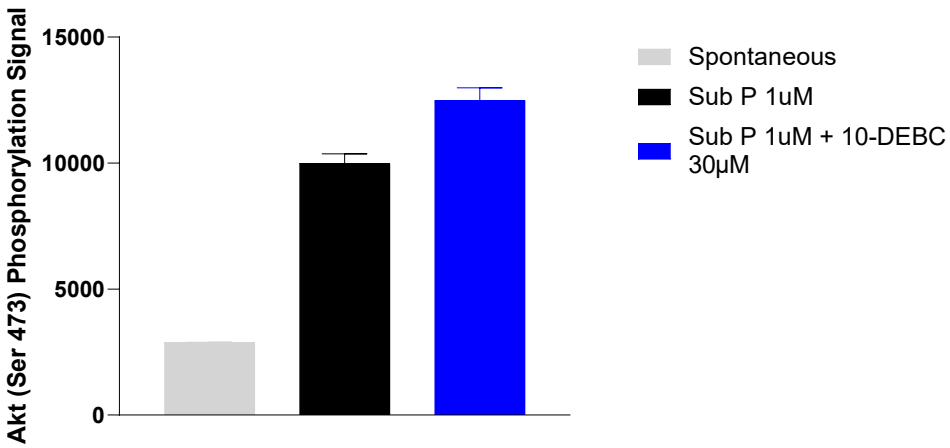

Figure 40: The effect of 10-DEBC on the substance P-induced Akt (Serine 473) phosphorylation in LAD2 cells. Experiment was performed once in duplicates (pilot data).

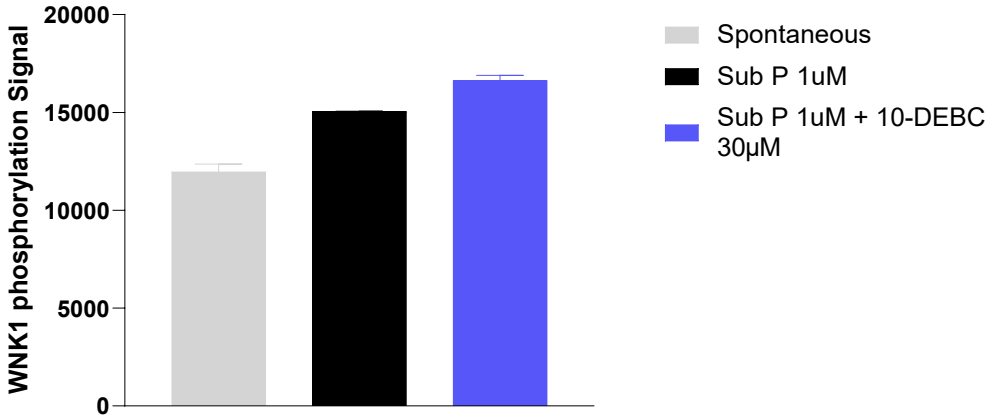

Figure 41: The effect 10-DEBC on the substance P-induced WNK1 phosphorylation in LAD2 cells. Experiment was performed once in duplicates (Pilot data).

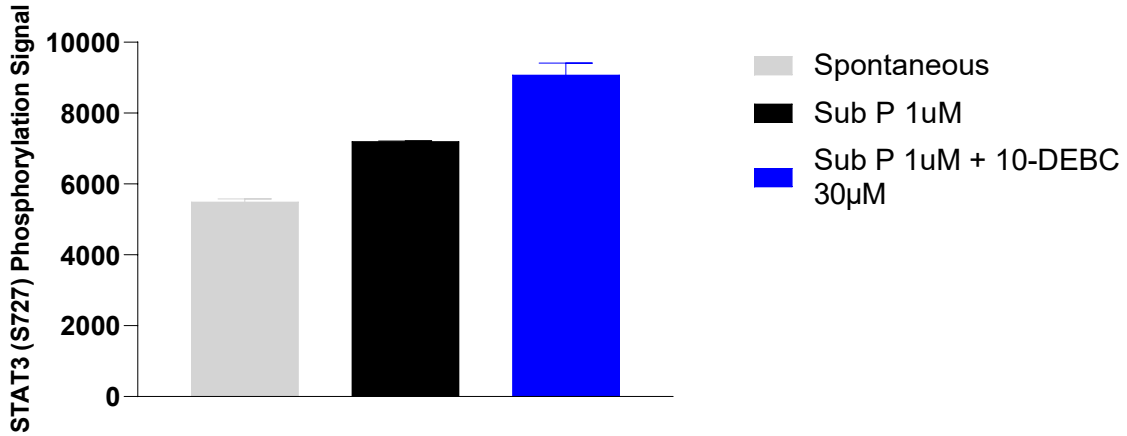

Figure 42: The effect 10-DEBC on the substance P-induced STAT3 (serine 727) phosphorylation in LAD2 cells. Experiment was performed once in duplicates (Pilot data).

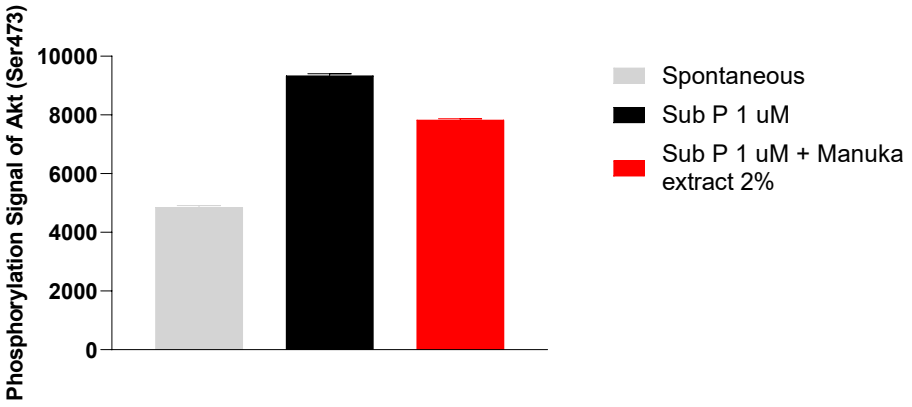

Figure 43: The effect the Manuka honey extract (2%) on the substance P-induced Akt (serine 473) phosphorylation in LAD2 cells. Experiment was performed once in duplicates (Pilot data).

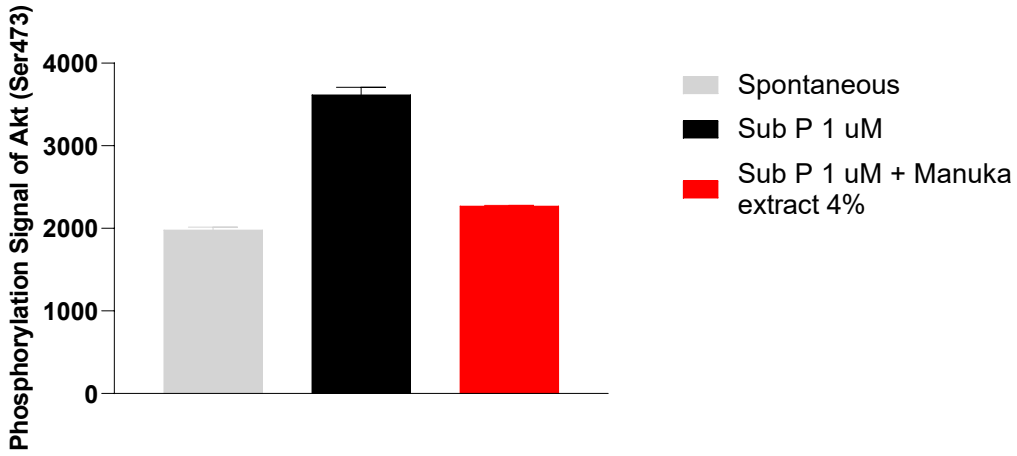

Figure 44: The effect the Manuka honey extract (4%) on the substance P-induced Akt (serine 473) phosphorylation in LAD2 cells. Experiment was performed once in duplicates (Pilot data).

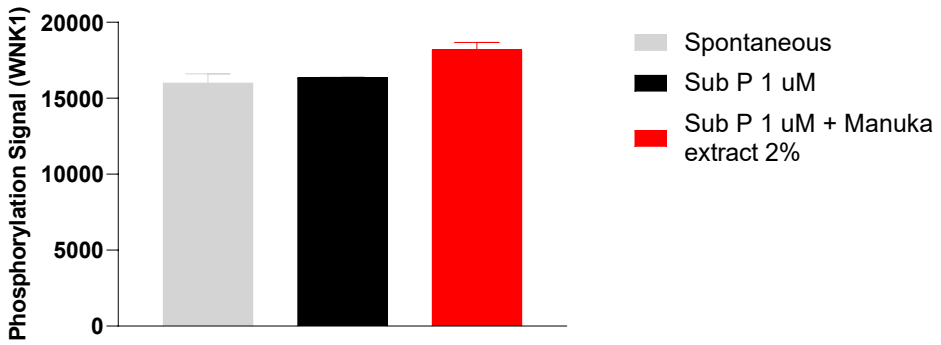

Figure 45: The effect the Manuka honey extract (2%) on the substance P-induced WNK1 phosphorylation in LAD2 cells. Experiment was performed once in duplicates (Pilot data).

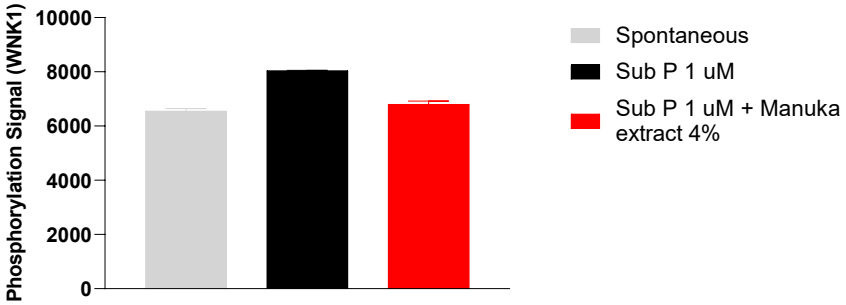

Figure 46: The effect the Manuka honey extract (4%) on the substance P-induced WNK1 phosphorylation in LAD2 cells. Experiment was performed once in duplicates (Pilot data).

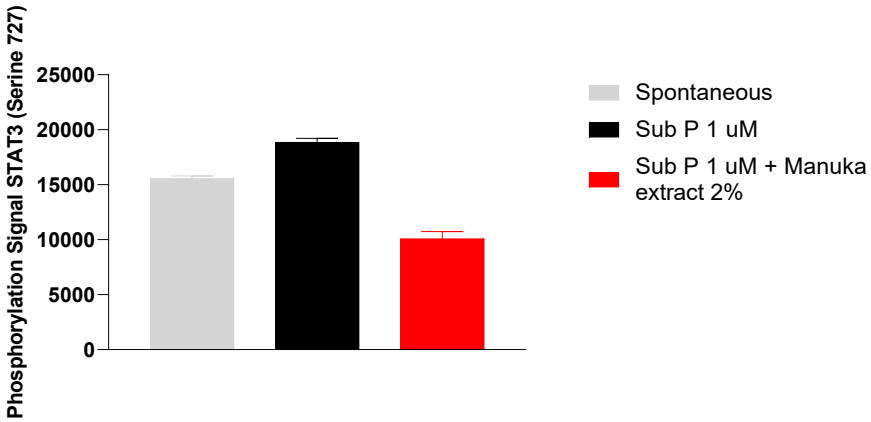

Figure 47: The effect the Manuka honey extract (2%) on the substance P-induced STAT3 (serine 727) phosphorylation in LAD2 cells. Experiment was performed once in duplicates (Pilot data).

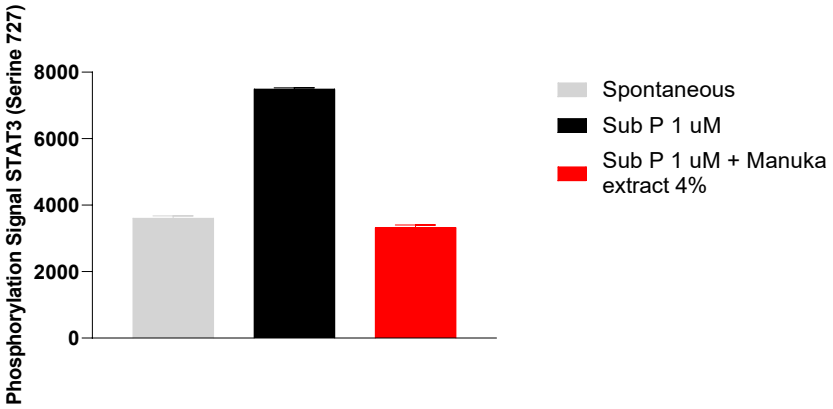

Figure 48: The effect the Manuka honey extract (4%) on the substance P-induced STAT3 (serine 727) phosphorylation in LAD2 cells. Experiment was performed once in duplicates (Pilot data).

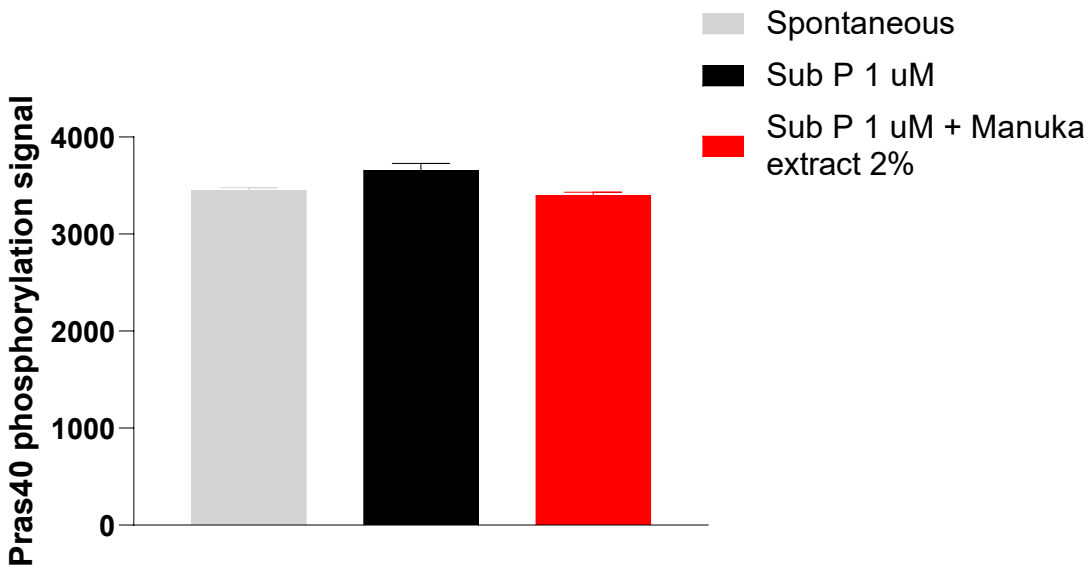

Figure 49: The effect the Manuka honey extract (4%) on the substance P-induced PRAS40 (Threonine 246) phosphorylation in LAD2 cells. Experiment was performed once in duplicates (Pilot data).

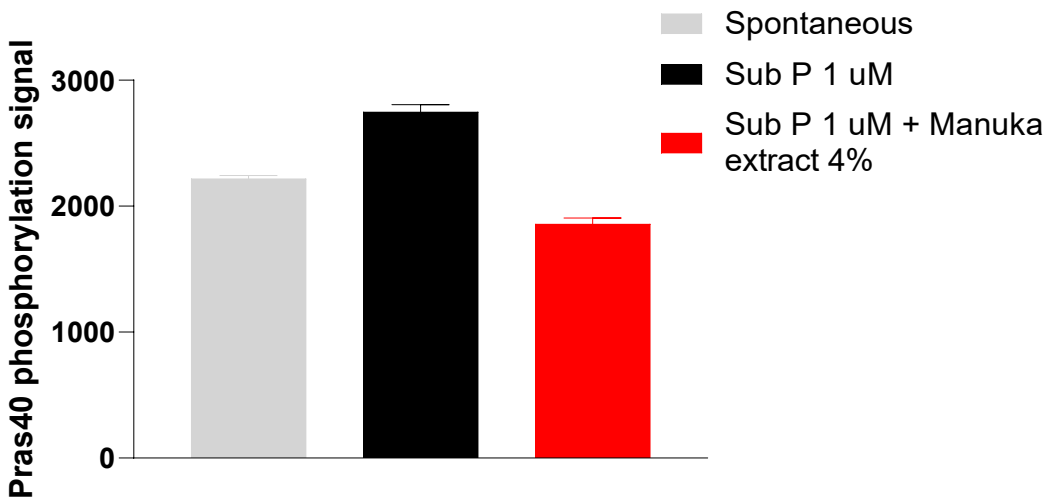

Figure 50: The effect the Manuka honey extract (4%) on the substance P-induced PRAS40 (Threonine 246) phosphorylation in LAD2 cells. Experiment was performed once in duplicates (Pilot data).

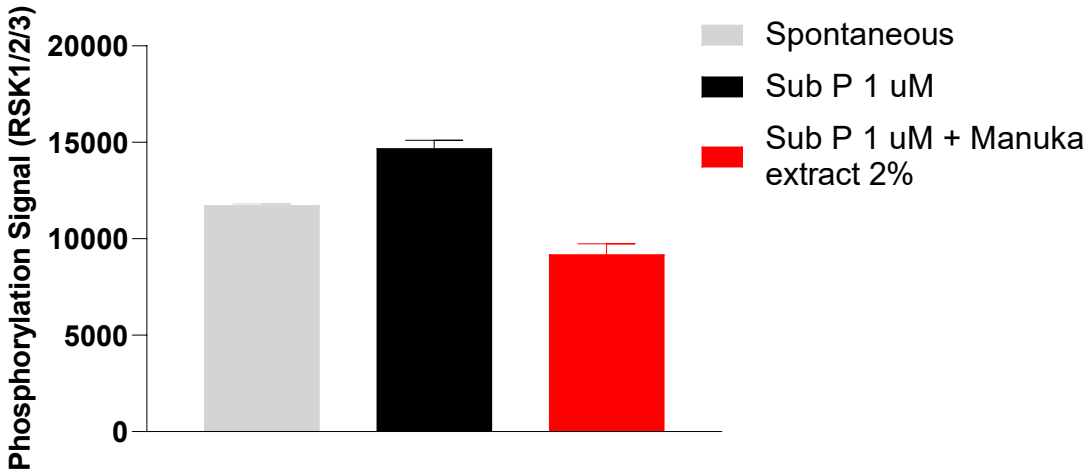

Figure 51: The effect the Manuka honey extract (2%) on the substance P-induced RSK1/2/3 phosphorylation in LAD2 cells. Experiment was performed once in duplicates (Pilot data).

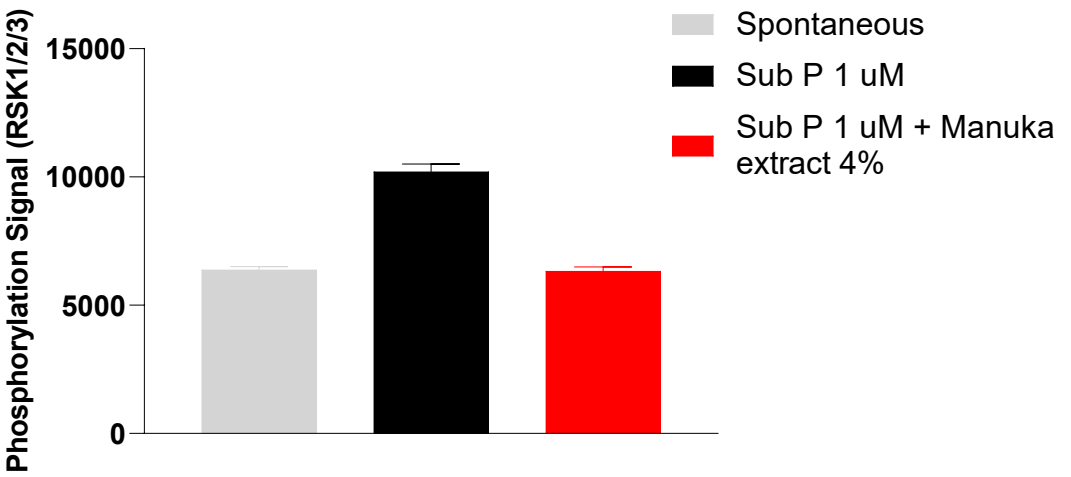

Figure 52: The effect the Manuka honey extract (4%) on the substance P-induced RSK1/2/3 phosphorylation in LAD2 cells. Experiment was performed once in duplicates (Pilot data).

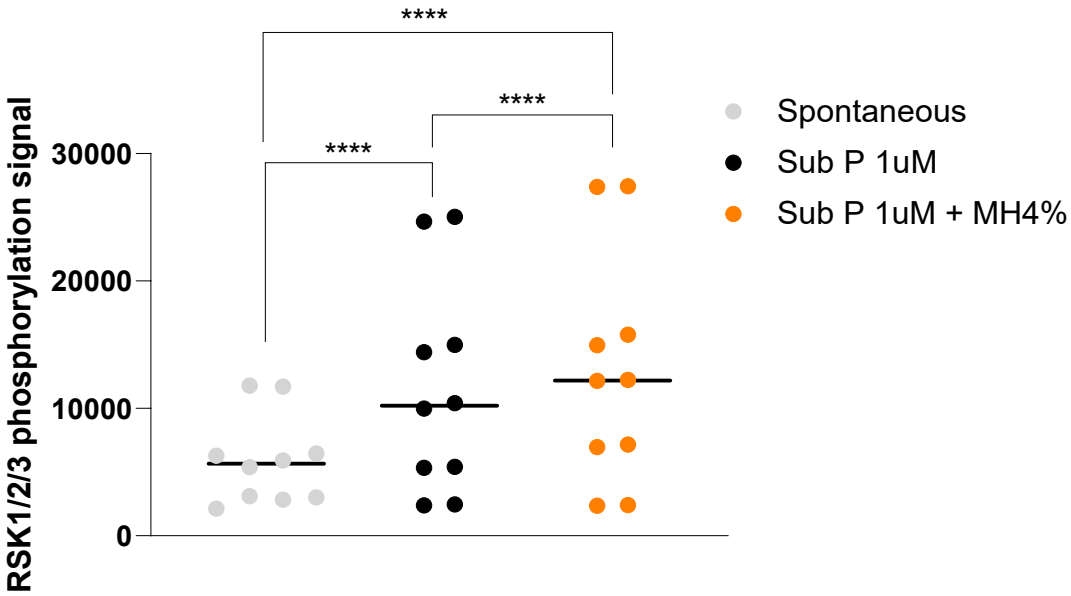

Figure 53: Scatter plot represents the effect the Medihoney (4%) on the substance P (10 minutes)-induced RSK1/2/3 phosphorylation in LAD2 cells. Experiment was performed 5 times in duplicates (**** P value <0.0001, n=10). Statistical differences were analysed by two-way ANOVA using GraphPad Prism 9.

7.4.4 The assessment of the phosphorylation of protein kinase B (Akt) at position serine 473 in LAD2 cells using Human Phospho (Ser473)/Total Akt Whole Cell Lysate Kit (Cat# K15100D-1, MSD, USA).

7.4.4.1 The effect of substance P 1µM, with or without pre-incubation with MH4% and 10-DEBC 30µM, on the phosphorylation of Akt at position serine 473 in LAD2 cells.

- Over the 3 different assays, MH 4%, but not the Akt inhibitor 10-DEBC HCL 30µM, induced a significant decrease in the levels of the phosphorylated Akt at position serine 473 (Akt 1).
- Consistent with our previous results, 10-minute substance P 1µM incubation induced a significant increase in the phosphorylated levels of Akt at position serine 473 (Akt 1) compared to the spontaneous untreated levels.
- 20-minute pre-incubation with MH4% induced a significant drop in substance P-induced phosphorylation of Akt (Figure 54).
- Interestingly, the Akt inhibitor 10-DEBC 30 µM did not induce any inhibition of Akt phosphorylation (Figure 54), which is consistent with the results of the 4th phospho MAPK array, where 10-DEBC significantly enhanced Akt phosphorylation induced by substance P (Figures 31 & 40).

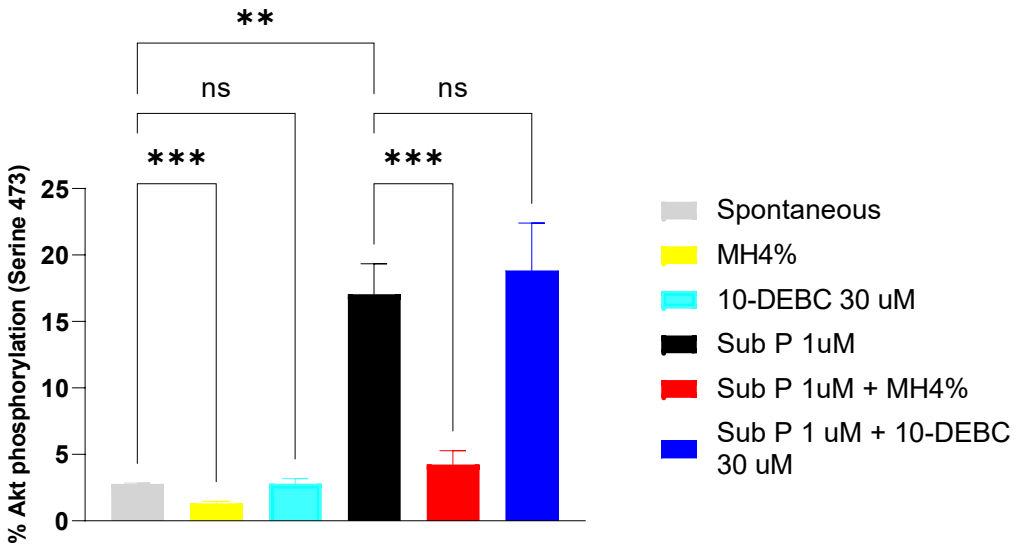

Figure 54: Percentage phosphorylation of protein kinase B/Akt at serine 473 position in the substance P-activated LAD2 cells as detected by the total/ser473 phospho Akt assay kit (MSD, USA). The graph represents the results of 3 independent assays, each performed in duplicates. Error bars represent means ± standard error means (** P value

<0.01, *** P value <0.001, n=6). Statistical differences were analysed by Repeated Measures one way ANOVA using GraphPad Prism 9.

7.4.4.2 The effect of Ca^{++} ionophore 1µM, with or without pre-incubation with MH4% and 10-DEBC 30µM, on the phosphorylation of Akt at position serine 473 in LAD2 cells.

- 10-minute incubation of the LAD2 cells with Ca^{++} ionophore 1µM induced a significant increase in Akt (Serine) 473 phosphorylation compared to the spontaneous untreated control ($P < 0.05$).
- 20-minute pre-incubation with MH4% induced a significant drop in the Ca^{++} ionophore-induced Akt phosphorylation, ($P < 0.05$).
- Interestingly, 10-DEBC pre-incubation induced significant elevation of the Ca ionophore-induced Akt phosphorylation, which was consistent with its effect on the substance P-induced Akt phosphorylation (Figure 55).

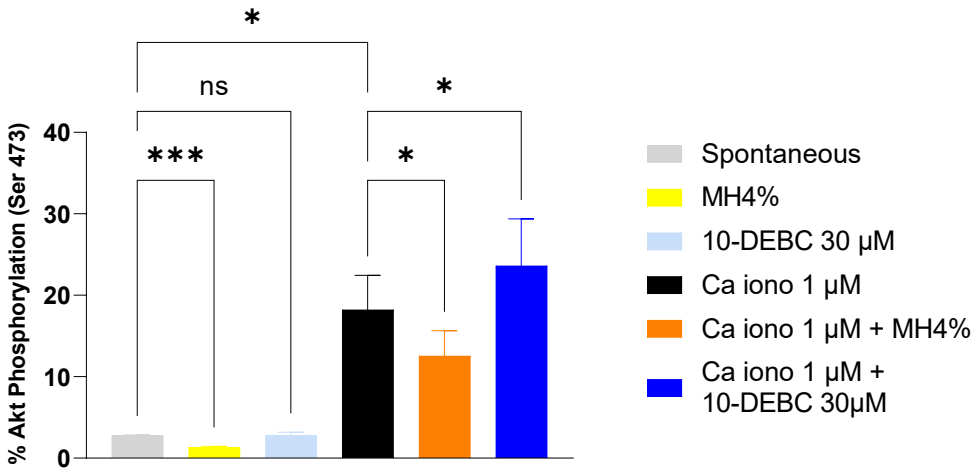

Figure 55: Percentage phosphorylation of protein kinase B/Akt at serine 473 position in Ca^{++} ionophore-activated LAD2 cells as detected by the total/ser473 phospho Akt assay kit (MSD, USA). The graph represent the results of 3 independent assays, each performed in duplicates. Error bars represent means **±** standard error means (* P value <0.05, *** P value <0.001, n=6). Statistical differences were analysed by Repeated Measures one way ANOVA using GraphPad Prism 9.

7.5 Discussion

Previous evidence has highlighted the effect of Manuka honey as an anti-inflammatory agent acting via the inhibition of mast cell degranulation. Medihoney, a medical grade Manuka honey, inhibited Ca^{++} ionophore-induced mast cell degranulation and histamine

release (Birch *et al.*, 2011; Alangari *et al.*, 2017a). Through our β-hexosaminidase studies (see chapter 3), we have observed a dose dependant inhibition of the substance P-induced mast cell degranulation by Medihoney. This study was designed to explore the effects of Medihoney treatment on intracellular signalling pathways well known to regulate many cellular functions, including growth, division, maturation, chemotaxis, degranulation, gene expression and exocytosis.

In the current study, as expected, following 10-minute incubation, substance P induced a significant increase in the levels of the phosphorylated kinases Akt (Protein kinase B) at position serine 473, ERK1/2, the transcription factor STAT3 at position serine 473 and the lysine deficient protein kinase (WNK1).
Importantly, the intracellular signalling was assessed using a validated phospho-MAPK array kit (ARY003C, R&D systems). The produced results were graphically presented in scatter plots. These scatter plots showed a wide range of separation between the dots. This separation can be explained by several factors that work collectively.
Firstly, LAD2 cells tend to age in the cell culture. This aging can be easily noticed by the stronger cell clumping and adherence to the walls of the cell culture flask. This aging is associated with significant drop in the responsiveness of mast cell as clearly shown by the significant decline in the percentage release upon mast cell degranulation.

Moreover, this protein phosphorylation assay requires huge numbers of cells (5-10 million cells) per condition. In order to generate cell counts enough for a single assay, we had to wait for around one month. During these long periods, the LAD2 cell responsiveness tend to drop, resulting in decrease in the intensity of the protein phosphorylation signal. Accordingly, the strongest signals were obtained using fresh cells lines that has just been retrieved from liquid nitrogen, while the as the cells advance in the cell culture, the signal intensity tends to drop. However, despite that drop, the patterns of activation and inhibition of protein phosphorylation were consistent throughout the repeated assays, resulting in high statistical significance.

7.5.1 The effect of Medihoney on the PI3K/Akt signaling pathway in LAD2 mast cells

7.5.1.1 The role of the PI3K/Akt pathway in mast cell degranulation

Using 9 independent assays, Substance P was shown to induce Akt phosphorylation in LAD2 cells utilizing two different protein phosphorylation assessment techniques

(Electroluminescence ELISA, MSD, and immunoblotting, R&D Systems). Similarly, Akt (serine 473) was phosphorylated upon Ca^{++}ionophore challenge, indicating that Akt serine 473 phosphorylation is a common event underlying mast cell degranulation induced by several secretagogues. Such is consistent with various previous reports (Gilfillan and Tkaczyk, 2006; Gilfillan, Austin and Metcalfe, 2011). Moreover, other reports highlighted that substance P-induced degranulation in LAD2 cells was strongly inhibited by the PI3K blockers Wortmannin and 3-MA (Lazki-Hagenbach, Ali and Sagi-Eisenberg, 2021).

On binding of the neuropeptide substance P to its G protein-coupled receptor (MRGPRX2), the PI3K/Akt signalling pathway is activated (Wang *et al.*, 2022) in an event that is essential for the pro-inflammatory responses of mast cells (Kimata *et al.*, 2000; Gilfillan and Tkaczyk, 2006). Upon substance P mast cell activation, $PI3K_{\gamma}$-produced PI3P phosphorylates the enzyme Bruton's tyrosine kinase (BTK), which phosphorylates the enzyme PLC-beta, augmenting the cytoplasmic Ca^{++} mobilization, and subsequently, mast cell activation and degranulation (Gilfillan and Tkaczyk, 2006; Gilfillan, Austin and Metcalfe, 2011).

In RBL-2H3 cells, both dexamethasone and the PI3K inhibitor LY294002 lowered the levels of the phosphorylated PI3K, Akt and membrane PI3P following Ag-IgE-Fc_{ε}RI reaction. Such an effect was associated with lowered mRNA levels of variable pro-inflammatory cytokines and chemokines (Andrade, Hiragun and Beaven, 2004).

In rat peritoneal mast cells derived from rats overexpressing micro RNA-126, Ag-IgE challenge of these cells induced significantly higher levels of beta-hexosaminidase and histamine release compared to cells derived from wild type rats. These effects were associated with the promotion of Ca^{++} influx and Akt phosphorylation, and were inhibited by the PI3K inhibitor LY294002 (Bao *et al.*, 2018).

Kefiran, the fermented milk beverage produced by the lactobacillus kefiranofaciens in kefir grains, inhibited the antigen-induced degranulation (β-hexosaminidase release) and TNF-α release from mouse bone marrow-derived mast cells (BMMCs) and RBL-2H3 mast cells. These effects were associated with decreased phosphorylation of Akt and ERK (Furuno and Nakanishi, 2012). Additionally, Adenine inhibited antigen/IgE and Ca^{++}

ionophore-induced Ca^{++} mobilisation, β-hexosaminidase release, histamine release and TNF-α production. The anti-inflammatory effect of adenine was associated with inhibition of Syk, Akt and ERK phosphorylation (Hosoi *et al.*, 2018).

The depletion of the $PI3K_{\delta}$ regulatory subunit ($P85_{\alpha}$) and the $PI3K_{\gamma}$ catalytic subunit ($P110_{\gamma}$) in mouse models resulted in severe inhibition of mast cell degranulation, associated with inhibited phosphorylation of Akt and ERK. Such a defect was partially corrected with the expression of constitutively active Akt (Takayama *et al.*, 2012). In mouse bone marrow-derived mast cells, Akt signalling was responsible for production of the pro-inflammatory mediator PGD2 through the phosphorylation of the enzyme cyclooxygenase-2 (COX-2) (Lu *et al.*, 2011). NVP-BEZ235, a strong inhibitor of the PI3K/Akt/mTOR pathway inhibited degranulation and histamine release in human cord blood-derived and lung primary cultured mast cells (Blatt *et al.*, 2012).

The previous data provide strong evidence for the crucial role of the PI3K/Akt pathway in mast cell activation and the generation of the immediate (degranulation) and late (cytokines and chemokines) pro-inflammatory mast cell responses. In our report, both Medihoney and the Manuka extracts strongly and significantly inhibited mast cell degranulation. Interestingly, both honey preparations inhibited the substance P-induced Akt phosphorylation. Such evidence provides a valid and robust explanation for the anti-inflammatory properties underlying the use of these Manuka honey preparations.

7.5.1.2 The effect of the PI3K/Akt pathway and mast cell survival and maturation

PI3K-dependant Akt activation is responsible for the normal growth and maturation of bone marrow mast cell progenitors into mature KIT^{+VE}, $Fc_{\varepsilon}RI^{+VE}$ mast cells (Ma *et al.*, 2011; Blatt *et al.*, 2012). Akt promotes mast cell survival by preventing apoptosis via several mechanisms. Akt phosphorylates the pro-apoptotic enzyme BAD, blocking its inhibitory effects on the anti-apoptotic activity of the enzymes BCL-2 and BCL-X_L (Datta *et al.*, 1997). Akt also phosphorylates the pro-apoptotic enzyme Caspase-9, blocking its pro-apoptotic activity (Cardone *et al.*, 1998). Finally, Akt phosphorylates the members of the forkhead family of transcription factors FKHRL1, AFX, and FKHR1, blocking the transcription of their pro-apoptotic genes (Brunet *et al.*, 1999).

Closer to our experimental model, activation of the RBL-2H3 rat mast cell line with agonists of the A3 adrenergic receptor ($G\alpha_i$ protein-coupled receptor), inosine (10μM) and IB-MECA (10 nM), induced a more than 50% inhibition of mast cell apoptosis following UV light exposure. This effect was associated with increased phosphorylation of Akt, which was $G\alpha_i$ and PI3K-dependant, as shown by sensitivity to pertussis toxin and wortmannin, respectively (Gao *et al.*, 2001).

SHIP is an inositol 5 phosphatase enzyme, which hydrolyses the PI3K-produced PI3P and, subsequently, inhibits PI3P-induced Akt phosphorylation. In a mouse model, knocking out the enzyme SHIP resulted in myeloid hyperplasia with systemic myeloid infiltration. Bone marrow derived mast cells from this model ($SHIP^{-/-}$) were associated with prolonged PI3K-induced PI3P accumulation, and, subsequently, stronger Akt phosphorylation upon treatment with the growth factors IL-3 and GM-CSF, displaying more resistance to apoptosis following growth factor withdrawal, when compared to wild type mouse derived cells (Liu *et al.*, 1999).

Abnormal Akt signaling is involved in other pathological processes involved in the pathophysiology of BPS/IC. For example, the slowly proliferating bladder urothelial cells in BPS/IC patients exclusively produce a small frizzled 8-related peptide molecule called Anti-proliferative factor (Krapf and Goldstein) (Keay *et al.*, 2004; Zhang *et al.*, 2005). APF treatment of primary cultured human urothelial cells from the bladders of healthy controls induced a marked decrease in their proliferation rate (Zhang *et al.*, 2005). This was associated with increased numbers of tetraploid and hyper-tetraploid cells indicating cell arrest at the G2 and M phases of the cell cycle (Rashid *et al.*, 2004). The anti-proliferative and pro-apoptotic effects of APF were associated with its ability to inhibit the serine 473-phosphorylation of Akt (Keay and Zhang, 2016).

These data provide strong evidence for the importance of PI3K/Akt signalling in promoting the development and survival of mast cells.

Excitingly, despite the strong inhibitory effect of both Medihoney and the Manuka extract on Akt phosphorylation, both honey preparations were well tolerated by the LAD2 cells with no element of cytotoxicity.

7.5.2 The effect of Medihoney on STAT3 phosphorylation

Importantly, Both Medihoney and the Manuka honey extract consistently and strongly inhibited the phosphorylation the transcription factor STAT3 at position serine 727. The phosphorylation of STAT 3 at position serine 727 has been linked to the regulation of essential cellular metabolic processes. For example, upon growth factor or insulin stimulation, the activated STAT3 translocates to the nucleus where it forms a complex with the enzyme pyruvate dehydrogenase, enhancing its activity. Such is manifested by enhancement of the conversion of pyruvate to acetyl Co-enzyme A, resulting in increased ATP and energy production (Xu *et al.*, 2016b).

STAT 3 phosphorylation (serine 727) is an essential event underlying mast cell degranulation and pro-inflammatory cytokine release in both rat basophilic leukaemia cells (RBL2H3) (Qin *et al.*, 2016) and human LAD2 cells (Siegel *et al.*, 2013). IgE-FCεRI activation of LAD2 cells was associated with a rapid increase in the phosphorylated levels of STAT 3 (serine 727), while silencing of STAT3 by short interference RNA inhibited both mast cell degranulation and the degranulation-related signalling events such as the phosphorylation of the protein kinases Akt, ERK and $PLC_{\gamma}1$ (Siegel *et al.*, 2013). The accelerated energy production by STAT3 phosphorylation can give a valid explanation of the importance of STAT3 phosphorylation in mast cell pro-inflammatory responses.

7.5.3 P38 MAPK and mast cell biology

Substance P induced phosphorylation of P38 α and γ isoforms in only a single assay, while P38 was not phosphorylated in other 2 assays. Adding to the fact that the P38 MAPK inhibitor SB203580 did not inhibit mast cell degranulation, the involvement of P38 MAPK phosphorylation in substance P-induced LAD2 cell degranulation is strongly doubted. Many studies highlight the involvement of the activated MAPKs in the nuclear expression of mast cell pro-inflammatory mediators following exposure to variable secretagogues. The human defence peptide AG-30/5C induced production of the chemokines MCP-1, MCP-3, IL-8, MIP-1α and MIP-1β, the cytokines GMCSF and TNF-α by the human LAD2 mast cell line. Such a pro-inflammatory effect was G-α_i dependant (as denoted by the sensitivity to pertussis toxin), and included the phosphorylation of the mitogen activated protein kinases P38, ERK and JNK (Kanazawa *et al.*, 2016). In $FC_{\varepsilon}RI$-mediated IgE-anti IgE challenge of two mast cell lines (Human MC15C6 and murine MC/9), the resultant IL-6

production was associated with increased phosphorylation of P38 MAPK. Such an effect was augmented by pre-incubation with the protein phosphatase 2A inhibitor, Okadaic acid, resulting in marked increased in both the anti IgE-induced IL-6 production and P38 phosphorylation (Boudreau, Hoskin and Lin, 2004).

In the HMC1 human mast cell line, the gene expression of the cytokines IL-1β, IL-6, IL-8 and TNF-α (by Reverse Transcription Polymerase Chain Reaction), following Ca^{++} ionophore and phorbol 12myristate 13-acetate (PMA) (Protein kinase C activator) challenge was significantly inhibited with pre-incubation with 30 µM Quercetin (a Flavonoid with kinase inhibition activity) or the selective P38 MAPK inhibitor SB-203580 (5 µM). Such an effect was associated with a marked decrease in the phosphorylation of P38 MAPK and NF_K B (Min *et al.*, 2007).

Additionally, Roxatidine, an h2 receptor antagonist, inhibited PMA (Protein kinase C activator) and Ca^{++} ionophore (binds to and transfers divalent cations such Ca^{++} and Mg^{++} across the cell membrane)-induced IL-1β, IL-6 and TNF-α production by HMC-1 mast cells through the inhibition of the P38 MAPK pathway and nuclear translocation of NF_K B (Lee *et al.*, 2017a). The ability of the P1 and P2 peptides, derivatives of the enzymatic hydrolysis of the cornynebacterium Spirulina, to inhibit the IgE/Fc_εRI-mediated degranulation and IL-13 production in RBL-2H3 rat mast cells was associated with its ability to inhibit the phosphorylation of MAPK ERK1/2, P38 and JNK (Vo *et al.*, 2018). Besides the pro-inflammatory mediator release, P38 MAPKs are involved in the SCF-induced mast cell chemotaxis (Sundström *et al.*, 2001), which, as previously described, drives the extravasation and localization of mast cells in the bladders of BPS/IC patient (Theoharides, Kempuraj and Sant, 2001; Sant *et al.*, 2007b). P38 MAPK alpha also drives the IL 3-induced development and maturation of bone marrow derived mast cells progenitors and positively regulates SCF-induced mast cell chemotaxis through increasing phosphorylation of the cyclic AMP response element binding protein (CREB) and expression of the transcription factor MITF (Hu *et al.*, 2012).

The previous data provide strong evidence for the importance of P38 MAPK signalling as a positive regulator in mast cell maturation, growth, and pro-inflammatory mediator release. However, despite the previous evidence of a positive regulatory role of P38

MAPK on mast cell production of pro-inflammatory cytokines and chemokines, other studies have highlighted a negative regulatory role of P38 on pro-inflammatory mediator release.

P38 MAPK inhibits the release of the growth factor GMCSF by human cord blood-derived mast cells upon IgE/$FC_{\varepsilon}RI$ interaction through the inhibition of the JNK pathway (Kimata *et al.*, 2000). In the rat mast cell line RBL-2H3, P38 inhibitor SB205380 showed a concentration dependant inhibition of IL-4 release, while it rendered a 25% inhibition of TNF-α release only at 0.3 μM concentration, and not at higher concentrations of the drug (Koranteng *et al.*, 2004). Additionally, SB203580 enhanced IL-8 production from the human LAD2 mast cells upon challenge with IL-37, a cationic human defence peptide well known to activate mast cells through MRGPCRX2 (Yu *et al.*, 2017). The lack of clarity regarding the role of this kinase in mast cell signalling will require further exploration of the effect of its inhibition on the substance P-induced mast cell degranulation and pro-inflammatory cytokine release, and how can that relates to the effects of Medihoney.

7.5.4 Extracellular signal-regulated kinases (ERK1/2)

Medihoney pre-incubation inhibited substance P-induced ERK1/2 phosphorylation, however, that was only seen in 2 out of the three assays. However, western blotting results from our lab indicates significant inhibition of substance P-induced ERK1/2 phosphorylation by Medihoney pre-treatment. The specific ERK1/2 inhibitor FR18084 did not inhibit mast cell degranulation, while the MEK1/2 inhibitor U0126 inhibited substance P-induced ERK1/2 phosphorylation and degranulation in LAD2 cells (Lazki-Hagenbach, Ali and Sagi-Eisenberg, 2021). This gives an indication that despite the obvious inhibitory effect of Medihoney against SP-induced ERK1/2 degranulation, such an effect might not be responsible for its mast cell stabilising effect.

Mitogen activated protein kinase MAPK, alternatively termed ERK1/2, has been described as being involved in various aspects of mast cell function in response to a variety of stimuli. In murine bone marrow derived mast cells, IgE/$Fc_{\varepsilon}RI$-induced degranulation was impaired in bone marrow derived mast cells from modified mouse models with the depletion of either P85α (a regulatory unit of $PI3K_{\delta}$) or $P110_{\gamma}$ (The catalytic subunit of $PI3K_{\gamma}$). However, depletion of both P85α and $P110_{\gamma}$ induced total inhibition of mast cell

degranulation. In these models, IgE/FcεRI-dependant PI3K activation resulted in phosphorylation of the kinases AKT and ERK1/2. Additionally, mast cell degranulation was significantly inhibited by inhibitors of Akt and ERK1/2, while a lentivirus vector continuously expressing active Akt restored mast cell degranulation in PI3K-depleted mice models indicating AKT dependency (Takayama *et al.*, 2012).

Inhibition of ERK by U0126 resulted in significant inhibition of IgE/FcεRI-induced release of the pro-inflammatory mediators LTC4, PGD2 and GMCSF, by cultured human mast cells (Kimata *et al.*, 2000). Both Knocking down of ERK1/2 or its inhibition by U0126 (25μM) significantly inhibited FcεRI-induced degranulation and the production of the pro-inflammatory mediators, LTC4, PGD2, TNF-α and IL-6, by murine bone marrow derived mast cells (Hwang *et al.*, 2014). One hour pre-treatment of the HMC-1 human mast cells with 100μg/L of Ecklonia cava (EC), a marine abundant brown algae, inhibited both PMA and Ca^{++} ionophore-induced production of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6, which was associated with inhibition of the phosphorylation of ERK1/2 (Kim, 2014).

7.5.5 WNK1

Medihoney pre-incubation inhibited the substance P-induced phosphorylation of the kinase WNK1. WNK1 is a serine threonine protein kinase involved in intracellular signalling pathways responsible for the regulation of various cellular metabolic processes. WNK1 increases the membrane expression and trafficking of the constitutive glucose transporter (GLUT1) via the phosphorylation of TBC1D1 and TBC1D4, RAB-GTPase activating proteins, in a process that is essential for glucose uptake by the cells (Mendes *et al.*, 2010; Henriques *et al.*, 2020). Down regulation of WNK1 via short interference RNA resulted in a two-fold reduction in the membrane expression of GLUT1 associated with a 60% reduction in glucose uptake by HEK293 cells (Henriques *et al.*, 2020).

Interestingly, Akt like WNK1 phosphorylates TBC1D1, resulting in regulation of its activity, which is associated with increased membrane expression of GLUT1. GLUT1 membrane trafficking was also dependant on increased phosphorylation of m-TOR and P70-S6 kinase downstream of Akt phosphorylation as denoted by sensitivity to the m-TOR inhibitor rapamycin. Akt-induced GLUT membrane trafficking was also observed to enhance insulin-mediated de-oxy glucose uptake in 3T3-L1 mouse adipocytes (Zhou *et al.*, 2008). It

is worth mentioning that Akt can phosphorylate WNK1 (Cheng and Huang, 2011), which adds another possible explanation for its pro- GLUT1 membrane trafficking action.

GLUT1 expression is essential for glucose uptake and, consequently, its utilisation by mast cells. Both allergic (FcεRI-mediated) and pseudo allergic (MRGPRX2-mediated) mast cell pro-inflammatory responses (Immediate degranulation and late phase cytokine release) are dependent on glucose internalisation and metabolism, which can be either anaerobic glycolysis or aerobic mitochondrial oxidation, resulting in ATP production (Kitahata *et al.*, 2010; Caslin *et al.*, 2018; Mendoza, Fudge and Brown, 2021). In the current study, substance P-induced degranulation in LAD2 cells was associated with increased phosphorylation of Akt, WNK1 and the signal transducer and activator of transcription 3 (STAT3), an event that was inhibited through Medihoney 4% pre-incubation.
Akt and WNK1 phosphorylation are known to enhance the membrane trafficking of GLUT1 resulting in increased intracellular glucose uptake and the subsequent metabolic changes, which enhance mast cell activation and pro-inflammatory mediator release. At the same time, STAT3 enhances mitochondrial oxidative respiration enhancing mast cell activation (Bernier *et al.*, 2011a; Erlich *et al.*, 2014).

The previous evidence might provide a possible explanation for the mast cell stabilising effects of Medihoney through the inhibition of glucose uptake and metabolism in mast cells. However, such a conclusion is preliminary and requires further confirmation via studying the levels of GLUT1 membrane trafficking, lactic acid production and ATP production, in addition to oxygen consumption upon LAD2 cell activation with or without Medihoney. Such will be included in a future study aiming at further exploration of the mechanisms behind Medihoney's mast cell stabilising properties.

7.5.6 Glycogen Synthase Kinase (GSK3α/β)

GSK3 is a serine threonine protein kinase that is essential for human mast cell survival (Rådinger *et al.*, 2011). Several reports have linked its activity to pro-inflammatory mast cell responses (Rådinger *et al.*, 2010; Rådinger *et al.*, 2011; Ogawa *et al.*, 2014; Zain, Vidyadaran and Hassan, 2017). In resting cells, GSK3 is in a consistently active state as denoted by phosphorylation at the Y216 position (Hughes *et al.*, 1993). In resting cells, the active GSK3 phosphorylates many microtubule-binding proteins, limiting their ability to bind to microtubules and start micro tubular assembly formations (Kim *et al.*, 2011).

Such assembly is critical for the translocation of mast cell granules to the proximity of the plasma membrane, which is an essential prerequisite for mast cell degranulation (Ogawa *et al.*, 2014). Upon IgE-$Fc_{\varepsilon}RI$ activation of both human (HMC-1) mast cells and C57BL/6 mouse bone marrow derived mast cells, the PI3K-Akt pathway is activated. As a result, the Serine 473 phosphorylated Akt phosphorylates GSK3 at positions serine 21 and serine 9, resulting in inhibition of its activity. As a result, the inhibitory effect of GSK3 3α/β on the assembly of the microtubular proteins is relieved, which allows for proper degranulation and pro-inflammatory cytokine release (Rådinger *et al.*, 2010; Ogawa *et al.*, 2014).

In our experiments, the resting LAD2 cells displayed strong GSK3 phosphorylation levels at positions serine 9 and serine 21. Despite the fact that these levels were not elevated upon substance P activation, MH4% pre-incubation significantly reduced serine 9 and serine 21 GSK3 phosphorylation, contributing to enhancement of its activity. Such may partially explain its mast cell stabilising effects in LAD2 cells.

7.5.7 PRAS40 and Akt

The 40 kDa proline-rich Akt substrate PRAS40 is a protein kinase that has been linked to multiple roles in many physiological and pathological processes. PRAS 40 is both a component and substrate of the mTORC1 complex. Within the mTORC1 complex, PRAS40 binding to mTOR results in an inhibitory effect on mTORC1 complex activity (Wiza, Nascimento and Ouwens, 2012). Phosphorylated Akt and mTORC1 complex Phosphorylate PRAS40 at positions Threonine 246 and Serine 183/212/221, respectively. Subsequently, the phosphorylated PRAS40 dissociates from the mTORC1 complex allowing it to phosphorylate several protein substrates such as 4E-BP1 and p70S6K (Sancak *et al.*, 2007; Zhu *et al.*, 2017).

mTOR is a serine/threonine kinase involved in many mast cell functions in response to a wide range of stimuli. mTOR is phosphorylated by activated AKT in a signalling pathway that is downstream of the PI3K activation seen in GPCR, FCεRI and C-KIT receptor-mediated mast cell activation. The enzyme mTOR has two molecular complexes, which are mTORC1 (rapamycin-sensitive) and mTORC 2 (non-rapamycin-sensitive). The downstream signalling pathway for mTORC1 includes the activation of the ribosomal

enzyme P70S6-kinase, and the transcription factor 4EBP1, resulting in nuclear gene expression regulating SCF-dependant mast cell survival and chemotaxis (Kim *et al.*, 2008; Blatt *et al.*, 2012). Both mTORC complexes are involved in de novo synthesis and release of cytokines and chemokines, but not the degranulation seen following mast cell challenge with IgE or the neuropeptide substance P (Kim *et al.*, 2008; Patel and Theoharides, 2017).

In various malignant conditions, elevated PI3K/Akt pathway activity induced increased phosphorylation of PRAS40, while specific PI3K and Akt inhibitors inhibited PRAS 40 phosphorylation (Yap *et al.*, 2011). The increased PRAS40 activity was associated with poor patient clinical condition and high mortality rates in malignant conditions such as lung adenocarcinoma and cutaneous melanoma (Lv *et al.*, 2017). Nevertheless, the constitutive binding between PRAS40 and P65, the nuclear factor κB (NFκB) subunit, results in the promotion of NFκB transcriptional activity and oncogenesis (Lv *et al.*, 2017). Nevertheless, the main signalling events downstream of PRAS40 activation, including enhancement of the NFκB and mTORC1 activities, have been reported to underlie mast cell pro-inflammatory responses (Patel and Theoharides, 2017; Taracanova *et al.*, 2017). Interestingly, in our mast cell model (LAD2 cells), substance P-induced Akt phosphorylation (serine 473) was accompanied by phosphorylation of PRAS40 (Threonine 246), a finding that is consistent with the previous reports. Interestingly, Medihoney pre-incubation resulted in strong inhibition of the substance P-induced phosphorylation of PRAS40, which might give a valid explanation for Medihoney's mast cell stabilising properties. Moreover, these findings indicate that Medihoney might be able to reproduce the same intracellular signalling events in cancer cells, which may potentially enable it to play a positive role as an anti-cancer agent. Such assumption is supported by several reports highlighting the anti-cancer properties of several honeys of different floral origins (Ahmed and Othman, 2013).

7.5.8 The effect of 10-DEBC on LAD2 cell signalling

10-DEBC HCL is known to be a selective inhibitor of Akt phosphorylation and downstream signalling (Thimmaiah *et al.*, 2005). Accordingly, it was anticipated that pre-incubation of LAD2 cells with 10-DEBC should partly or completely inhibit degranulation, a finding that was seen to be both strong and dose dependent in the current study. Upon further exploration of the effects of 10-DEBC on LAD2 cell signalling, contrary to the effects of

Medihoney, 10-DEBC, at 30 µM concentration, enhanced both substance P and Ca^{++} ionophore-induced Akt phosphorylation at position serine 473 (Figures 36, 39 & 40). This effect was associated with enhancement of substance P-induced phosphorylation of WNK-1 and STAT3 (Figures 37 & 38).

Unlike Medihoney, 10-DEBC pre-incubation failed to negatively regulate the phosphorylation of GSK at positions serine 9/ serine 21, which are the events underlying the inhibition of GSK activity upon mast cell activation. Accordingly, at the most potent mast cell stabilising concentration (30 µM), 10-DEBC, surprisingly, did not inhibit degranulation via the inhibition of the PI3K/Akt signalling pathway but inhibition was instead produced via a strong cytotoxic effect on LAD2 cells. Moreover, 10-DEBC was highly cytotoxic to LAD2 cells producing LDH activity comparative to that seen in the 100% cytotoxicity control, while all Medihoney treatments did not induce any cytotoxicity when compared to untreated controls (Chapter 2, Figures 22 & 23).
Considering these events, it has been suggested that alteration of Akt signalling activity, whether positively (10-DEBC) or negatively (Medihoney), might inhibit LAD2 cell degranulation. Moreover, despite the fact that Akt activity is essential for cell growth and replication, the enhancement of Akt phosphorylation by 10-DEBC was accompanied by severe cytotoxicity, which was not found with any Manuka honey treatments, all of which inhibited Akt phosphorylation.

7.5.9 The effects of the Manuka honey extract on the substance P-induced Akt phosphorylation

Interestingly, a newly developed sugar-free Manuka honey extract, kindly developed by COMVITA, New Zealand, induced a strong dose dependent inhibition of LAD2 cell degranulation culminating at 4% concentration. In a similar fashion to Medihoney, the mast cell stabilising effect of the Manuka extract was accompanied by inhibition of substance P-induced phosphorylation of the protein kinases Akt (Serine 473), STAT3 (Serine 727), PRAS40 (Threonine 246) and WNK1. By way of contrast to Medihoney, the Manuka extract inhibited the substance P induced phosphorylation of the P90 S6 kinase (RSK1/2/3) at serine 380/386/377. It is important to mention that these are the results of 2 assays using 2 and 4% concentrations and further repeats are needed to obtain a robust conclusion.

RSK1/2/3 is a serine threonine protein kinase which is functionally a substrate for and downstream effector molecule of ERK1/2. RSK has 2 protein kinase catalytic domains (phosphorylation sites) at both the C and N-terminal domains. Upon ERK1/2 activation, the phosphorylation of mitogen-regulated domains in the C-terminal domain results in auto phosphorylation of RSK at position Serine 380. This, in turn, creates a docking site for PDK-1, which further phosphorylate RSK at the N-terminal domain. Upon activation, RSK phosphorylates several cytoplasmic and nuclear effectors such as cyclic-AMP response element binding protein (CREB), IκB and c-FOS (Xing, Ginty and Greenberg, 1996; Schouten *et al.*, 1997). Through the phosphorylation of various nuclear and cytoplasmic substrates, RSK can modulate the growth survival and proliferation of many cell lines (Richards *et al.*, 1999). A recent report has highlighted its potential implication in breast cancer cell progression (ZHAO *et al.*, 2016a).

The role of RSK activation in mast cell pro-inflammatory responses is still unclear, and interestingly, RSK phosphorylation at position serine 380 was significantly inhibited upon 2 and 4% Manuka extract pre-incubation, while 4% Medihoney consistently and significantly enhanced this phosphorylation. Accordingly, further studies are required to explore the exact contribution of this kinase in mast cell mediated inflammation.

It is worth mentioning that Manuka extract is sugar-free. This gives it a clear therapeutic advantage over Medihoney in the case of diabetic patients, especially as it possess nearly equivalent anti-inflammatory mast cell stabilising potencies to whole MH preparations. However, the anti-microbial effects of the Manuka extract are yet to be explored.

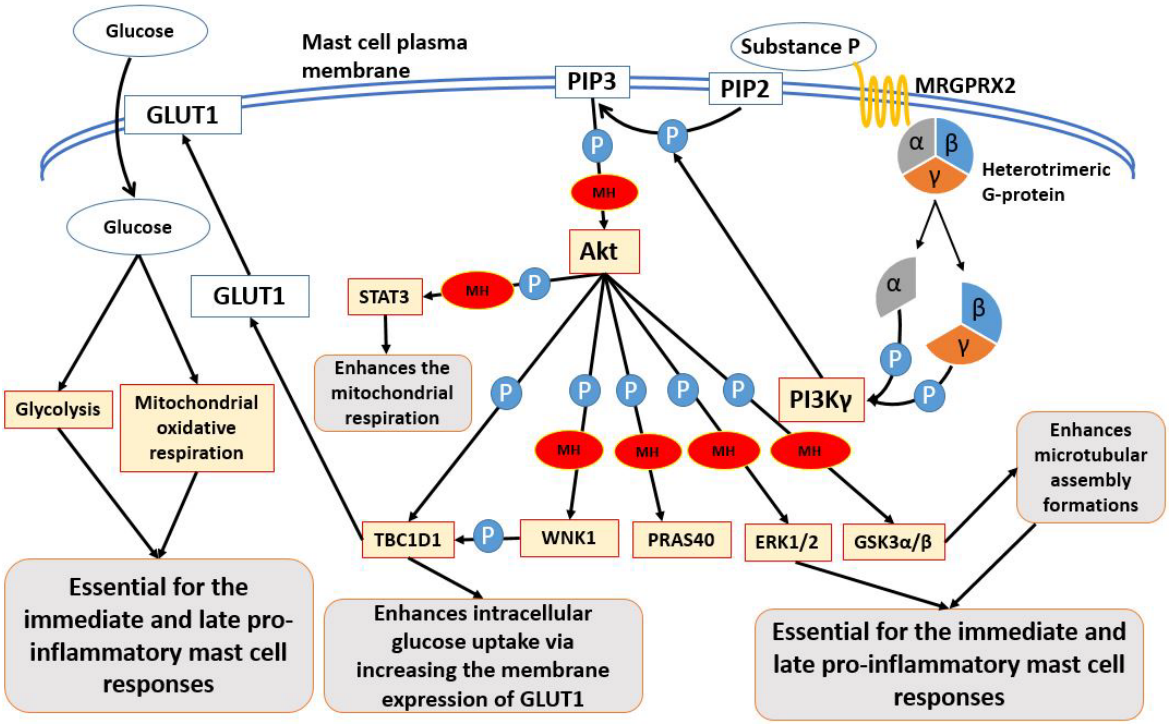

Figure 56: The modulatory effects of Medihoney on the intracellular signalling events underlying the substance P-induced mast cell metabolism and activation. P stands for phosphorylation and MH stands for inhibition of phosphorylation by Medihoney.

7.6 Conclusion

Medihoney has strong anti-inflammatory properties acting as a mast cell stabilizer. This effect is brought about mainly through the inhibition of phosphorylation of protein kinase B (Akt) at position serine 473 and the transcription factor (STAT3) at position serine 727, in addition to the inhibition of phosphorylation of MAPK ERK1/2. Such findings provide the first scientific explanation for the strong mast cell stabilizing properties of Medihoney. Such properties support a possible role for Medihoney as an anti-inflammatory agent in various mast cell driven chronic inflammatory conditions.

8 Chapter Four: Immunohistochemical analysis of mast cell density and their potential involvement in neurogenic inflammation in the bladder of IC/BPS patients

Mast cell numbers and activity are significantly elevated in the bladders of patients with interstitial cystitis (IC/ BPS). This condition is associated with an increased density of bladder sensory nerve endings, which release neuroactive substances, e.g. substance P (SP), thereby inducing mast cell degranulation and tissue inflammation. However, the responsiveness of mast cells to SP varies between different tissues depending on the local cellular microenvironment. MRGPRX2 is a recently identified G-protein coupled receptor involved in mast cell responsiveness to SP in different chronic inflammatory conditions, and its expression is associated with increased tissue inflammation. The responsiveness of urinary bladder mast cells to SP has not yet been explored, which makes theories related to neurogenic inflammation in the pathogenesis of BPS/IC uncertain. In the current study, the density of the bladder mast cells and their potential responsiveness to the neuropeptide SP was investigated through studying the expression of MRGPRX2 on BPS/IC bladder mast cells. For such purpose, urinary bladder biopsies, from 18 consented non-Ulcerative BPS/IC patients and 16 non-inflammatory controls, were serially sectioned and stained with antibodies for the mast cell-specific proteases (Tryptase and Chymase), in addition to MRGPRX2. Interestingly, all the BPS/IC biopsies consistently co-expressed MRGPRX2 as well as Tryptase and chymase, in the detrusor layers of the bladder wall. Mast cell density and % of MRGPRX2 expression was significantly higher in the BPS/IC group compared to controls. This evidence strongly supports the narrative that neurogenic inflammation caused by SP-induced mast cell degranulation is the driving engine for the chronic tissue inflammation in BPS/IC patients. Blocking SP-MRGPRX2 signalling could potentially alleviate longstanding bladder inflammation and pain in this group of patients. Moreover, other MRGPRX2 agonists including morphine, antibiotics e.g. fluoroquinolones and vancomycin, neuromuscular blockers e.g. Atracurium, and bradykinin B2 receptor antagonists e.g. Icatibant, may potentially induce mast cell degranulation and subsequent bladder wall inflammation.

8.1 Introduction and aims of the study

As previously described, neurogenic inflammation, through the interaction between desensitized nociceptive sensory nerve endings and tissue resident mast cells is believed by some experts to play an important role as a driving engine for the initiation and/or maintenance of the chronic bladder wall inflammation seen in the bladders of BPS/IC patients (Ke and Kuo, 2015). However, the narrative of neurogenic inflammation in the pathophysiology of the disease is still doubted by many experts. This is due to the uncertainty regarding some factors essential for the development of neurogenic inflammation in the bladders of the patients. Many reports linked BPS/IC with a characteristic increase in the density of mast cells in the detrusor layer of the bladders of the patients (>32 cells/mm2) (Larsen *et al.*, 1982; Kastrup *et al.*, 1983; Sant and Theoharides, 1994; Yamada *et al.*, 2000; Theoharides, Kempuraj and Sant, 2001; van de Merwe *et al.*, 2008; Liu *et al.*, 2012a; Malik *et al.*, 2018b). However, other reports stated that there is no significant difference in the mast cell numbers between patients compared to controls (Dundore, Schwartz and Semerjian, 1996; Gamper *et al.*, 2015).

Moreover, the neurogenic inflammation theory is based on positive responsiveness of mast cells to the neuroactive agents released by the desensitized sensory nerve endings, including the neuropeptide substance P. Such responsiveness is based on the expression of the neuropeptide receptor MRGPRX2, responsible for substance P mediated mast cell activation in humans, on the surface of mast cells. However, such expression is variable between different microenvironments (Varricchi *et al.*, 2019), and the expression of the receptor on the surfaces of the urinary bladder mast cells has not been explored yet.

The **aim** of the current study was to further explore the importance of the neurogenic inflammation theory in the pathophysiology of the disease. For such purpose, the numbers of mast cells and their expression of the neuropeptide receptor MRGPRX2 in the urinary bladders of BPS/IC patients compared to healthy bladder tissue controls were studied and analysed using an immunohistochemistry approach.

8.2 Methodology

8.2.1 Ethical approval

- Ethical approval for the study was granted by the Research Ethics Committee (REC) (IRAS ID 246784).

8.2.2 Urinary Bladder biopsies

- Formalin Fixed Paraffin embedded urinary bladder biopsies from BPS/IC patients and non-inflammatory controls were kindly provided by the human tissue archive, clinical pathology department, University Hospital Southampton, NHS FT.

8.2.3 Patient and control list preparation

- According to the ethical approval terms, Mr Brian Birch, UHS consultant urologist, and Dr Hannah Markham, UHS clinical pathologist, performed a search through the clinical pathology files to identify patients with the clinical diagnosis of BPS/IC, whose biopsies had been taken for histopathological diagnostic analysis.
- Also, Dr Hannah Markham prepared the control list using the clinical pathology data files.
- After issuing a student research account for Mr Omar Abdelwahab, both lists were carefully reviewed by Mr Abdelwahab to ensure the exclusion of biopsies from patients who met any exclusion criteria or belonged to patients who had not given consent for the use of their tissues for medical research purposes.

8.2.3.1 Inclusion and Exclusion Criteria

8.2.3.1.1 Inclusion Criteria

Samples included are Formalin Fixed Paraffin Embedded (FFPE) urinary bladder biopsies from BPS/IC, which have been excised for diagnostic purposes after taking patient consent. Patients were diagnosed as having BPS/IC according to the following clinical symptoms:

- Chronic Pain (more than 6 months), perceived to be related to the urinary bladder.
- Strong urge to void.
- Day time and night-time frequency
- Absence of exclusion criteria

8.2.3.1.2 Exclusion Criteria

- Urinary tract infection.
- Overactive Bladder
- Carcinoma and carcinoma in situ
- Radiation cystitis
- Chemotherapy with cyclophosphamide

- Immunotherapy, including anti-inflammatory therapy with tiaprofenic acid
- Bladder stone
- Lower ureteric stone
- Urethral diverticulum
- Urogenital prolapse
- Endometriosis
- Vaginal candidiasis
- Cervical, uterine, and ovarian cancer
- Incomplete bladder emptying (retention)

8.2.3.1.3 Control Criteria

As a routine practice in carcinoma in situ patients, FFPE bladder biopsies excised from both the tumour mass and the surrounding non-malignant healthy bladder tissue for optimal analysis. Dr Markham suggested the use of the healthy bladder tissue biopsies of these patients as controls. In addition, another control group included FFPE bladder biopsies excised on top of non-specific chronic bladder inflammatory conditions that did not fall under the clinical diagnosis of BPS/IC.

8.2.3.1.4 Sample Size Calculation

- Sample size calculation was performed in collaboration with Dr Sean Ewings, Research Fellow in the Southampton Statistical Sciences Research Institute, University of Southampton.
- The sample size was calculated as follows:
    (A) A sample of size 68 patients (34 non-Ulcerative BPS/IC patients and 34 histologically normal controls) would allow detection of an effect size of 0.7 (with 80% power and 5% type I error rate). This was deemed sufficient for detection of differences between the non-ulcerative and control groups.
    (B) A sample of size 21 patients in the ulcerative group would provide 80% power to detect an effect size of 0.8 (assuming 80% power and 5% type I error rate). This was deemed sufficient for detecting differences between the ulcerative and each of the non-ulcerative and control groups. The lower sample size for the ulcerative group reflects the larger anticipated differences in mast cell numbers (in the majority of the studies) between

the ulcerative group and the two other groups, in addition to the limited number of people in this group (10 to 16% of BPS/IC patients).

- Based on the sample size calculation, the targeted numbers of biopsies to be included for study was 55 Bladder Pain Syndrome patients (ulcerative (21) and non-ulcerative (34)) and 34 inflammation free controls. These biopsies have been taken for diagnostic purposes and stored in the cellular pathology tissue bank, Level E, University Hospital Southampton.

8.2.3.2 Immunohistochemical analysis

8.2.3.2.1 Antibodies included.

As all of the mast cells express the enzyme tryptase, while only a subtype of mast cells express both the enzymes tryptase and chymase, mast cells have two main subtypes which are mast cell tryptase (MC_T) and mast cell tryptase chymase (MC_{TC}) (Saito *et al.*, 2006). Consequently, mast cells were identified through tryptase and chymase immunostaining using the following antibodies:

- Mouse monoclonal anti human mast cell chymase antibody (CC1) (ab2377, abcam, USA).
- Mouse monoclonal anti human mast cell tryptase antibody (CC1) (ab2378, abcam, USA).

In addition, in order to study the expression of the substance P receptor MRGPRX2, the bladder biopsies were stained with mouse monoclonal anti human MRGPRX2 antibody (ab167125, abcam, USA).

8.2.3.2.2 Immunostaining design

The purpose of using the previously mentioned antibodies is to study the following:

- The density of MC_T
- The density of MC_{TC}
- The percentage of MC_T expressing MRGPRX2.
- The percentage of MC_{TC} expressing MRGPRX2.
- In order to achieve this purpose, 4 serial sections (4µm thickness each) were cut from each bladder biopsy. As mast cell size ranges between 10 and 20 microns (Schulman *et al.*, 1983), Each 2 successive sections will be comparable to each other.
- The first section will be stained with CC1 antibody (anti-Chymase),

- The second will be stained with anti-MRGPRX2 antibody.
- The third section will be stained with AA1 (anti-Tryptase) antibody.
- The fourth section will be stained with CC1 antibody (anti-Chymase) (See table 9).
- Such a design allows for studying the co-expression of MRGPRX2 with both Tryptase and chymase, in addition to the co-expression between tryptase and chymase.
- Finally, another set of 4 serial sections (4µM thickness each), were stained using the same design as described above. To ensure a representative assessment of the bladder tissue in each bladder biopsy, 7 serial sections were cut and discarded before the second set of 4 serial sections to be included in the study were cut. This means that each set of 4 serial sections stained in the study were 7 sections away from the other set (Larsen *et al.*, 2008).

Table 9: The study design for immunostaining of a set of 4 serial sections from the bladder biopsies.

	Slide 1	Slide 2	Slide 3	Slide 4
Thickness	4µm	4µm	4µm	4µm
Antibody	CC1	Anti-MRGPRX2	AA1	CC1

8.2.3.2.3 Antibody optimisation

- Antibody concentrations were titrated and optimised using both control tissues and BPS/IC urinary bladder biopsies. Tonsil tissue was used as a control for AA1 and CC1 antibodies, while bone marrow tissue was used as a control for anti-MRGPRX2 antibody (Figures 57, 58 & 59).

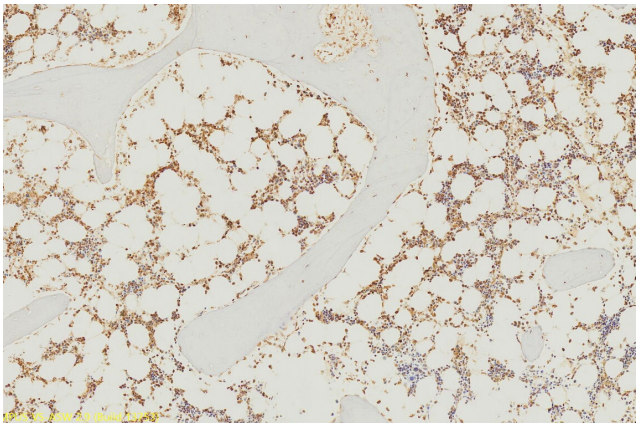

Figure 57: Section of bone marrow tissue biopsy (control tissue) from systemic mastocystosis patient stained with anti-MRGPRX2 antibody (1:100) concentration.

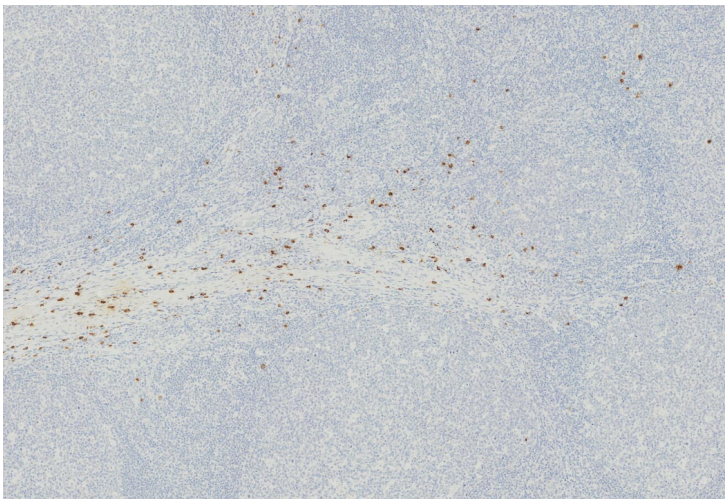

Figure 58: Section of tonsil tissue biopsy (control tissue) stained with CC1 antibody (1:5000) concentration.

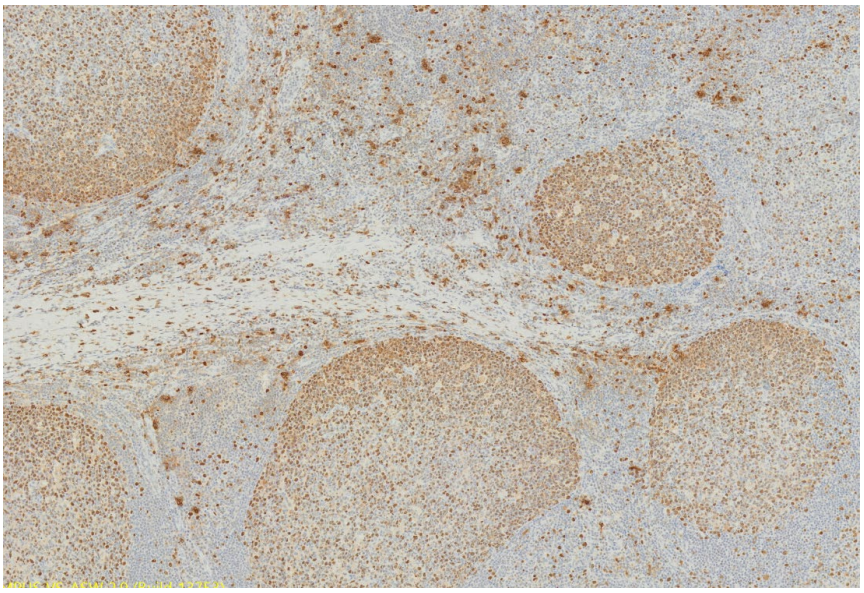

Figure 59: Section of tonsil tissue biopsy (control tissue) stained with AA1 antibody (1:20,000) concentration.

- The optimal concentration for AA1 (anti-Tryptase) antibody was 1:20,000 without mouse linker and low PH.
- The optimal concentration for CC1 (anti-Chymase) antibody was 1:500 and low PH.
- The optimal concentration for the anti-MRGPRX2 antibody was 1:100.
- It is worth mentioning that the anti-MRGPRX2 antibody used in the study is the only monoclonal antibody that is suitable for immunohistochemistry of formalin fixed paraffin embedded (FFPE) tissues, hence the reason it was chosen.
- Anti-MRGPRX2 antibody requires enzymatic antigen retrieval by proteinase K, while the AA1 and CC1 antibodies require heat-mediated antigen retrieval. This does not allow for multiplex staining using MRGPRX2 with CC1 or AA1 antibodies in the same slide. As a result, single staining of serial 4µm thickness sections was the best option to be implemented. Moreover, the staining de-staining technique used in the multiplex staining of the same slide might affect the target epitopes on the mast cells.

8.2.3.3 Immunohistochemistry staining protocol.

- FFPE urinary bladder biopsies were cut into 4µm thickness sections using microtome, then, the sections were mounted on glass slides.
- Dewaxing was started in oven at 60°C for a minimum of one hour.

- In case of heat-mediated antigen retrieval (AA1 and CC1 antibodies), dewaxing was continued in a PT Link pre-treatment platform (Aloe and Chaldakov), which also performs heat-mediated antigen retrieval in low PH (6) at 97°C for 20 minutes.
- For enzymatic antigen retrieval (anti-MRGPRX2 antibody), the slides were dewaxed through 4 rounds of washing in xylene followed by 4 rounds of washing in 100% alcohol, and finally, rehydration in water to prevent drying (1 minute per round).
- Regarding slides stained with anti-MRGPRX2 antibody, the enzymatic antigen retrieval by proteinase K was performed in the autostainer platform.
- Finally, all the slides were stained using an autostainer platform based in the clinical pathology department, UHS, NHS FT. The standardised staining process was performed according to the following steps:
    (a) Wash buffer rinse for 5 minutes.
    (b) Peroxidase block, which blocks any enzyme that can interact with the antibody or the chromogen (DAB) (20 minutes).
    (c) Another Wash buffer rinse.
    (d) Proteinase K antigen retrieval for the anti-MRGPRX2 stained slides (20 minutes).
    (e) Adding primary antibodies (20 minutes).
    (f) Wash buffer rinse (5 minutes).
    (g) Adding Mouse linker (20 minutes), which acts as a secondary antibody giving a stronger staining pattern. Mouse linker was not used with the AA1 antibody to avoid excessive background staining.
    (h) Adding Horse radish peroxidase for 20 minutes followed by another 5 minutes rinse with the wash buffer.
    (i) Adding the chromogen (DAB) for 2 washes, 5 minutes each. DAB was used fresh in each staining session to ensure proper blocking and minimise background staining.
    (j) Another 5-minute rinse with the wash buffer followed by adding the counter stain (Haematoxylin).
    (k) Finally, slides were washed with deionised water for 5 minutes.

8.2.3.4 Image Analysis

8.2.3.4.1 Slide scanning

- Slides were microscopically scanned at a maximal magnification power of 20X in the biomedical imaging Unit, Faculty of Medicine, University of Southampton.
- The produced vsi (visual studio installer) files of the scanned slides were viewed using OLYMPUS OLyVIA software, where smaller views at the same positions in each 4 successive sections were cropped to create new comparable tiff (tagged image file format) files for further analysis (Figure 60).

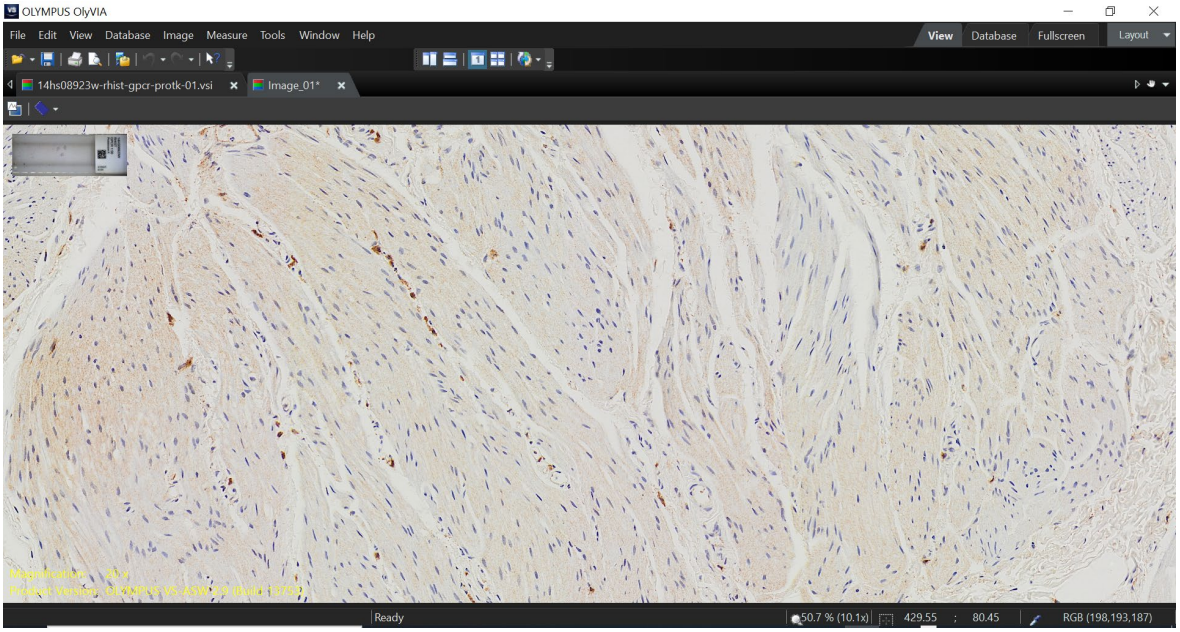

Figure 60: A cropped view (tiff file) taken from a vsi file of a BPS/IC bladder biopsy stained with anti-MRGPRX2 (slide 2) using OLYMPUS OLyVIA software.

- Each set of 4 tiff files representing the same view on 4 successive sections of the same bladder biopsy were opened by Adobe Photoshop software and copied as 4 layers in a single Photoshop document (PSD) file (Figures 61 & 62). Using the “Free Transform” option, the 4 layers can be aligned with each other to create new aligned 4 tiff files, which are nearly identical images of each other, allowing for proper analysis of the co-expression patterns between the different antibodies on these successive slides.

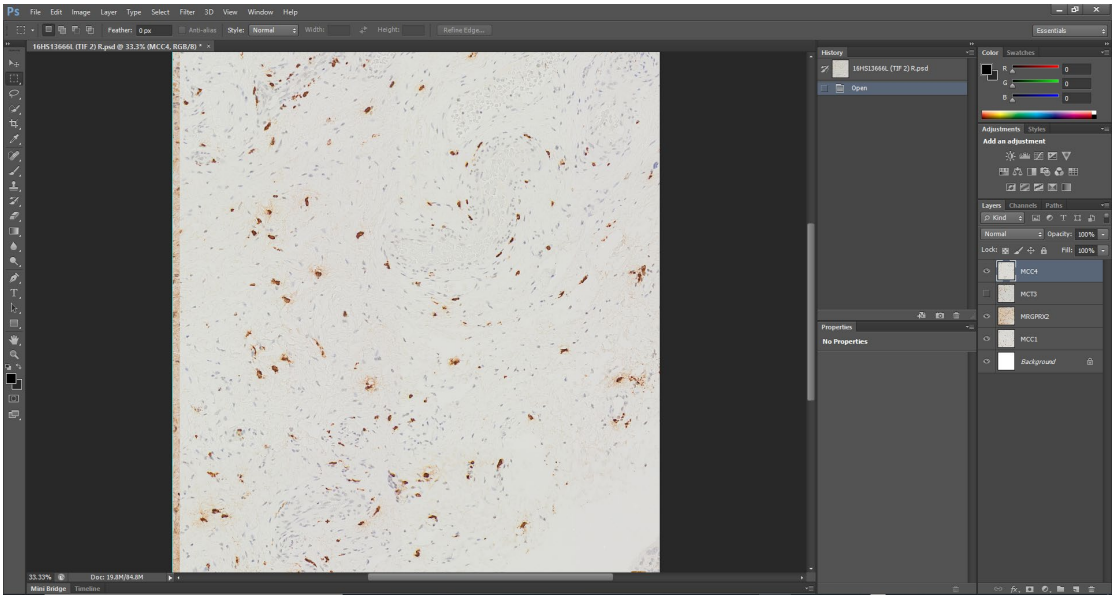

Figure 61: Layer 4 (Chymase) view of a 4-layered Photoshop (psd) file.

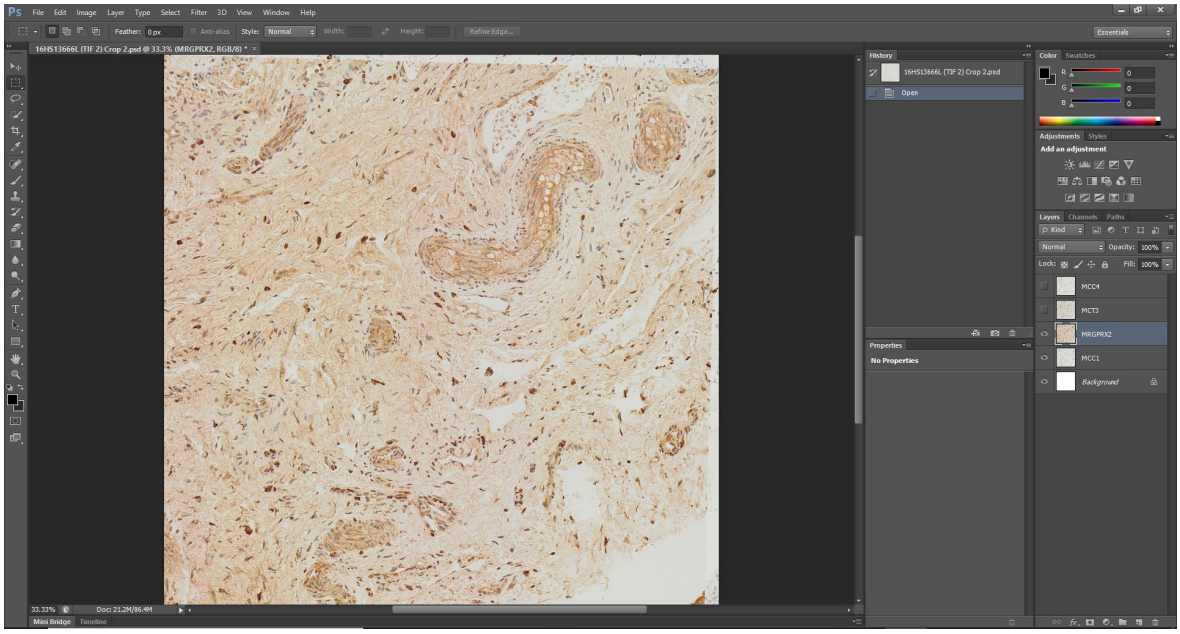

Figure 62: Layer 2 (MRGPRX2) view of the same 4-layered Photoshop (psd) file featured in figure 61.

- Finally, the co-expression patterns were analysed in the newly aligned tiff files using Fiji software. The position of the mast cells stained with AA1 (anti-tryptase) antibody will be marked in the third section (tiff file), creating a template marking the positions of mast cells stained with tryptase (all mast cells). This template was superimposed onto the two successive (neighbouring) slides, which are slides number 2 and 4, allowing for studying the co-expression of tryptase with MRGPRX2 or chymase, respectively (Figure 64).

- Similarly, another template was created in slide 1 to mark the positions of mast cells stained with chymase (MC_{TC}), which was superimposed onto slide 2 to allow the analysis of the co-expression between MCC1 and MRGPRX2 (Figure 63).
- In addition, the numbers of mast cells stained with tryptase and chymase were quantified by the cell counter in the Fiji software.

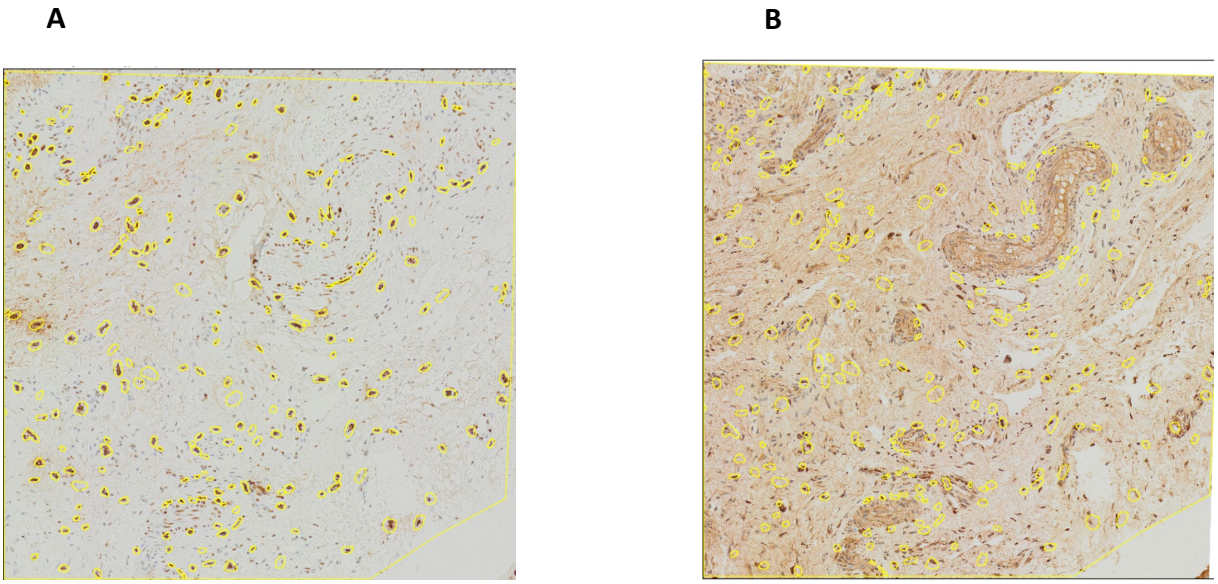

Figure 63: (a) Slide number 3 (stained with tryptase) from a urinary bladder biopsy of BPS/IC patient showing a template marking the positions of mast cells stained with tryptase using Fiji software (ROI). (b) The same template (ROI) is superimposed to slide number 2 to study the co-expression between tryptase and MRGPRX2.

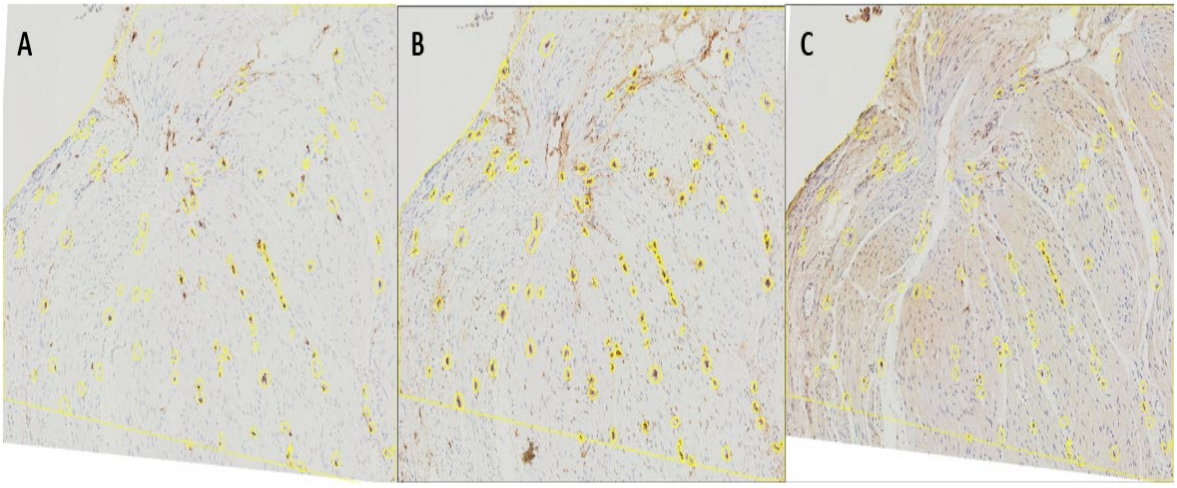

Figure 64: Three serial sections from BPS/IC bladder biopsy stained with anti-chymase (A), anti-tryptase (B) and anti-MRGPRX2 (C). The same ROI (region of interest) marking the positions of the tryptase-stained mast cells in section (B) is superimposed on the slides (A) and (C) to study the co-expression of tryptase with chymase and MRGPRX2, respectively. ROI was established using Fiji Image J software.

- It is worth mentioning that cases and controls were stained on two separate sessions. In both sessions, all the conditions were standardised using the same antibody lots, the same equipment and autostainer platform, and control tissue was stained in each staining session to provide a consistent and comparable staining pattern (Figures 65, 66 & 67).

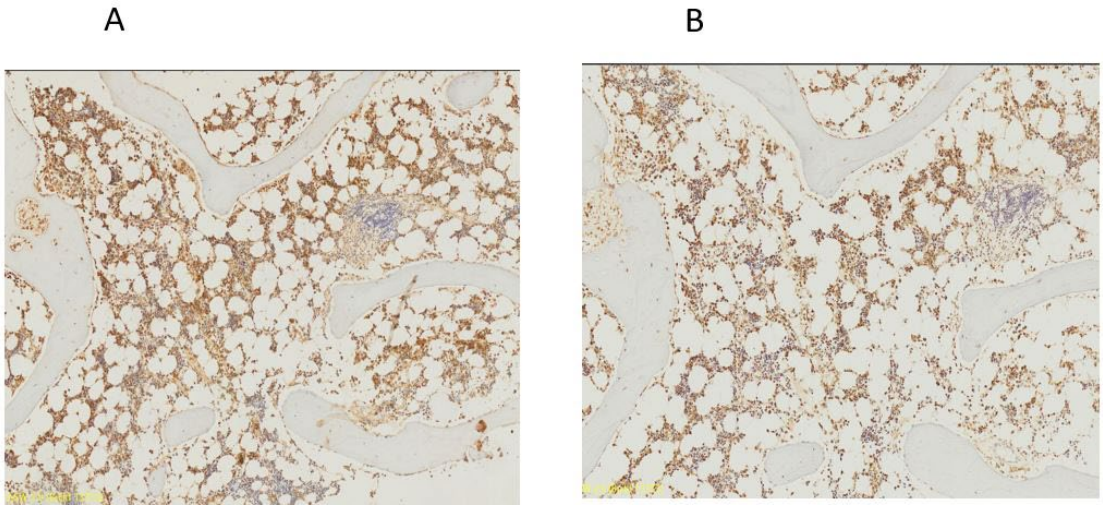

Figure 65: Bone marrow tissue from the same systemic mastocystosis patient (Control tissue) stained with anti-MRGPRX2 antibody in the cases (A) and the controls (B) staining sessions. In both images, mast cells stained positively for MRGPRX2 indicating consistent staining pattern in both the patients and the controls staining sessions.

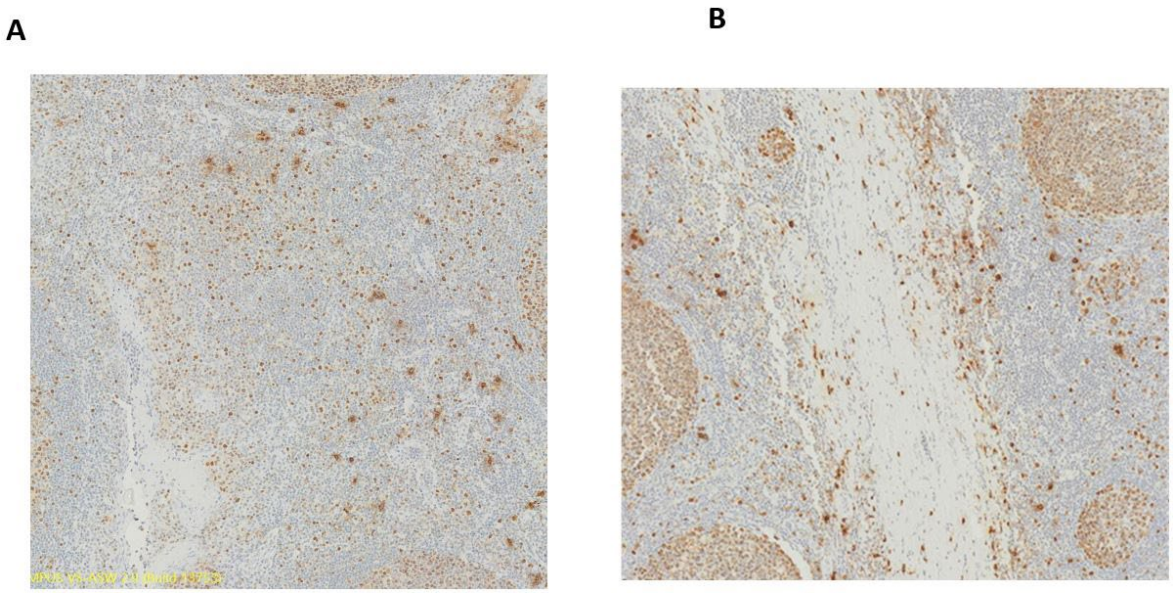

Figure 66: Tonsil tissue (Control tissue) stained with AA1 in the cases (B) vs the controls (B) staining sessions. In both images, mast cells stained positively for tryptase indicating consistent staining pattern in both the patients and the controls staining sessions.

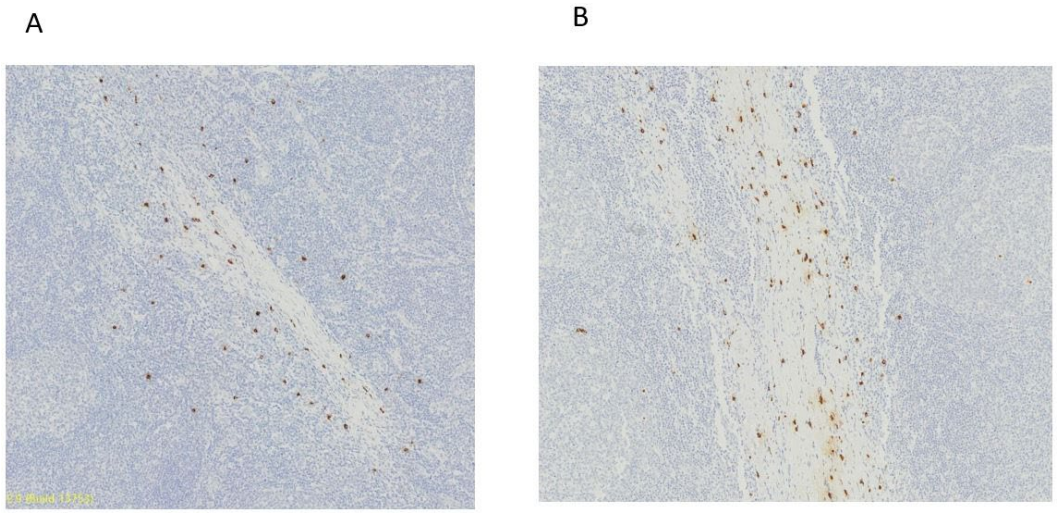

Figure 67: Tonsil tissue (Control tissue) stained with CC1 antibody in the cases (a) vs the controls (b) staining sessions. In both images, mast cells stained positively for chymase indicating consistent staining pattern in both the patients and the controls staining sessions.

- Elevation of the mast cell density above 32 cells/mm2 (detrusor mastocystosis) is considered as one of the histological diagnostic criteria of BPS/IC (van de Merwe *et al.*, 2008). Accordingly, differences in the mast cell density and MRGPRX2 expression between biopsies of the patients and controls were analysed in the detrusor layer, especially as the detrusor is more intact and subject to less

fragmentation compared to the lamina propria, keeping the cells in their original positions.

- In the current study, the density of both MC_T and MC_{TC}, in addition to the MRGPRX2 expression by both MC_T and MC_{TC} was calculated and compared between the patients and the controls.
- Statistical analysis was performed using SPSS 27 software.

8.2.4 Study Sample

8.2.4.1 BPS/IC biopsies

In the current study, only 18 cases and 16 controls were involved. This was the result of many factors that resulted in very low tissue availability, which included:

(a) Despite the recently increased prevalence and incidence rates of BPS/IC (Curhan *et al.*, 1999; Mishra, 2015; Vella, Robinson and Cardozo, 2015), the practice of taking urinary bladder biopsies for histopathological analysis is not recommended, being an invasive technique that may carry the risk of urinary tract infections, excessive bladder bleeding and bladder perforation (Cox *et al.*, 2016) . Consequently, the numbers of the BPS/IC urinary bladder biopsies available for the research purposes are much lower compared to the patients diagnosed with BPS/IC.

(b) This was made worse by the fact that a number of patients do not approve their surplus tissues to be used in research studies after the end of the histopathological diagnostic analysis. In the current study, more than half of the BPS/IC patient biopsies available in the human tissue archive were not granted approval for research use.

(c) Many of the urinary bladder biopsies from BPS/IC patients that have been granted research use approval have been extensively used in diagnostic analyses. As a result, many of the bladder biopsies utilised in the current study did not include enough tissue to repeat the staining.

(d) Many bladder biopsies from BPS/IC patients were severely deformed with a lot of artefacts due to the waxing and dewaxing processes, especially as the bladder tissue is thin and friable. As a result, many bladder biopsies were excluded despite their clinical history suggesting that they would have been useful for the study.

(e) Collectively, all these factors inflected negatively upon the study sample size, where the targeted sample size could not be achieved. As a result, only 18 BPS/IC biopsies were involved in the current study.

(f) Importantly, all of these cases belonged to the non-ulcerative group, as there was no urothelial ulceration on cystoscopy with hydrodistension (van de Merwe *et al.*, 2008; Malde and Sahai, 2020).

8.2.4.2 Controls

- Based on the fact that bladder biopsy excision is an invasive and risky technique, it is impossible to obtain a perfectly healthy bladder tissue as an ideal control tissue for urinary bladder research purposes. In the current study, the best approach that could be taken was kindly suggested by Dr Hannah Markham, who recommended the use of healthy bladder tissues excised from patients with bladder pathologies. Such a control group included 16 bladder biopsies.

8.2.4.3 Age and gender differences between the study groups

- As a result of the above-described limitations of the tissue availability, it was not possible to perform a proper age and gender matching between the groups, which varied between the study groups according to the nature of the pathological condition of each group.

8.2.4.4 Gender differences between the study groups

- Regarding the gender, about 15 out of the 18 BPS/IC bladder biopsies belonged to female patients, while only 3 biopsies belonged to male patients. Such is consistent with the previous reports stating that BPS/IC is 10 times prevalent in females than in males (Nickel *et al.*, 2005b).
- Regarding the control group, 11 out of 16 bladder biopsies belonged to male patients.

8.2.4.5 Age differences

- Regarding the age, the age of the BPS/IC group averaged at 51.06, which was significantly lower when compared to the control group, which averaged at 66 years ($P<0.05$).
- Generally speaking, the limitations in tissue availability for both BPS/IC patients and the controls made a proper age and sex matching an impossible task.

- However, going through the literature, there was no evidence suggesting significant gender-based differences in the mast cell counts between males and females.

8.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9. Normality of the data were assessed using Anderson-Darling test, while statistical differences were analysed using unpaired t-test and Mann-Whitney U test.

8.3 Results

- 18 biopsies from the BPS/IC group and 16 control biopsies were involved in the current study. The BPS/IC patients were diagnosed based on the 2008 criteria of the European Society of the Study of Interstitial cystitis (Préfontaine *et al.*). The control group involved healthy bladder tissue from patient with bladder pathologies that did not fall under the diagnosis of BPS/IC. The study focused on the analysis of the mast cell numbers and their expression of the MRGPRX2 receptor in both the detrusor and the lamina propria layers of the bladder wall, knowing that detrusor mastocystosis is considered a histological diagnostic criterion for BPS/IC (van de Merwe *et al.*, 2008; Nordling, Fall and Hanno, 2012).

8.3.1 The Density of mast cells stained with tryptase and chymase and their expression of MRGPRX2 in the detrusor layer of the BPS/IC patient group compared to the controls

8.3.1.1 Density of the tryptase^{+ve} mast cells in the detrusor layer of the BPS/IC patients vs controls

In the BPS/IC group, mast cells were abundant in the detrusor layer. The mean (± SEM) density of the Tryptase^{+ve} mast cells (all mast cells) was 166.1 ± 21.02 cells/mm^2, which was significantly higher compared to the control group (72.64 ± 9.150; Figure 68).

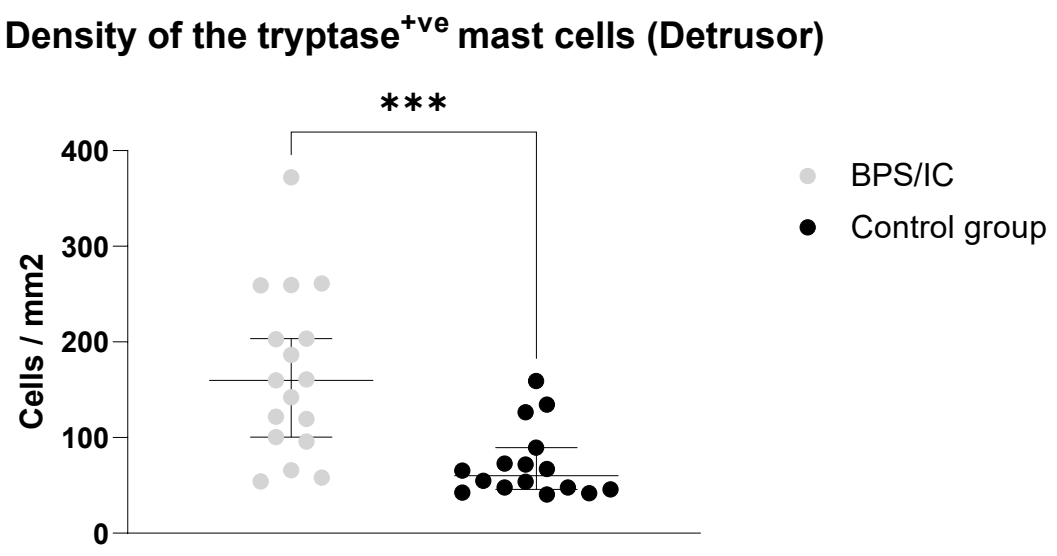

Figure 68: Scatter plot representing the density of mast cells stained with tryptase in the urinary bladder detrusor layer of the patients vs controls. Each bar represents the median with 95% confidence interval. Statistical differences were analysed by Mann-Whitney U test using GraphPad Prism 9 software (*** P value <0.0005).

8.3.1.2 Density of the chymase^{+ve} mast cells in the detrusor layer of the BPS/IC patients vs controls

In the BPS/IC group, the mean (± SEM) density of the Chymase^{+ve} mast cells was 155.7 ± 23.86, which was significantly higher compared to the control group (59.83 ± 9.153; Figure 69).

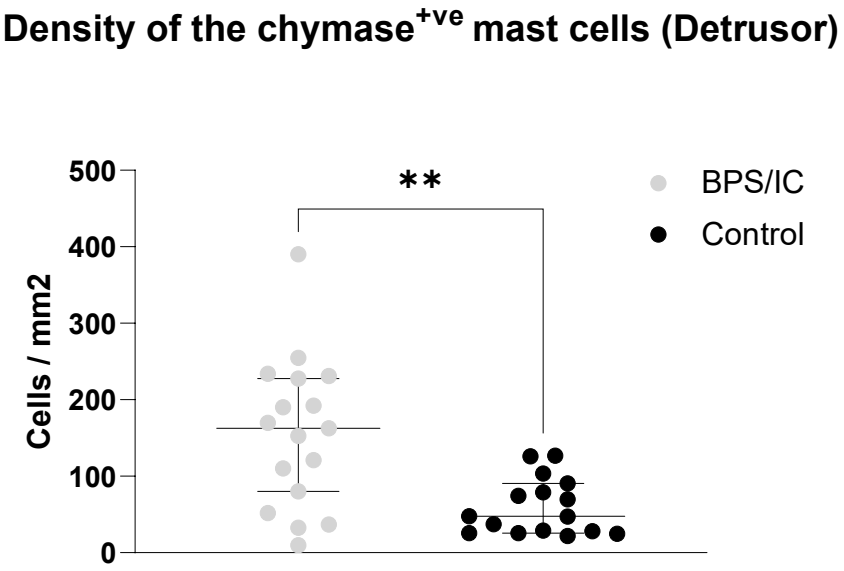

Figure 69: Scatter plot represent the density of the chymase^{+ve} mast cells in the urinary bladder detrusor layer of the patients vs controls. Each bars represents the median with

95% confidence interval. Statistical differences were analysed by Mann-Whitney U test using GraphPad Prism 9 software (**P<0.005).

8.3.1.3 The percentage of MRGPRX2 expression by the tryptase^{+ve} mast cells in the detrusor layer of the BPS/IC patients vs controls

- In the BPS/IC group, the mean (± SEM) of the percentage MRGPRX2 expression by the Tryptase^{+ve} mast cells was 67.99 ± 2.537, which was significantly higher compared to the control group (28.38 ± 6.004; Figure 70).

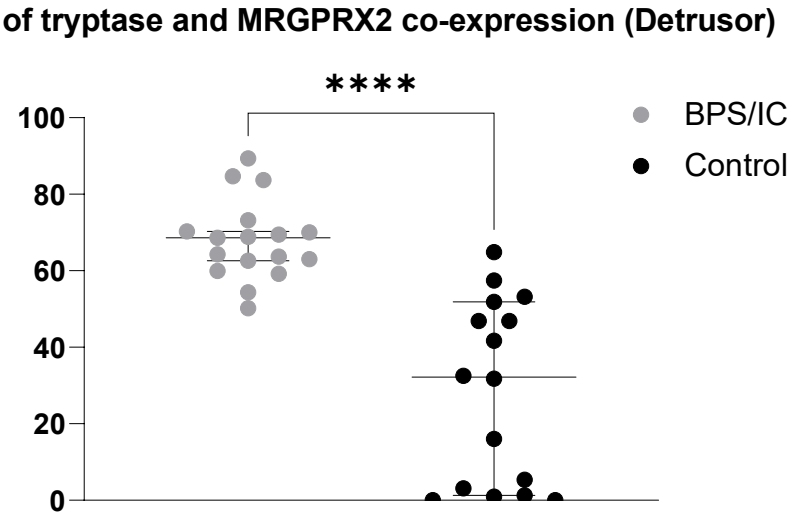

Figure 70: Scatter plot representing the percentage of MRGPRX2 expression by the Tryptase^{+ve} mast cells in the urinary bladder detrusor layer of the patients vs controls. Each bar represents the median with 95% confidence interval. Statistical differences were analysed by Mann-Whitney U test using GraphPad Prism 9 software (**** P value <0.0001).

8.3.1.4 The percentage of MRGPRX2 expression by the chymase^{+ve} mast cells in the detrusor layer of the BPS/IC patients vs controls

- In the BPS/IC patient group, the mean (± SEM) percentage of MRGPRX2 expression by the Chymase^{+ve} mast cells was 66.66 ± 2.629 , which was significantly higher compared to the control group (31.44 ± 6.046; Figure 71).

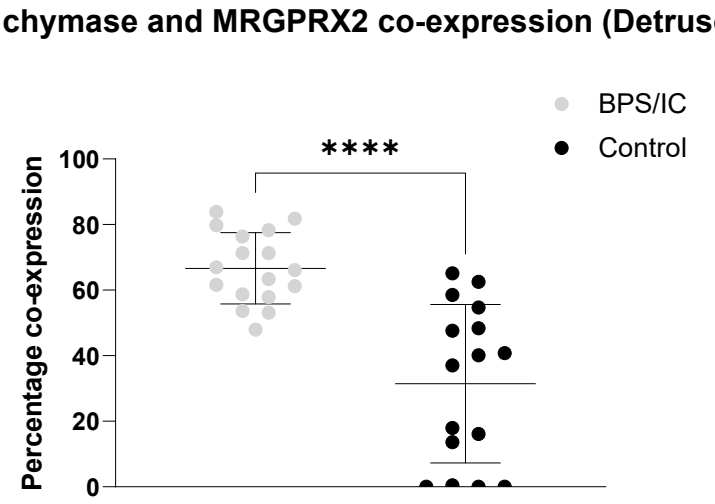

Figure 71: Scatter plot representing the percentage of MRGPRX2 expression by the chymase^{+ve} mast cells in the urinary bladder detrusor layer of the patients vs controls. Each bar represents the mean ± standard deviation. Statistical differences were analysed by unpaired t-test using GraphPad Prism 9 software (** P value <0.01 and **** P value < 0.0001).

8.3.1.5 The percentage of chymase expression by the tryptase^{+ve} mast cells in the detrusor layer of the BPS/IC patients vs controls

- In the BPS/IC group, the means (± SEM) percentage of chymase expression by the tryptase^{+ve} mast cells was 70.95 ± 5.371, which was significantly higher compared to the control group 1 (54.45 ± 4.546; Figure 72).

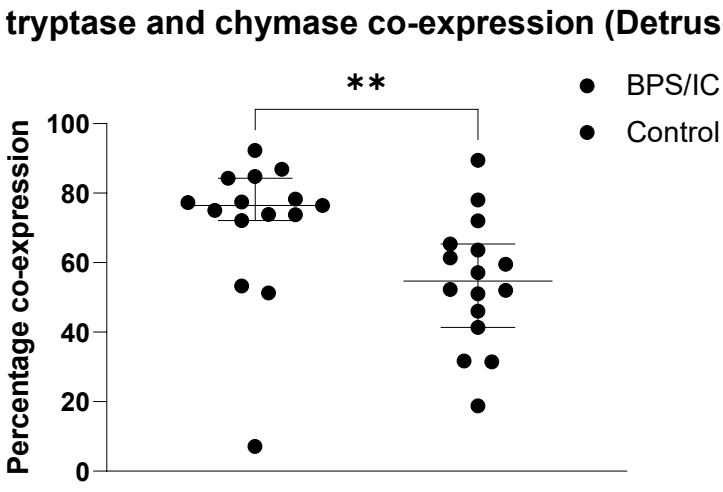

Figure 72: Scatter plot representing the percentage of the tryptase^{+ve} mast cells that express chymase in the urinary bladder detrusor layer of the patients vs controls. The bars represent median with 95% confidence interval. Statistical differences were analysed by Mann-Whitney U test using GraphPad Prism 9 software (** P value <0.01).

8.3.2 The Density of mast cells stained with tryptase and chymase and their expression of MRGPRX2 in the lamina propria of the BPS/IC patient group compared to the controls.

8.3.2.1 Density of the tryptase^{+ve} mast cells in the lamina propria of the BPS/IC patients vs controls

- Similar to the detrusor layer, mast cells were abundant in the lamina propria of the BPS/IC group. In the lamina propria of the BPS/IC group, the mean (± SEM) density of the tryptase^{+ve} mast cells was 215.0 ± 19.05 cells/mm2, which was significantly higher compared to the control group (84.88 ± 9.212; Figure 73).

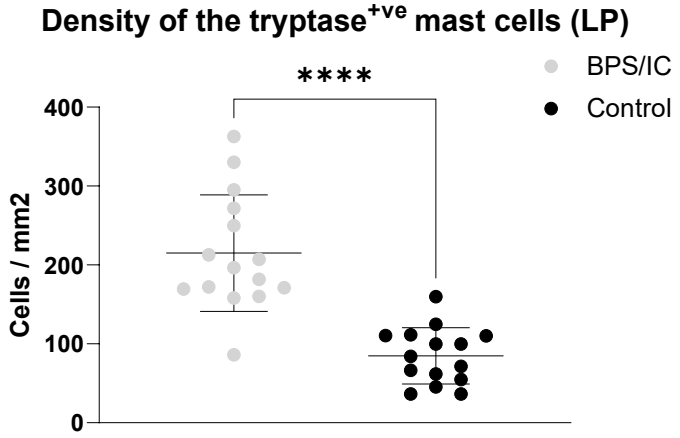

Figure 73: Scatter plot representing the density of the tryptase^{+ve} mast cells in the urinary bladder lamina propria (LP) of the BPS/IC patients vs controls. Each bar represents the mean ± standard deviation. Statistical differences were analysed by unpaired t-test using GraphPad Prism 9 software (**** P value = 0.0001).

8.3.2.2 Density of the chymase^{+ve} mast cells in the lamina propria of the BPS/IC patients vs controls

- In the lamina propria of the BPS/IC group, the mean (± SEM) density of the chymase^{+ve} mast cells was 181.6 ± 17.09, which was significantly higher compared to the control group 1 (65.27 ± 8.710; Figure 74).

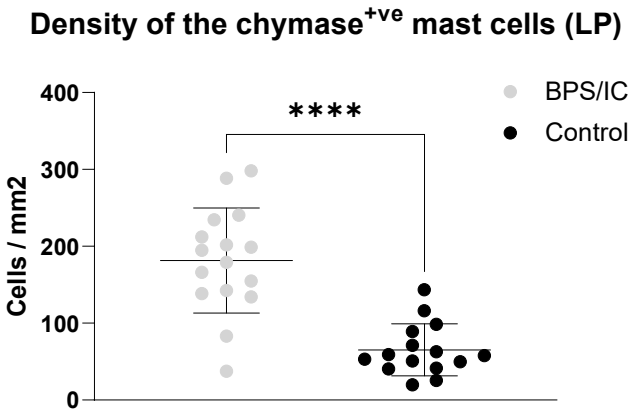

Figure 74: Scatter plot represent the density of the Chymase^{+ve} in the urinary bladder lamina propria (LP) of the patients vs controls. Each bars represents the mean ± standard deviation. Statistical differences were analysed by unpaired t-test using GraphPad Prism 9 software (**** P value = 0.0001).

8.3.2.3 The percentage of MRGPRX2 expression by the tryptase^{+ve} mast cells in the lamina propria of the BPS/IC patients vs controls

- In the lamina propria of the BPS/IC group, the mean (± SEM) percentage MRGPRX2 expression by the tryptase^{+ve} mast cells was 61.75 ± 3.366, which was significantly higher compared to the control group (29.97 ± 6.352; Figure 75).

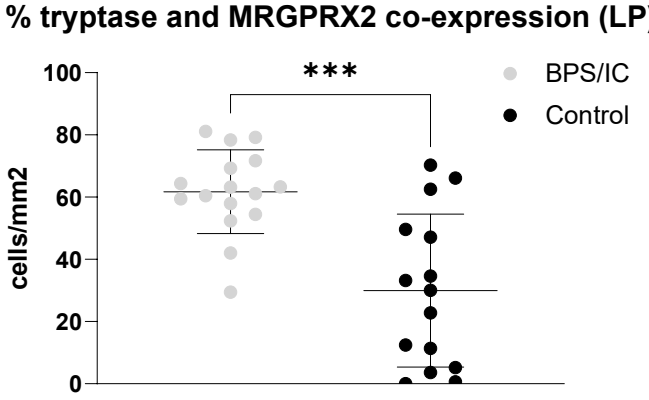

Figure 75: Scatter plot representing the percentage of the tryptase^{+ve} mast cells expressing MRGPRX2 in the urinary bladder lamina propria of the patients vs controls. Each bar represents the mean ± standard deviation. Statistical differences were analysed by unpaired t-test using GraphPad Prism 9 software (*** P value 0.0001).

8.3.2.4 The percentage of MRGPRX2 expression by the chymase^{+ve} mast cells in the lamina propria of the BPS/IC patients vs controls

- In the lamina propria of the BPS/IC patient group, the mean (± SEM) percentage of the chymase^{+ve} mast cells that expressed MRGPRX2 was 61.64 ± 3.975, which was significantly higher compared to the control group (24.99 ± 7.296; Figure 76).

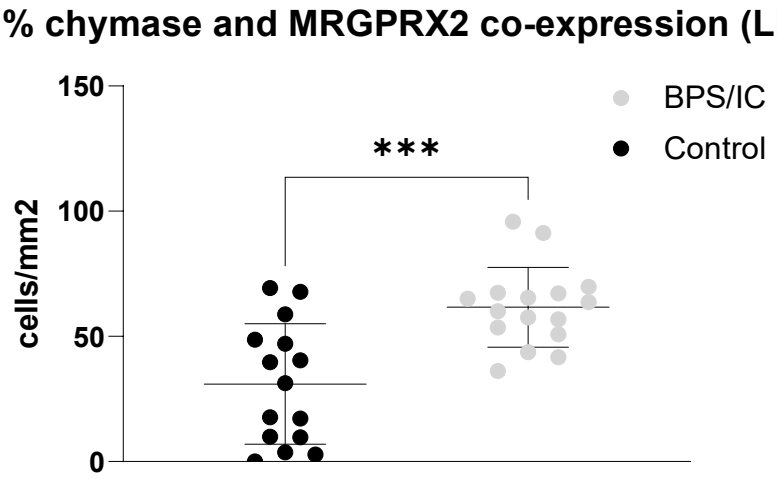

Figure 76: Scatter plot representing the percentage of MRGPRX2 expression by the chymase^{+ve} mast cells in the urinary bladder lamina propria (LP) of the patients vs controls. Each bar represents mean ± standard deviation. Statistical differences were analysed by unpaired t-test using GraphPad Prism 9 software (*** P value 0.002).

8.3.2.5 The percentage of Chymase expression by the tryptase^{+ve} mast cells in the lamina propria of the BPS/IC patients vs controls

- In the lamina propria of the BPS/IC group, the mean (± SEM) percentage of chymase expression by the tryptase^{+ve} mast cells was 84.47 ± 4.209, which was significantly higher compared to the control group (55.21 ± 4.332; Figure 77).

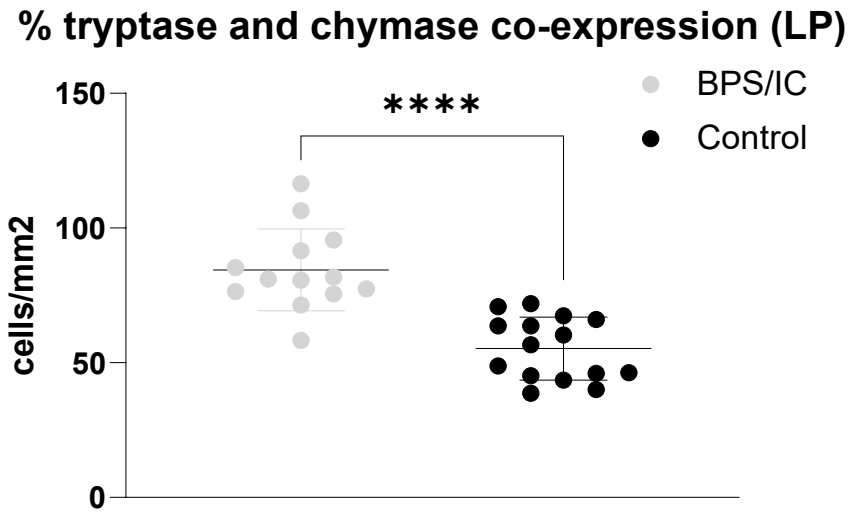

Figure 77: Scatter plot representing the percentage of the tryptase^{+ve} mast cells that express chymase in the urinary bladder lamina propria (LP) of the patients vs controls. The bars represent the mean ± standard deviation. Statistical differences were analysed by unpaired t-test using GraphPad Prism 9 software (**** P value <0.0001).

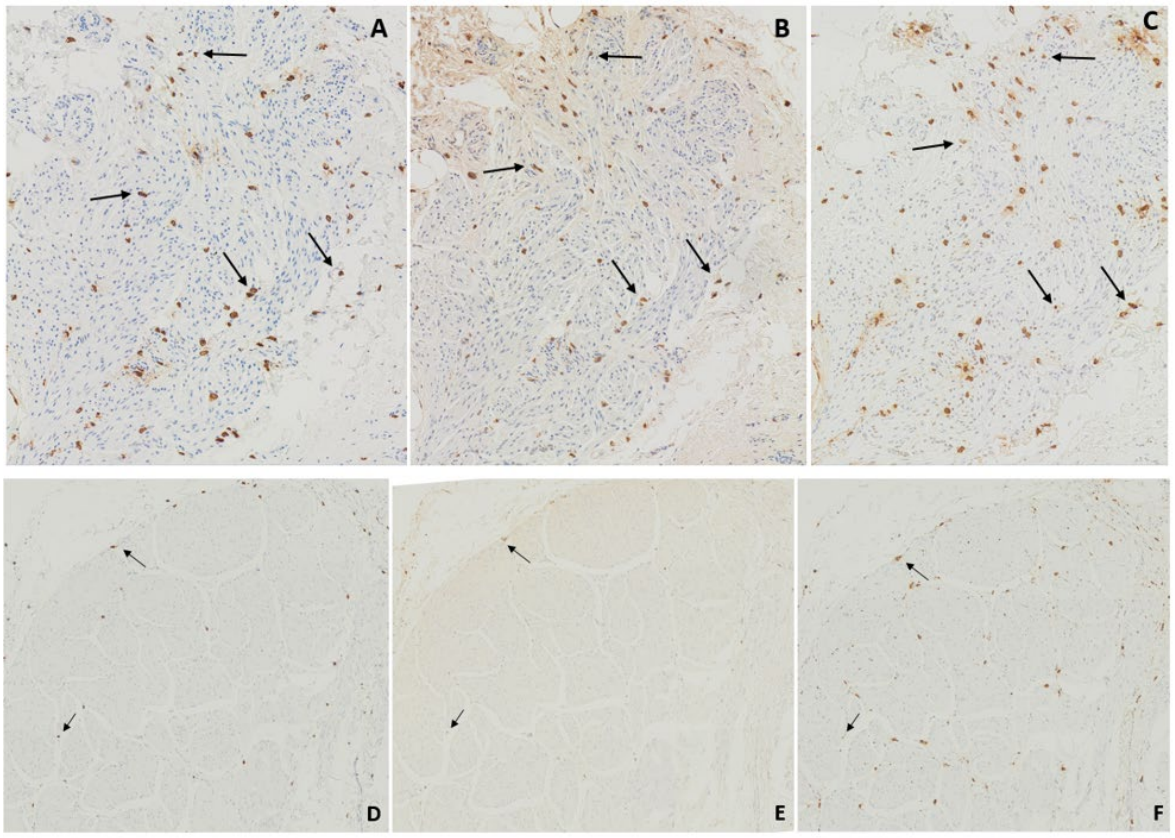

Figure 78: Serial sections of 2 urinary bladder biopsy from an BPS/IC patient (A, B and C), and healthy bladder tissue control (D, E and F) showing mast cells in the detrusor layer, some are indicated in arrows, stained with antibodies for mast cell chymase (A & D), MRGPRX2 (B & E) and mast cell tryptase (C& F).

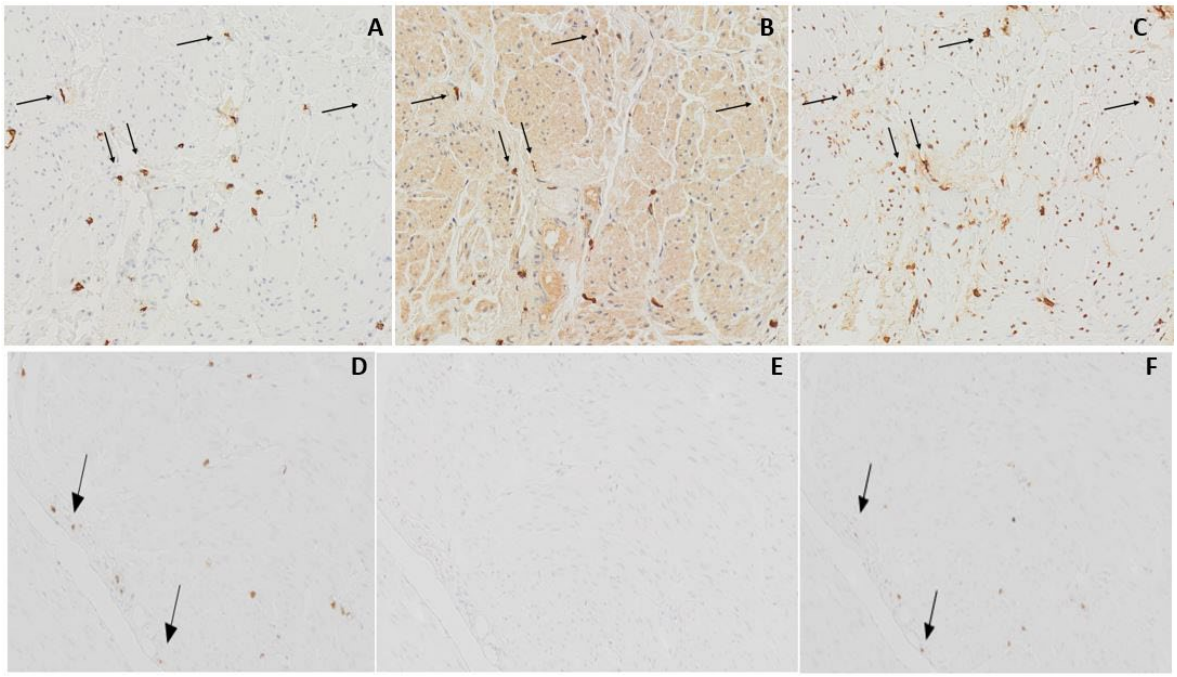

Figure 79: Serial sections of 2 urinary bladder biopsy from an BPS/IC patient (A, B and C), and healthy bladder tissue control (D, E and F) showing mast cells in the detrusor layer, some are indicated in arrows, stained with antibodies for mast cell chymase (A & D), MRGPRX2 (B & E) and mast cell tryptase (C& F).

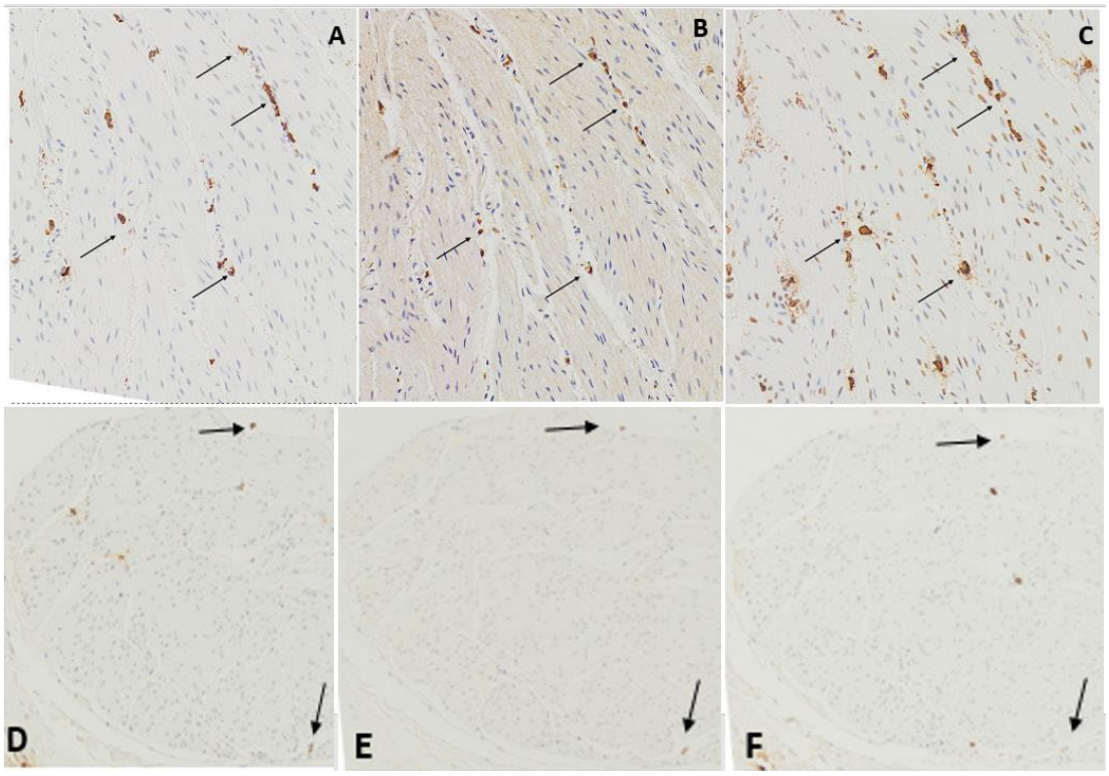

Figure 80: Serial sections of 2 urinary bladder biopsy from an BPS/IC patient (A, B and C), and healthy bladder tissue control (D, E and F) showing mast cells in the detrusor layer, some are indicated in arrows, stained with antibodies for mast cell chymase (A & D), MRGPRX2 (B & E) and mast cell tryptase (C& F).

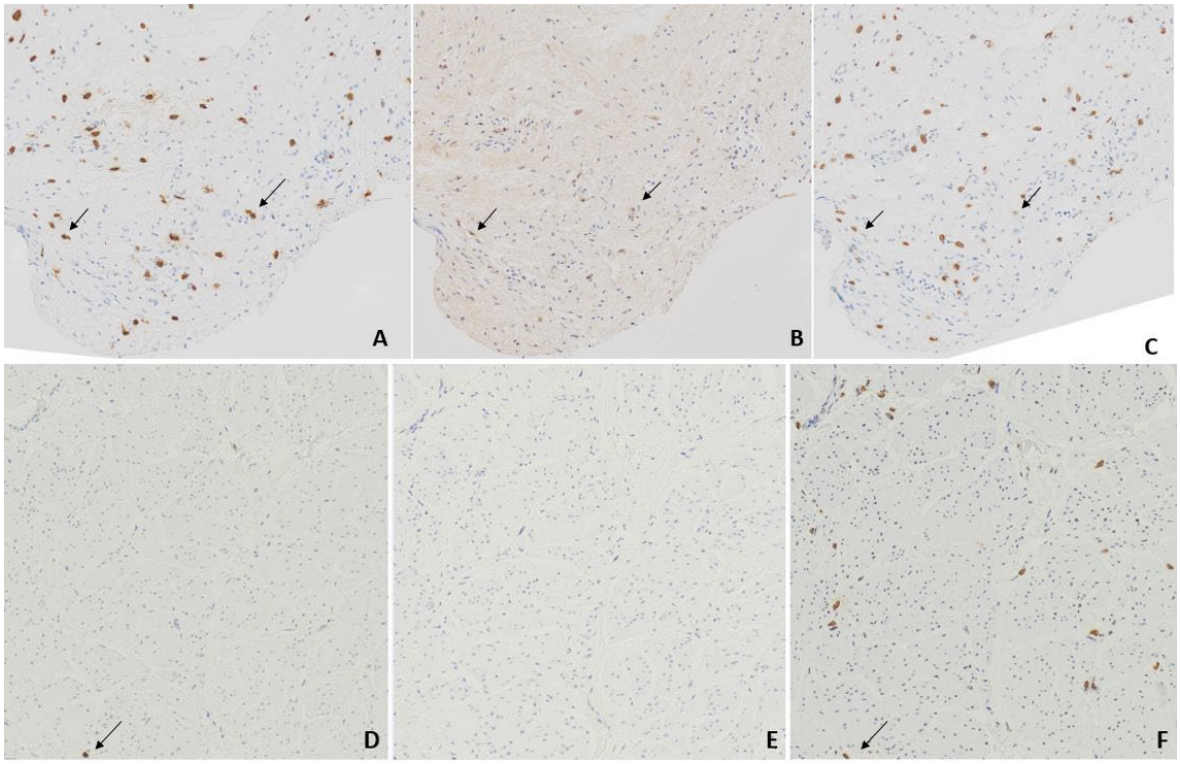

Figure 81: Serial sections of 2 urinary bladder biopsy from an BPS/IC patient (A, B and C), and healthy bladder tissue control (D, E and F) showing mast cells in the detrusor layer, some are indicated in arrows, stained with antibodies for mast cell chymase (A & D), MRGPRX2 (B & E) and mast cell tryptase (C& F).

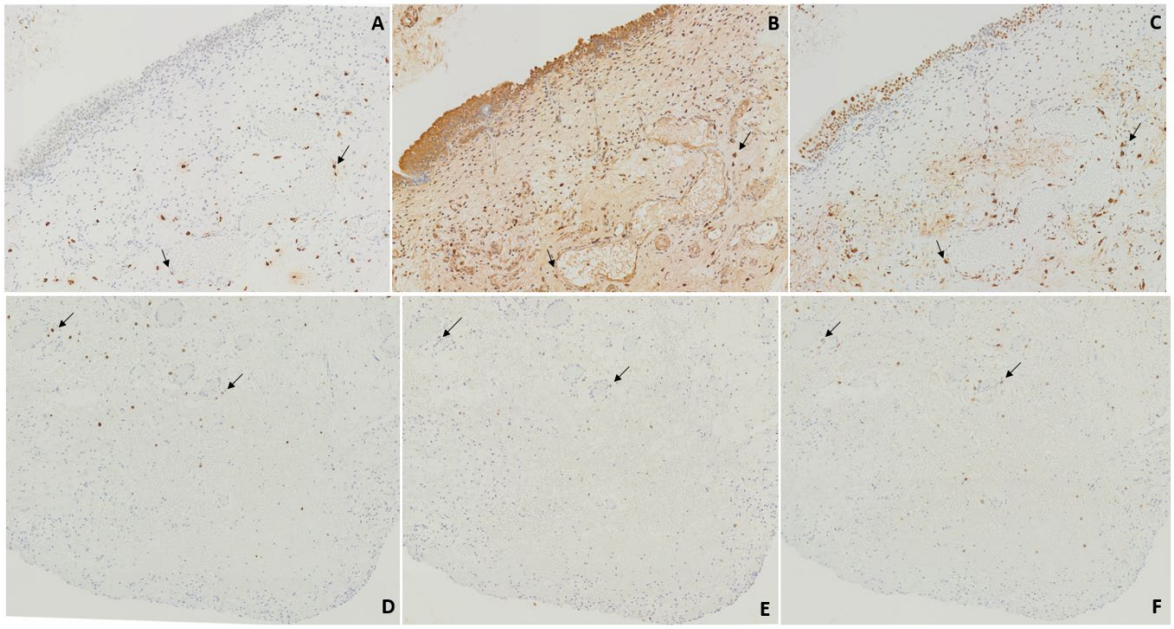

Figure 82: Serial sections of 2 urinary bladder biopsy from an BPS/IC patient (A, B and C), and healthy bladder tissue control (D, E and F) showing mast cells in the lamina propria, some are indicated in arrows, stained with antibodies for mast cell chymase (A & D), MRGPRX2 (B & E) and mast cell $_{\text{tryptase}}$ (C& F).

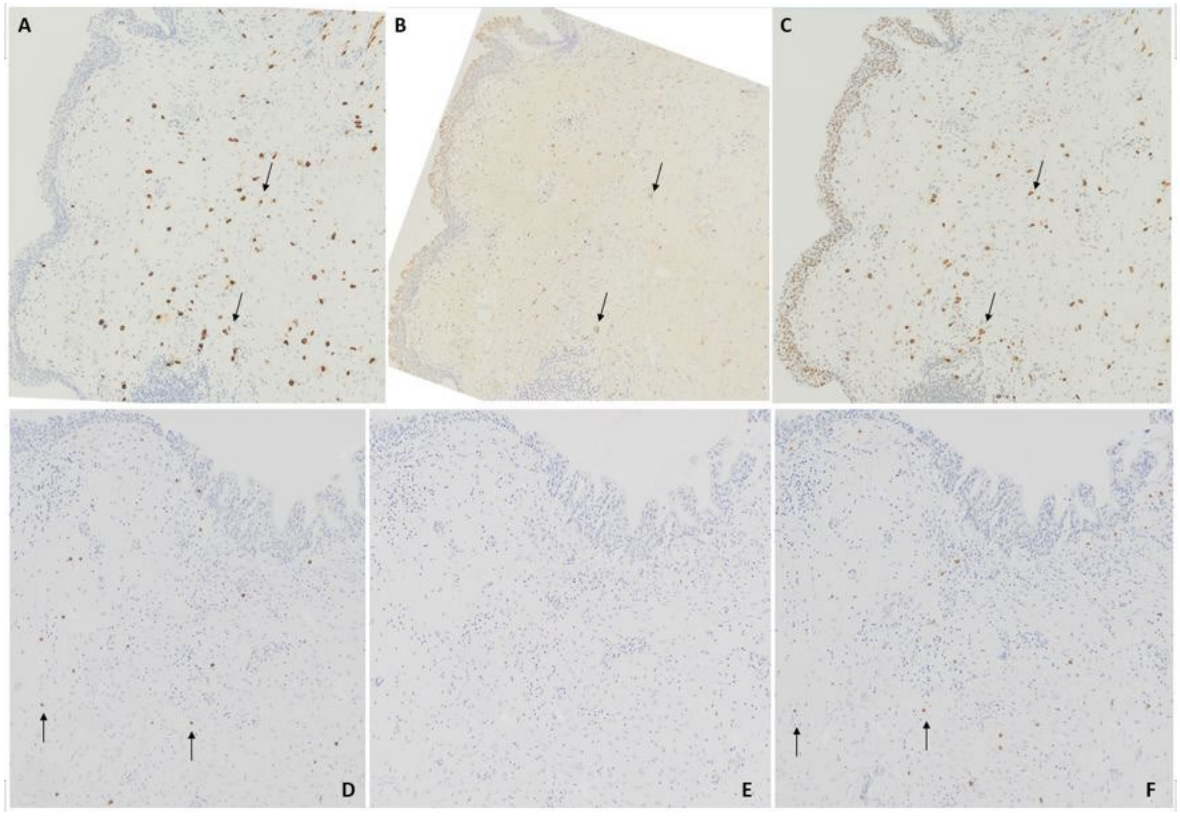

Figure 83: Serial sections of 2 urinary bladder biopsy from an BPS/IC patient (A, B and C), and healthy bladder tissue control (D, E and F) showing mast cells in the lamina propria, some are indicated in arrows, stained with antibodies for mast cell chymase (A & D), MRGPRX2 (B & E) and mast cell tryptase (C& F).

8.3.3 The expression of MRGPRX2 in the urinary bladder tissue

In both in the IC/BPS and the control biopsies, the expression of MRGPRX2 was almost co-localized with tryptase and chymase expression, where MRGPRX2 expression was upregulated in IC/BPS in association with increased mast cell density. It is worth mentioning that there was an occasional faint staining pattern on MRGPRX2 staining in the urinary bladder urothelium. However, as per confirmed by our pathologist, Dr Hannah Markham, this is a non-specific background.

8.4 Discussion

Abnormal activation of mast cells is believed by some experts to play a role in maintaining the chronic bladder wall inflammation seen in BPS/IC (Pang *et al.*, 1996b; Theoharides, Kempuraj and Sant, 2001; Sant *et al.*, 2007b; Wang *et al.*, 2016). Many studies report a significant increase in the density of mast cells in the detrusor layer compared to controls

(Peeker *et al.*, 2000a; Liu *et al.*, 2012b; Malik *et al.*, 2018a). It is believed that a positive feedback circle between depolarised substance P-positive nerve endings and mast cells maintains bladder wall inflammation (Sant *et al.*, 2007b).

However, mast cell responsiveness to the neuropeptide substance P in humans depends on their expression of MRGPRX2, a recently identified G protein coupled receptor (Ali, 2017). The expression of such a receptor on mast cells is variable between different tissue micro-environments and its expression on urinary bladder mast cells has not been explored (Varricchi *et al.*, 2019). MRGPRX2 is expressed on the surfaces of the skin mast cells (MC_{TC} subtype), but not on the pulmonary mast cells (MC_T subtype) (Fujisawa *et al.*, 2014). Several studies have reported the increased expression of MRGPRX2 in chronic tissue inflammation. MRGPRX2 expression on skin mast cells is upregulated in severe chronic urticaria, which is associated with increased tissue resident mast cells (Fujisawa *et al.*, 2014). In the lungs, the expression of MRGPRX2 on mast cells is upregulated in biopsies from patients who died with severe chronic asthma, and this was associated with a shift towards MC_{TC} dominance (Balzar *et al.*, 2011; Manorak *et al.*, 2018).

8.4.1 Mast cell density in BPS/IC vs the control group

In the current study, the average density of mast cells stained with tryptase or chymase in the detrusor layer of the BPS/IC group was 166 and 155 cells / mm^2, respectively. Consistent with the detrusor layer, the average density of mast cells stained with tryptase or chymase in the lamina propria in the BPS/IC patients was 215 and 181 cells / mm^2, respectively. Such evidence is consistent with previous reports highlighting the increased numbers of the tissue resident mast cells in the bladders of BPS/IC patients (Peeker *et al.*, 2000a; Yamada *et al.*, 2000; Kim *et al.*, 2017; Malik *et al.*, 2018a).

Regarding mast cell subtypes, about 70% of the tryptase^{+ve} mast cells in the detrusor layer of the BPS/IC group co-expressed chymase, indicating that nearly 70% of these tissue resident mast cells were of the MC_{TC} subtype.

Consistently in the lamina propria, about 84% of the tryptase^{+ve} mast cells co-expressed chymase, indicating that nearly 85% of the mast cells in the lamina propria were of the MC_{TC} subtype. Interestingly, the percentage of the MC_{TC} subtype out of all mast cells in the BPS/IC patients was significantly higher compared to healthy bladder tissue controls,

where the MC_{TC} subtype accounted for around 50% of all the tissue resident mast cell population. Such evidence suggests a shift of the mast cell subtype from MC_T to MC_{TC} in the BPS/IC group, which is consistent with other report highlighting the shift of the MC subtype from MC_T to MC_{TC} in the pulmonary mast cells of severe asthma patients (Balzar *et al.*, 2011).

Based on previous reports highlighting the pro-inflammatory and cell lysis effects of mast cell chymase (Wernersson and Pejler, 2014; Choi *et al.*, 2016), the shift in the mast cell subtype to MC_{TC} might, in part, explain the presence of the urothelial barrier defect seen in the bladders of the BPS/IC patients.

8.4.2 MRGPRX2 expression by the bladder mast cells.

In the detrusor layer of the BPS/IC group, about 68% of each of the tryptase^{+ve} and the chymase^{+ve} mast cells expressed MRGPRX2 receptor. Consistent with the detrusor layer, in the lamina propria of the BPS/IC group, about 60% of each of the tryptase^{+ve} and the chymase^{+ve} mast cells expressed MRGPRX2 receptor. To our knowledge, this is the first evidence confirming the expression of MRGPRX2 on the mast cells in a urinary bladder tissue and, specifically, in BPS/IC patients.

Nevertheless, the percentage of both the tryptase^{+ve} and the chymase^{+ve} mast cells that express MRGPRX2 was significantly higher in the patients when compared to the controls, where that percentage averaged around 30% in both the detrusor and the lamina propria layers. Such data indicates the upregulation of MRGPRX2 receptor expression by mast cells in BPS/IC.

The results of the current study, where the shift of the mast cell subtype towards MC_{TC} in the BPS/IC group was associated with an interesting increase in the expression of MRGPRX2 by both the tryptase^{+ve} and the chymase^{+ve} mast cells, are consistent with recent reports highlighting the exclusive expression of MRGPRX2 on the MC_{TC} subtype of the mast cells (Babina, 2020). In addition, upregulation of MRGPRX2 expression on mast cells was linked to severe tissue inflammatory conditions (Balzar *et al.*, 2011; Fujisawa *et al.*, 2014). Based on the fact that MRGPRX2 is responsible for neuropeptide-induced mast cell activation and pseudo-allergic reactions (Green *et al.*, 2019), the current evidence

supports the narrative of a possible role for neurogenic inflammation in the pathophysiology of BPS/IC.

In contrast to a previous report from our lab which stated that MC_T was the dominant mast cell subtype (about 80% of all mast cells) in the bladders of BPS/IC patients, in the current study, MC_{TC} was the dominant subtype (around 70% of all mast cells). This finding is supported by the MRGPRX2 expression, which is believed by many to be exclusively expressed by connective tissue mast cells (MC_{TC}) (Balzar *et al.*, 2011; Fujisawa *et al.*, 2014).

Comparable to other studies comparing mast cell density in BPS/IC bladder biopsies to controls, it was extremely difficult to obtain healthy uninflamed bladder biopsy tissue to use as a control. This is due to the invasive nature of the bladder biopsy, which means that it is usually only requested by doctors to exclude bladder malignancies (Cox *et al.*, 2016). However, the abnormal and imperfect nature of the control tissue utilized in the current study might carry an advantage in that the density of mast cells in this control tissue might, if anything, be higher when compared to normal tissue (Serel, Soyupek and Çandir, 2004; Moradi Tabriz *et al.*, 2021). Thus, the real differences seen between cases and controls might be underestimated in the current study.

Despite the various reports supporting the current study findings, other studies reported no statistically significant difference in mast cell density between BPS/IC and other forms of chronic cystitis (Gamper *et al.*, 2015; Akiyama *et al.*, 2018). However, the abundant nature of mast cells in the bladder walls of BPS/IC patients and their expression of MRGPRX2 is strong evidence supporting a role for neurogenic inflammation theory in the pathophysiology of the chronic inflammation seen in the disease.

Beyond neurogenic inflammation, the fact that MRGPRX2 is a multi-ligand receptor that can bind to several agents resulting in mast cell activation and degranulation, might provide a basis for new causative agents causing bladder wall inflammation in BPS/IC patients and other chronic inflammatory bladder conditions. For example, several molecular agents such as neuropeptides (substance P and homokinin-1), eosinophil peroxidase, and host defence peptides such as (Human β-defensins) and cathelicidin (LL-

37) can activate mast cells via MRGPRX2. Likewise, many therapeutic agents such as Atracurium and Cisatracurium (skeletal muscle relaxants), morphine (pain killer), Icatibant (Bradykinin B2 receptor antagonist), Mastoparan (a peptide toxin from wasp venom) and the antibiotics Vancomycin and fluoroquinolones can elicit the same effect (Subramanian *et al.*, 2011; McNeil *et al.*, 2015; Ali, 2017; Che *et al.*, 2018). As a result, the use of these therapeutic agents in the treatment of patients suffering from chronic bladder wall inflammation should be avoided or at the very least patients should be carefully tested for allergy to these drugs before starting treatment.

Based on our results showing that Medihoney has proven to have a strong mast cell stabilizing effect against MRGPRX2 agonists such as the neuropeptide substance P and Compound 48/80, we have strong evidence that aqueous Medihoney preparations used as intravesical agents might exert a favourable anti-inflammatory effect against the neurogenic inflammation seen in both BPS/IC and other non-BPS/IC forms of chronic cystitis. In this setting Medihoney preparations could be introduced into the bladder via a catheter either alone or at the time of routine diagnostic or therapeutic cystoscopy.

8.4.3 Study limitations

From our perspective, the study has four main limitations. These are as follows:

8.4.3.1 Lack of the Ulcerative BPS/IC phenotype

Firstly, due to the limitation to tissue availability in the human tissue archive, University Hospital Southampton, the study only included non-hunner (non-ulcerative) BPS/IC patients, while there was only one confirmed case of the hunner (ulcerative) BPS/IC, but that was not enough to create a comparison group. As previously described in chapter 1, the Ulcerative (Hunner type) is characterised by cracking and ulceration of the urothelium upon cystoscopy with hydrodistension at 100 cm H_2O pressure. Importantly, the hunner (ulcerative) BPS/IC is considered a different phenotype where the patients represent the more sever inflammatory form of the disease and their treatment should be more focused on the urinary bladder (Hanno *et al.*, 2011; Belknap, Blalock and Erickson, 2015; Cox *et al.*, 2016). This is associated with significant histological differences between the two BPS/IC subtypes. It is generally accepted that mast cell counts in ulcerative BPS/IC are higher than those in non-ulcerative BPS/IC, while in both BPS/IC subtypes mast cell counts are significantly higher compared to controls (Peeker *et al.*, 2000a; Yamada *et al.*, 2000;

Gamper *et al.*, 2015; Akiyama *et al.*, 2018; Malik *et al.*, 2018a). Consistently, in all these studies, there was a significant difference in the mast cell density between the two BPS/IC phenotypes. These histological differences strongly support the evidence behind the narrative that Hunner and non-Hunner BPS/IC are two different entities. Accordingly, the outcomes of the study are only representative of the non-Hunner BPS/IC patients, while a similar study on the Hunner BPS/IC patients needs to be performed.

8.4.3.2 Lack of multiplexing in the co-expression analysis

Secondly, to study the co-expression patterns, it was ideal to perform two or more stains on the same tissue section (Multiplexing). However, due to the differences between the commercially available antibodies in their antigen retrieval step, it was impossible to multiplex. Alternatively, as described above, based on the fact that mast cell densities range between 10 and 20 microns, serial sections of 4µM thickness each were cut and stained with antibodies for tryptase, chymase and MRGPRX2, then the co-expression patterns were analysed by superimposition of the images obtained from these serial sections using adobe photoshop software.

8.4.3.3 Lack of analysis of the relationship between the histological findings and clinical data

Thirdly, due to the lack of accurate numerical clinical data, we were not able to analyse the relationship between the BPS/IC patients' clinical symptoms and both the mast cell density and their MRGPRX2 expression.

8.4.3.4 Gender and age differences between the BPS/IC and the control groups

Finally, as previously described in the methodology section, there was a significant age and gender differences between both the BPS/IC and the control groups. Due to the nature of each disease group, most of the BPS/IC biopsies belong to the female patients, while most of the control biopsies belong to male patients. Importantly, there was no evidence suggesting significant gender-based differences in the mast cell counts between males and females. However, in order to exclude any effect of the gender-based differences on the study outcomes, all the study outcomes were compared between males and females within the control group. Consistently, there was no Gender-based differences in mast cell densities, subtypes or MRGPRX2 expression within the control group, which adds more credibility to the augmented differences between the BPS/IC and the control groups (see figures 84 & 85).

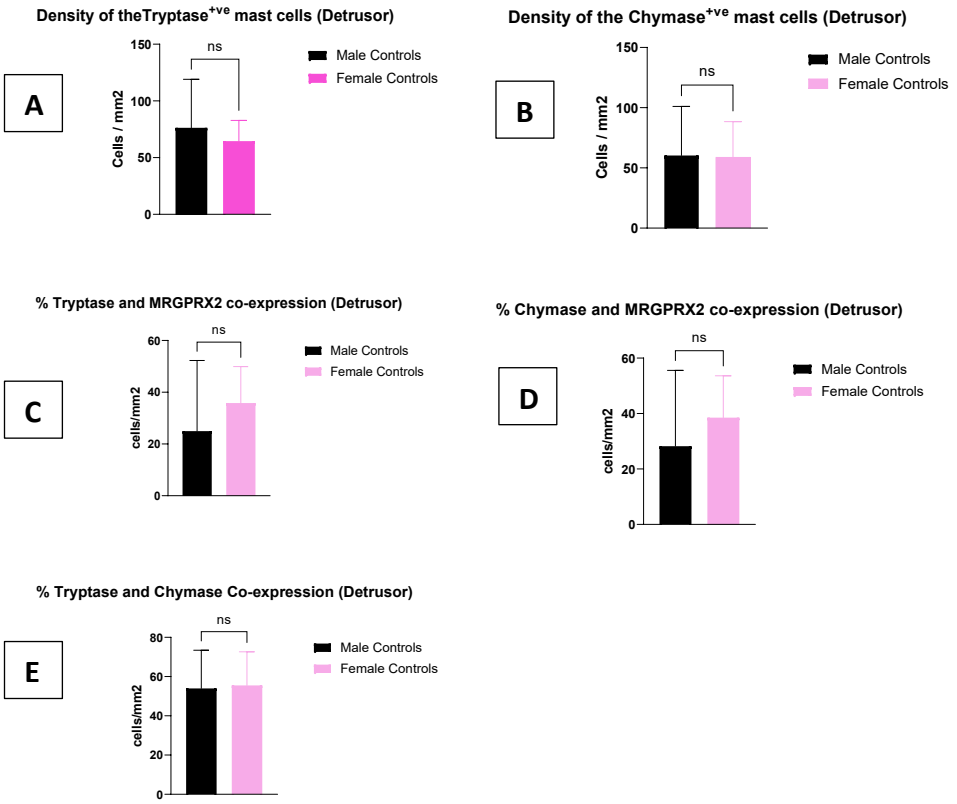

Figure 84. Graphical representation of the mast cell densities and their MRGPRX2 expression in the detrusor layer of the urinary bladders of male vs female controls

(A) & (B) are bar graphs representing the density of mast cells stained with tryptase or chymase, respectively, in the detrusor layer of the urinary bladders of the male controls vs female controls. (C) & (D) are bar graphs representing the percentage of expression of MRGPRX2 by the tryptase^{+ve} or chymase^{+ve} mast cells, respectively, in the detrusor layer of the urinary bladders of the male controls vs female controls. (E) is a bar graph representing the percentage of expression of chymase by the tryptase^{+ve} mast cells in the detrusor layer of the urinary bladders of the male controls vs female controls. Each bar represents the mean. Statistical differences were analysed by Mann-Whitney U test using GraphPad Prism 9 software.

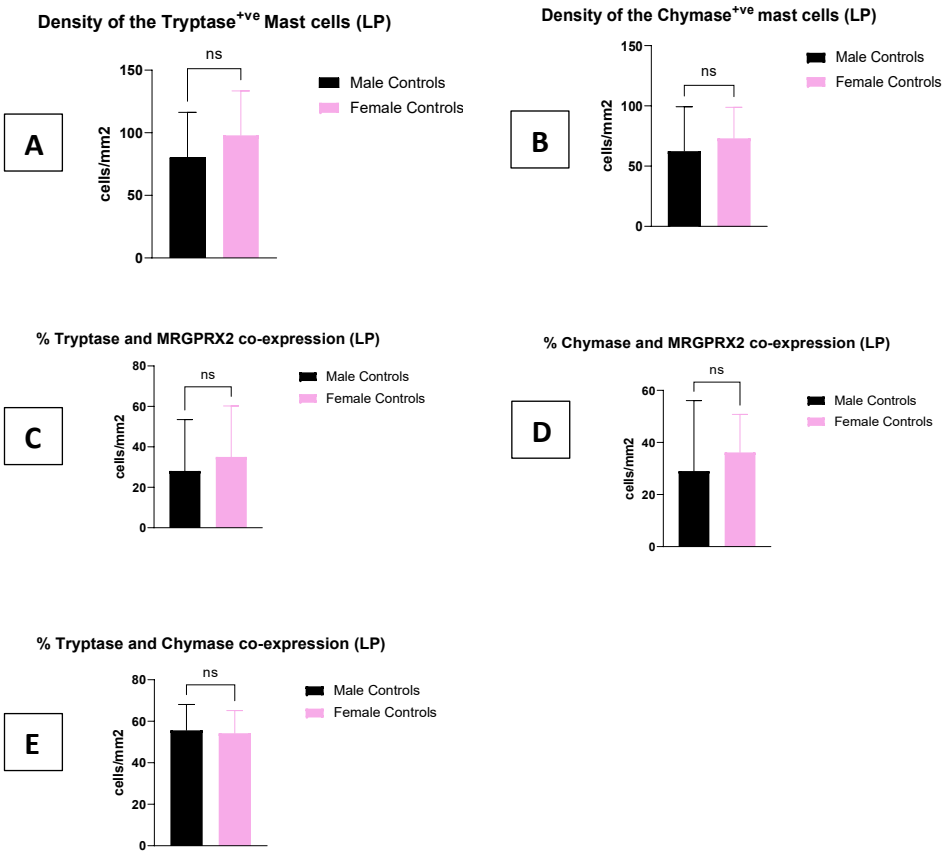

Figure 85. Graphical representation of the mast cell densities and their MRGPRX2 expression in the lamina propria (LP) of the urinary bladders of male vs female controls

(A) & (B) are bar graphs representing the density of mast cells stained with tryptase or chymase, respectively, in the lamina propria of the urinary bladders of the male controls vs female controls. (C) & (D) are bar graphs representing the percentage of expression of MRGPRX2 by the tryptase^{+ve} or chymase^{+ve} mast cells, respectively, in the lamina propria of the urinary bladders of the male controls vs female controls. (E) is a bar graph representing the percentage of expression of chymase by the tryptase^{+ve} mast cells in the lamina propria of the urinary bladders of the male controls vs female controls. Each bar represents the mean. Statistical differences were analysed by Mann-Whitney U test using GraphPad Prism 9 software.

8.5 Conclusion

The bladder biopsies of the non-ulcerative BPS/IC patients show a consistently high density of mast cells with a predominance of the connective tissue mast cell (MC_{TC}) subtype positive for the pro-inflammatory receptor MRGPRX2. Such evidence supports a positive role for neurogenic inflammation in the pathophysiology of the disease. The anti-inflammatory mast cell stabilising properties of Medihoney make it well suited to play a beneficial role in the bladders of this patient group when used as an intravesical agent introduced into the bladder via a urinary catheter.

9 Chapter Five: General Discussion, conclusions, and future work

9.1.1 Bladder Pain Syndrome/Interstitial Cystitis (a major health problem)

BPS/IC is a chronic debilitating inflammatory condition of the urinary bladder. It is defined as “chronic pelvic pain, pressure or discomfort perceived to be related to the urinary bladder, accompanied by at least one of the lower urinary tract symptoms such as urge to void, frequency and nocturia” (van de Merwe *et al.*, 2008). Recent reports show a progressive increase in the prevalence of BPS/IC (i.e. 52 to 500/100,000 in females and 8 to 41/100,000 in males), accompanied by an increase in the incidence rate across genders (Mishra, 2015; Vella, Robinson and Cardozo, 2015). The incidence is estimated at 1.2/100,000. This places BPS/IC among conditions that merit research to bridge the wide gaps that exist between diagnosis and treatment. Most of the currently avaialble conventional treatments for BPS/IC show variable, short-term and often unsatisfactory improvements in symptom scores (Vella, Robinson and Cardozo, 2015; Cox *et al.*, 2016). It is believed that the efficiency of current treatments is negatively impacted by several factors including failure of identification of a definite etiological factor and treatment side effects. These, in addition to the multifactorial nature of the disease, highlight the need to develop targeted, more effective treatments (Belknap, Blalock and Erickson, 2015; Pazin *et al.*, 2016).

9.1.2 Neurogenic inflammation theory in the elusive pathophysiology of bladder pain syndrome/interstitial cystitis

The current study was based on the elusive and multifactorial pathophysiology of the sterile, non-infection-related bladder wall inflammation seen in the bladders of a group of patients suffering from a chronic inflammatory debilitating condition known as bladder pain syndrome/ interstitial cystitis (BPS/IC) (Dasgupta and Tincello, 2009; Ke and Kuo, 2015; Cho, 2016; Jhang and Kuo, 2016). The bladders of these patients consistently display a thin and atrophic lining urothelium that allows urine to penetrate the bladder wall and depolarize nociceptive sensory nerve endings (Graham E. and C., 2006; Keay *et al.*, 2011; Keay, Birder and Chai, 2014; Hurst *et al.*, 2015; Jhang, Hsu and Kuo, 2016). In turn, these nerve endings release neuroactive substances such as neuropeptide substance P (SP) that induce degranulation of tissue resident mast cells, creating a vicious cycle of sensory nerve desensitization, mast cell activation, inflammatory cell chemo-attraction and chronic bladder wall inflammation (Pang *et al.*, 1996b; Theoharides,

Kempuraj and Sant, 2001; Batler *et al.*, 2002; Sant *et al.*, 2007b; Wang *et al.*, 2016). Such a process is termed neurogenic inflammation, which despite being scientifically convincing, remains unproven.

Several reports have highlighted the high density of mast cells in the bladders of BPS/IC patients compared to controls, and which lie in close proximity to SP^{+ve} nerve endings (Yamada *et al.*, 2000; Liu *et al.*, 2012b; Kim *et al.*, 2017; Malik *et al.*, 2018a). However, recent reports have highlighted the fact that mast cell responsiveness to SP is variable between different tissue microenvironments and that such responsiveness depends on the expression of MRGPRX2, a recently identified G-protein coupled receptor (Varricchi *et al.*, 2019). To our knowledge, such expression has not been explored in the urinary bladder tissue generally, and in the bladder of BPS/IC patients specifically.

As a result, we decided to study the expression of MRGPRX2 receptor on urinary bladder mast cells in BPS/IC urinary bladder biopsies via an immunohistochemistry approach. Such would allow a better understanding of the pathophysiology of the disease, which might pave the way for optimally directed treatment options.

Our results have shown that the about 65% of the urinary bladder mast cells in BPS/IC patients are connective tissue mast cells (MC_{TC} subtype), which express MRGPRX2, a finding that provides further supports for the neurogenic inflammation theory in the pathophysiology of the disease.

9.2 Medihoney, a medical-grade Manuka honey, as an anti-inflammatory agent against neurogenic inflammation involved in the pathophysiology of BPS/IC

One of the main reasons why honey has endured as a medicinal agent is its significant antibacterial activity. This can be exploited in the clinical setting where honey can be used as an alternative to traditional antibiotics without the risk of developing drug resistance. Thus, Manuka honey possesses strong antimicrobial activity against a broad spectrum of bacterial strains including those that are resistant to antibiotics (Halstead *et al.*, 2016; Girma, Seo and She, 2019), and strongly inhibits bacterial biofilm formation in a dose-dependent manner (Emineke *et al.*, 2017). Both antibiotic-sensitive and antibiotic-non

sensitive bacterial strains show sensitivity to medical-grade Manuka honey preparations. These include variable *Staphylococcus aureus* species such as methicillin-resistant *Staphylococci aureus* (MRSA), methicillin-sensitive staphylococci (MSSA), and coagulase-negative staphylococci (Girma, Seo and She, 2019). Manuka honey also inhibits the growth of gram negative organisms such as *E. coli*, *Pseudomonas aeruginosa* and Enterobacteriaceae (Girma, Seo and She, 2019).

One of the most common and persistent therapeutic uses of honey has been as a wound dressing. Initially, the work on honey started with cell culture, moving on to animal models and has now become one of the main wound treatment strategies, especially for pressure ulcers. Honey impregnated wound dressings significantly reduce the healing time for bed sores and partial thickness burns (Molan, 1998). Furthermore, honey induced partial or total autolytic debridement of necrotic or infected tissue, promoting angiogenesis and tissue regeneration, as well as combating tissue infection and inflammation (Evans and Mahoney, 2013; Girma, Seo and She, 2019).

The anti-inflammatory properties of honey were noted in aseptically inflicted wounds in animal models, suggesting that its anti-inflammatory effect is independent of its anti-microbial properties (Molan, 2002). Indeed, in animal models of open wounds and burns, the use of honey impregnated-dressings was associated with faster healing rates and reduced numbers of acute inflammatory cells compared to other standard dressings (Molan, 1998).

The anti-inflammatory effect of Manuka honey is attributed to the inhibition of the production of reactive oxygen species by activated polymorphonuclear neutrophils. Honey also scavenges the superoxide anion produced by activated polymorphonuclear neutrophils at the wound site (van den Berg *et al.*, 2008). In addition, honey extracts exerted 40 to 80 % inhibition of nitric oxide production by both lipopolysaccharide and IFNγ-activated macrophages as well as significantly inhibiting the cytotoxicity produced by TNF-α on fibroblast L929 cell lines (Kassim *et al.*, 2010).

In atopic dermatitis patients, medical grade manuka honey (MH) decreased inflammation compared to controls. It also inhibited the production of IL-4 and CCL-26 from the

keratinocyte cell line HaCaT and in the human mast cell line LAD2 stabilised mast cell degranulation and histamine release (Alangari *et al.*, 2017a). This effect was seen with different honey formulations, which were noted to inhibit calcium Ionophore-induced mast cell degranulation and histamine release (Birch *et al.*, 2011). Moreover, honey is well tolerated by urothelial cells and can protect them from harmful chemical injuries (Lwaleed *et al.*, 2014).

Taken together, the above studies demonstrate that MMH possesses three properties that could be exploited medically. Thus, it is:
anti-microbial
pro-angiogenic
anti-inflammatory
This "triple action" would appear ideal for the treatment and management of BPS/IC. Therefore, a logical extension of the above is to investigate the use of MMH as an application for use within the urinary bladder. There are sound reasons to believe it might be of use in this location to treat BPS/IC, especially as no side effects have been reported with MMH use in other clinical conditions e.g. eczematous skin lesions in babies and young children. Guidelines also recommend honey for acute cough in children.

In the current study, the potential inhibitory effects of Medihoney against pro-inflammatory mast cell activation and the degranulation induced by neuropeptide SP were investigated using a highly reliable and representative in vitro mast cell model (LAD2 cells). Consistently, pre-incubation of the LAD2 cells with 4% Medihoney strongly and significantly inhibited mast cell degranulation induced by various secretagogues including SP, Ca^{++} ionophore, and the MRGPRX2 Agonist compound 48/80. This was associated with the inhibition of SP-induced phosphorylation of several protein kinases, most importantly, Akt (serine 473) and STAT3 (serine 727). Interestingly, 10-DEBC HCL, a specific Akt inhibitor, inhibited the SP-induced mast cell degranulation at 20 and 30 µM concentrations, indicating that Medihoney inhibits mast cell degranulation through the inhibition of Akt phosphorylation.

9.3 The potential of Medihoney as an antimicrobial agent in urology

As previously described, Manuka honey is a potential multi-modal therapeutic agent with strong antimicrobial, anti-inflammatory, pro-angiogenic and pro-healing

properties. Generally speaking, urinary tract infections are one of the most common outpatient infections that is associated with a significant impact on the general health, work productivity and quality of life (Medina and Castillo-Pino, 2019). Between the years 2009 and 2019, urinary tract infections accounted for 404 million cases and 236,790 deaths worldwide (Yang *et al.*, 2022). Importantly, due to a shorter and wider urethra, the incidence of urinary tract infections is 3.6-fold higher in females than in males, where about 50-60% of adult women experience urinary tract infections (Medina and Castillo-Pino, 2019; Yang *et al.*, 2022). It is well established that there is no difference in the urinary microbiome between BPS/IC patients and non-BPS/IC controls (Bhide, Tailor and Khullar, 2020), where traditional antibiotic regimens fail to control the patients' symptoms. However, recent reports highlighted that the use of traditional culture media and short incubation periods results in underdiagnosis of UTI in a significant proportion of IC/BPS patients (Aydogan *et al.*, 2019; Malde and Sahai, 2020).

Importantly, superimposed urinary tract infections can strongly trigger the BPS/IC flares, which will necessitate the use of antibiotics. This in turn increases the value of Manuka honey, where it can both downregulate the neurogenic inflammation and prevent superimposed urinary tract infections, especially with repeated catheterisation.

Based on its broad-spectrum antimicrobial properties, Medihoney, as a medical-grade Manuka honey, has the potential to prevent or even limit the incidence of superimposed urinary tract infections in BPS/IC patients. In addition, catheter introduced Medihoney may potentially be used as an alternative or an adjuvant to the systemic antibiotics used to treat urinary tract infections. In such case, the use of Medihoney as an adjuvant may potentially allow to reduce the doses of systemic antibiotics in case of urinary tract infections. Accordingly, this will strongly contribute to reducing the development of side effects associated with antibiotic use.

Fluoroquinolones, including ciprofloxacin and levofloxacin, are not commonly used for the treatment of urinary tract infections, where their side effects outweigh their therapeutic benefits (Chao and Farrah, 2019). Their side effects include arthritis, tendon swelling, neuritis, mood changes and low blood sugar, so they are not

recommended for use in diabetic patients, cardiac patients, nerve problems, in addition to those with liver and kidney dysfunction. Accordingly, the use of fluoroquinolones is limited to the sever complicated cases of urinary tract infection (Kang *et al.*, 2018).

Vancomycin is a glycopeptide antibiotic that works via destruction of the bacterial cell membrane integrity of both the aerobic and anaerobic gram^{+ve} bacteria. Typically, it is used orally to treat sever pseudomembranous colitis induced by clostridium difficile. In addition, vancomycin is used intravenously to treat sever microbial infections in resistant to other antibiotics such as Methicillin-resistant staph. Aureus (MRSA) and multidrug-resistant S. epidermidis (MRSE). Intravenously, vancomycin is used for the treatment of sever bacterial infections in different parts of the body. However, the side effects of vancomycin include pain, redness, or swelling at the injection site, vancomycin flushing syndrome (Azimi, 2017 #1275), thrombophlebitis of the injection site, nephrotoxicity, ototoxicity and sever anaphylaxis.

As described above, both groups of antibiotics, especially fluoroquinolones, are used to treat sever resistant urinary tract infections, however, that does not come without a price, where significant health problems are likely to develop. Moreover, both antibiotics have the tendency to activate the mast cell receptor MRGPRX2 (Ali, 2017 #1278), which can give a valid justification for the allergic side effects associated with their use. Accordingly, these antibiotics might potentially induce flares of BPS/IC upon their usage in the treatment of superimposed urinary tract infection in BPS/IC patients.

Moreover, long term antibiotics are used as a prophylactic treatment against the development of urinary tract infections in patients with history of recurrent urinary tract infections (Jent, 2022 #6952). In such case, an adjuvant treatment of catheter-introduced intravesical Medihoney may potentially reduce the antibiotic doses used or even completely replace the antibiotic regimen. Nevertheless, repeated catheterisation in BPS/IC patients can increase the incidence of urinary tract infections, where intravesical Medihoney (one a week) might limit or totally prevent the incidence of urinary tract infections.

Based on the above evidence, we strongly believe that Medihoney has a strong potential to enhance the therapeutic efficacy of the current treatment options not only for BPS/IC patients, but also resistant urinary tract infections. Clinical trials including both BPS/IC and urinary tract infection patients are needed to ensure that the therapeutic potentials of the Manuka-derived therapeutic agents, such as Medihoney, are maximally exploited.

9.4 Conclusion

Based on the findings of the current study, it was concluded that Medihoney has the potential to combat the neurogenic inflammation proposed to underly the pathophysiology of variable chronic inflammatory conditions, including BPS/IC.

9.5 Future work

(1) We plan to further study the effect of Medihoney on the phosphorylation (activation) of the pro-inflammatory mast cell receptor MRGPRX2. For such purpose, the HEK-293 cell line will be transfected to express the receptor. Then, the activation of the receptor induced by both SP and compound 48/80 will be studied using both calcium signalling and western blotting.

(2) Using western blotting and protein kinase inhibitors, we plan to further assess the exact relationship between the different protein kinases which are affected by the Manuka honey derivatives and the effect of their inhibition on mast cell degranulation.

(3) As symptomatic flares in BPS/IC patients are commonly associated with stress, we plan to further study the effect stress on the intensity of the neurogenic bladder wall inflammation in BPS/IC patients. For such purpose, LAD2 mast cells will be incubated with corticotrophin releasing factor (CRF) for 6, 12 and 24 hours. Then, the expression of the pro-inflammatory receptor MRGPRX2 on the LAD2 mast cells will be assessed by flow cytometry in the CRF-treated vs non CRF-treated cells.

(4) Using a flow cytometry technique, we plan to study the effect of Medihoney on the membrane expression of the mast cell activation markers CD63 and CD203c upon mast cell activation with various secretagogues.

(5) Using a well validated protocol, we plan to use a flow cytometry technique to isolate mast cell progenitors from the peripheral blood and induce their maturation in vitro. Such cells will be used to replicate and confirm the current results obtained using LAD2 cells. It is worth mentioning that this approach was not part of the current PhD project because such technique requires a time consuming process of obtaining the required ethical approvals, in addition to the fact that using primary cells will have the limitation of limited number of replication cycles in vitro before these cells start to die. Accordingly, LAD2 cells were more practically suitable for the PhD project, which added to their well-established reliability.

(6) Using in vitro and ex-vivo models of human urinary bladder, human skin, human stomach and duodenum, the potential anti-inflammatory and cytoprotective effects of Medihoney against tissue inflammation seen in BPS/IC, atopic dermatitis and functional dyspepsia will be studied. It is worth mentioning that some of these models are immunocompetent and capable of developing a normal immune response when treated with the pathologically relevant pro-inflammatory agents.

(7) Our main future plan is to validate the use of Medihoney as an intravesical catheter-introduced agent through establishment of an experimental cystitis animal study to investigate therapeutic efficacy. This will be followed by the establishment of a pilot study to assess the safety and potency of Medihoney in the bladders of BPS/IC patients.

9.6 The effect of the COVID-19 pandemic on the whole PhD project

The current PhD project was based on two studies.

The first study was based on investigating of the possible involvement of the neurogenic inflammation theory in the pathophysiology of BPS/IC via immunohistochemical analysis of the patients' urinary bladder biopsies.

The second study was to investigate the potential anti-inflammatory properties of Medihoney against neurogenic inflammation in an in vitro model of inflammation using the human mast cell line (LAD2). Unfortunately, both studies were affected by the COVID-19 pandemic resulting in significant loss of resources and time. However, such loss did not affect the overall achievement of the aims and objectives of the study as follows:

9.6.1 The effect of COVID pandemic on the in vitro the Medihoney and LAD2 cell study

Upon political acknowledgement of the seriousness of the COVID-19 pandemic, the government issued a national lockdown as a result of which all laboratory activities were suspended for nearly 8 months starting from late March 2020 until late October 2020. Just before the lab closure, the LAD2 cells were frozen in liquid nitrogen to be able to reuse them after the end of the lockdown. Upon return to the lab, the LAD2 cells were thawed, and cell culture was established. It is worth mentioning that LAD2 cells are slowly growing cells that double every 2 weeks, especially after thawing where they double every 21 days. As a result, the cells were tested at 45 days from the start of culture. Unfortunately, at that time (mid November 2020), the cells were completely unresponsive to substance P, which made them useless for the project.

Accordingly, a new batch of LAD2 cells were kindly provided by Prof Kirshenbaum's lab, NIH, USA. Starting from January 2020, the new LAD2 cells were cultured for 45 days until the cell count allowed resumption of the study. Collectively, the COVID-19 pandemic resulted in the loss of whole year from March 2020 to March 2021. Such lost time was planned to be invested in a flow cytometry study, investigating the effect of various molecular agents on the responsiveness of the LAD2 cells to substance P through the expression of the substance P receptors MRGPRX2 and NK-1. Such time loss was accompanied by the loss of many resources such as protein kinase inhibitors and antibodies, which are time sensitive.

9.6.2 The effect of the COVID-19 pandemic on the immunohistochemistry study

Similarly, the COVID-19 lockdown resulted in 8-month delay in slide processing and immunostaining progress. In addition, the process of preparation of the patient and control lists was also delayed. This process was performed by an expert pathologist, Dr Hannah Markham, who was heavily engaged with clinical tasks after the pandemic. The study was originally planned to target the study of the 2 SP receptors MRGPRX2 and NK-1, but the severe time loss caused by the pandemic has forced us to prioritise the study of the expression of MRGPRX2 alone.

10 References

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11 Appendix 1: Paper submitted

Medical-Grade Manuka Honey Inhibits Mast Cell Degranulation through Downregulation of Protein Kinase-B/Akt Phosphorylation: Potential Intravesical Agent in the Management of Interstitial Cystitis/Bladder Pain Syndrome?

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Abstract

Aims: Mast cell numbers and activity are significantly elevated in several inflammatory conditions including interstitial cystitis/bladder pain syndrome (IC/BPS). This condition is associated with the release of neuroactive substances e.g. substance P (Sub P), inducing mast cell degranulation and tissue inflammation. Medihoney (MH) is a medical grade Manuka honey with strong anti-microbial properties. We recently reported its anti-inflammatory properties through inhibition of mast cell histamine release. In this study, we investigated the anti-inflammatory effect of MH against neurogenic inflammation via stabilisation of Sub P-induced mast cell degranulation. **Methods:** LAD2 human mast cells were activated by Sub P (1µM) for 40 minutes with or without 20-minute pre-incubation with MH. Degranulation was assessed by measuring release of the enzyme β-hexosaminidase, while the underlying intracellular signalling was assessed using protein phosphorylation assays. Statistical analysis was performed by one-way ANOVA and Friedman test using GraphPad Prism 9. **Results:** MH (4% and 6%) significantly inhibited mast cell degranulation by approximately 90%. MH (4%) inhibited Sub P-induced phosphorylation of the protein kinases Akt (serine 473), WNK1 and STAT3 (Serine 727). **Conclusion**: MH inhibits sub P-induced LAD2 cell degranulation possibly via downregulation of Akt activation. Our findings suggest that MH could potentially be of value as an intravesical anti-inflammatory agent in the management of the neurogenic bladder wall inflammation associated with IC/BPS. However, animal studies and clinical trials are required to further establish its efficacy and safety.

Key message:

Abnormal mast cell activation is implicated in chronic inflammatory conditions including BPS/IC. Medihoney inhibited non-specific mast cell activation in vitro.

Medihoney modulates intracellar signalling events to strongly inhibit the neurogenic degranulation induced by substance P in LAD2 mast cells.

As catheter-introduced wash, Medihoney might potentially alleviate bladder inflammation.

Introduction

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic debilitating inflammatory disease of the urinary bladder associated with chronic bladder pain, urge to void and both day- and night-time frequency (Nordling, Fall and Hanno, 2012). Several treatment guidelines have been established to treat this complex condition (Cox *et al.*, 2016). Most treatments show variable, short-term improvements, which can vary from one patient to another (Belknap, Blalock and Erickson, 2015) making IC/BPS difficult to diagnose and treat (Bosch and Bosch, 2014a). Thus, there is an unmet need for more effective therapeutic agents be they naturally derived or synthetic. Whilst the pathophysiology is elusive and multi-factorial (Patnaik *et al.*, 2017b), neurogenic inflammation is believed to underlie the chronic bladder inflammation seen in IC/BPS (Patnaik *et al.*, 2017b). Histology shows the urothelium in IC/BPS to be thin and denuded with or without ulceration (Hurst *et al.*, 2015). This allows harmful urinary constituents to leak into the sub-urothelial layer and depolarize bladder sensory nerve endings (Parsons, 2011; Hurst *et al.*, 2015), resulting in the release of neuroactive substances including substance P (Sub P) and calcitonin gene-related peptide. Supporting this theory are studies showing that the density of SP-positive nerve endings in IC/BPS is not only significantly higher compared to controls but are located in close proximity to mast cells (Pang *et al.*, 1995).

Sub P activates mast cells resulting in degranulation and the release of both pre-stored and de-novo synthesized pro-inflammatory mediators, which promote chronic bladder wall inflammation (Kulka *et al.*, 2008; Manning *et al.*, 2016). These include serine proteases, biogenic amines, arachidonic acid metabolites, cytokines and chemokines (da Silva, Jamur and Oliver, 2014), which generate chronic inflammation and pain in IC/BPS bladders (White, Bhangoo and Miller, 2005; Zhang and An, 2007). Upon binding to its G protein-coupled receptors (MRGPRX2 and NK-1), Sub P triggers intracellular signalling events underlying mast cell activation and degranulation. These include phosphorylation of the enzymes phospholipase C-β (PLC-β), phosphoinositide-3 kinase (PI3K), protein kinase B/Akt, extracellular signal-regulated kinase (ERK1/2) and the signal transducer and activator of transcription 3 (STAT3) (Vines and Prossnitz, 2004; Qin *et al.*, 2016).

Manuka honey is a unique type of honey from New Zealand with a wide range of properties including antimicrobial activity and biofilm inhibition (Emineke *et al.*, 2017) with recent reports highlighting its potential anti-inflammatory and anti-oxidant effects (van den Berg *et al.*, 2008). Interestingly, Medihoney (MH), a medical-grade Manuka honey, has been shown to inhibit IgE-dependent degranulation and histamine release in vitro using a human mast cell line (LAD-2) (Alangari *et al.*, 2017b).

The aim of the present study was to investigate the potential anti-inflammatory effect of MH against Sub P-induced mast cell degranulation (as a model for neurogenic

inflammation), and to explore the potential cellular mechanisms through which MH might exhibit such properties using an in vitro mast cell model.

Materials and Methods

Reagents

Medihoney (Comvita, New Zealand Ltd), Sub P (Sigma), *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (CAS # 3459-18-5, Sigma), Human phospho MAPK Array Kit (Cat# ary003c, R&D Systems). Human Phospho (Ser473)/Total Akt Whole Cell Lysate Kit (Cat# K15100D-1, MSD, USA). Lactate Dehydrogenase Activity Assay Kit (Cat# MAK066, Sigma).

Cell culture of the human LAD-2 mast cells

The human mast cell line (LAD2) - a kind gift from Professor Arnold Kirshenbaum (NIH, USA) - a well-established mast cell model for in vitro experiments (Kirshenbaum *et al.*, 2014) , was used in the current study. As per the source instructions, LAD2 cells were cultured in Stem Pro 34-Serum free medium (Invitrogen) enriched with 100 ng /ml recombinant human stem cell factor (Peprotech), 2mM L-glutamine with penicillin/streptomycin (Sigma Aldrich), and 1 ml of Stem Pro growth supplement (Invitrogen) (Kirshenbaum *et al.*, 2014).

β-hexosaminidase (degranulation) assay using LAD-2 cells

Following two wash in 1x PBS, the LAD2 cells were re-suspended in Tyrode's buffer at a density of 50,000 cells per 160 µL of Tyrode's buffer/ well, and the cell suspensions were transferred into a 96-well plate. The cells were challenged with 1µM Sub P or 10µg/ml Compound 48/80 for 40 minutes with or without 20-minute pre-incubation with MH at 2, 4 and 6% concentrations (W/V). All drugs were prepared in the reaction buffer (Tyrode's buffer) (Leung *et al.*, 2014). After incubation, the plates were centrifuged for at 693 g for 10 minutes at 4°C. Subsequently, 30µL of each well was transferred into the corresponding well of a 96 well assay plate. A 50 µL aliquot of beta-hexosaminidase substrate (*p*-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Aldrich) was added to each well, followed by one hour incubation. Finally, 100 µL of the stop solution 0.2mM glycine was added into each well, and the plate read by a plate reader at Endpoint L1-L2, wavelength 410 to 595 nm.

Lactate dehydrogenase (Cytotoxicity) assay using LAD2 cells

LAD2 cells in Tyrode's buffer (400,000 cells/well) were incubated at 37°C with 2% and 4% MH for 20 minutes. Then the cells were challenged for 1 hour with 2% and 4% MH (W/V) or for 40 minutes with 1µM Sub P. The plates were then centrifuged at 693 g for 10 minutes at 4°C. Cytotoxicity was measured through the quantification of lactate

dehydrogenase enzyme in the cell culture supernatant using a specific lactate dehydrogenase activity assay kit (MAK066, Sigma). 50 µL of the cell culture supernatant was added to 50 µL of the master reaction mix to each well of a 96-well assay plate. The total sample (100% cytotoxicity) was prepared by completely lysing the cells in 1% Triton-X in 1X PBS. Measurement were taken after 3 minutes (initial reading) and 13 minutes (final reading) from adding the substrate mix while incubating at 37°C and 5% CO_2. Finally, plates were read using a plate reader at Endpoint L1, wavelength 450 nm, and lactate dehydrogenase activity was calculated according to the manufacturer's instructions.

The effect of Sub P with or without MH pre-incubation on LAD2 cell mitogen-activated protein kinase activity

A. LAD2 cell challenge and lysate preparation

For each condition, 10 million cells were re-suspended in Tyrode's buffer and transferred into 2 ml Eppendorf tubes. The cells were challenged with 1µM Sub P for 15 minutes with or without 30-minute pre-incubation with 4% MH. After challenge, each tube was topped up with ice-cold 1x PBS, and washed twice at 4000 g at 4°C for 5 minutes each in cold micro centrifuge. 1ml of lysis buffer (R&D Systems) was added to each tube, followed by short vigorous vortexing and 30-minute rolling in an ice-filled bottle. This was followed by 5-minutes centrifugation at 4°C and 14,000 G to lyse the cells. Cell lysates were pipetted out and stored at -80^{o}C for a maximum of 5 days before used in the phosphorylation assay.

B. Assessment of the phosphorylation levels of mitogen activated protein kinases using Phospho MAPK array assay

The levels of 37 phosphorylated MAPKs in each cell lysate were quantified using Phospho-MAPK Array Kit (# ary003c, R&D Systems, USA) according to the manufacturer's protocol. Each kit contains four sets of two nitrocellulose membranes each, one set for each sample, where detection and control antibodies for each phosphorylated protein kinase are plated on a pair of dots (duplicates). After incubation with detection antibodies and streptavidin-HRP, membranes were visualised using Amersham^{TM} imager 600.

C. Assessment of the phosphorylation levels of protein kinase B/Akt at position serine-473 using a validated highly specific electroluminescent phosphorylation assay

Following cell challenge, lysates were prepared as previously described, but in this case, 8 million cells per condition were used, and cells were lysed in 150 µL of the full lysis buffer provided in the Akt phosphorylation Kit. Both the total and the serine-473 phosphorylated levels of Akt were evaluated in the cell lysates using Human Phospho (Ser473)/Total Akt Whole Cell Lysate Kit according to the manufacturer's protocol (Cat# K15100D-1, MSD, USA). The kit quantifies the levels of total Akt and the seine 473-phosphorylated Akt in each well of a 96-well plate via an electrochemoluminescent assay, allowing the calculation of a percentage phosphorylation in each sample.

Statistical Analysis

Data were included in a database and analysed using GraphPad Prism 9 software (GraphPad Software, San Diego, USA). Data normality was tested using the Shapiro-Wilk method. Results which were normally distributed are expressed as mean ± SEM. Differences between two or more groups were assessed by one-way ANOVA and Friedman tests. A *p value of <0.05* was taken as statistically significant.

Ethical Considerations

The study does not require specific ethical approval.

Conflict of Interest

All authors have no conflict of interest to declare.

Results

The effect of MH on LAD-2 cells

After one hour incubation of the LAD2 cells with 2, 4 or 6% concentrations of Medihoney, β-hexosaminidase levels remained very close to the spontaneous levels, indicating that Medihoney at all concentrations employed did not induce LAD2 cell degranulation. On the other hand, both the neuropeptide substance P and the MRGPRX2 agonist compound 48/80 induced 42% and 61% release, respectively (Figure 1 (A)).

The effect of MH on Sub P and compound 48/80-induced LAD2 cell degranulation

Pre-incubation with MH at 2%, 4% and 6% induced 79%, 97% and 94% inhibition of β-hexosaminidase induced by both Sub P or compound 48/80, respectively (Figure 1 (B) & (C)).

The effect of MH and substance P on the viability of LAD2 cells

MH at both 2% and 4% concentrations, in addition to substance P 1µM, did not caused any rise of the lactate dehydrogenase activity when compared to the spontaneous levels (controls), suggesting no cytotoxic effect on the LAD2 cells (Figure 2).

The effect of pre-incubation with or without MH on the Sub P-induced degranulation-linked to the intracellular signalling pathways in LAD2 cells

A 15-minute incubation with Sub P induced significant increase in the levels of phosphorylated protein kinases Akt, at the serine 473 position, lysine deficient protein kinase 1 (WNK1), at the threonine 60 position, and ERK1/2, in addition to STAT3 at the serine 727 position. Akt, WNK1 and STAT3 phosphorylation was significantly and consistently inhibited upon pre-treatment of the LAD2 cells with 4% MH (Figure 3).

The effect of pre-incubation with MH on the Sub P-induced phosphorylation of Akt at position serine 473 in the LAD2 cells

Sub P induced significant increase in the levels of the serine 473 phosphorylated Akt. This effect was inhibited upon pre-incubation with MH4% (Figure 4).

It is worth mentioning that this assay was repeated four times using two different batches of LAD2 cells.

Discussion

Mast cells are key effectors in both the innate and adaptive immune responses (Krystel-Whittemore, Dileepan and Wood, 2015). Their abnormal activation is believed to be involved in both the allergic and pseudo-allergic reactions implicated in the pathophysiology of many chronic inflammatory conditions (da Silva, Jamur and Oliver, 2014). As previously described, a mutual activation cycle between nerve-released neuropeptide Sub P and tissue resident mast cells is believed to maintain the chronic inflammatory state in IC/BPS. Such a Sub P and mast cell interaction has been implicated in the pathophysiology of many chronic inflammatory diseases such as atopic dermatitis, severe chronic urticaria, rheumatoid arthritis, irritable bowel syndrome and severe asthma (Ali, 2016b; Voisin, Bouvier and Chiu, 2017).

MH has been shown to inhibit Ca^{++} ionophore-induced mast cell degranulation and histamine release (Alangari *et al.*, 2017a). In the current study, MH has inhibited Sub P-induced β-hexosaminidase release from mast cells in a dose-dependent fashion. Maximal inhibition was achieved using 4% MH, inducing 95% inhibition. A similar effect was observed for 6% MH (Figure 2). In addition, 4% MH inhibited the Sub P-induced activation of the PI3K/Akt signalling pathway as evidenced by the inhibition of Akt phosphorylation at the serine 473 position (Figures 4 (a) & (b)) and Figure 5). MH also inhibited the Sub P-induced WNK1 and STAT3 phosphorylation, but it did not significantly inhibit ERK1/2 (Figure 4 (a), (c), (d) & (E)).

Besides its crucial role in the positive regulation of mast cell development and survival (Gao *et al.*, 2001), PI3K-mediated Akt activation is essential for the pro-inflammatory responses of mast cells upon activation by variable secretagogues (Zhang *et al.*, 2022), a finding which is consistent with our results. This is demonstrated by the increased PI3K and Akt phosphorylation associated with both degranulation and cytokine release (results not shown), in an effect that was sensitive to the PI3K inhibitor LY294002 (Kim, Rådinger and Gilfillan, 2008; Blatt *et al.*, 2012; Bao *et al.*, 2018). Other reports have highlighted the involvement of both Akt and ERK in the mast cell pro-inflammatory responses, as shown in figure 6 (Takayama *et al.*, 2012). Importantly, despite the strong inhibitory effect of MH of the Akt phosphorylation, as shown in figures 4 and 5, MH did not affect the viability of the LAD2 cells (Figure 3).

Interestingly, WNK1 increases the membrane expression and trafficking of the constitutive glucose transporter (GLUT1) via the phosphorylation of TBC1D1 and TBC1D4, RAB-GTPase activating proteins, in a process that is essential for glucose uptake by the cells (Mendes *et al.*, 2010; Henriques *et al.*, 2020). Downregulation of WNK1 via short interference RNA has resulted in a two-fold reduction in the membrane expression of GLUT1 associated with a 60% reduction in glucose uptake by HEK293 cells (Henriques *et al.*, 2020). Interestingly, Akt, like WNK1 phosphorylates TBC1D1, resulted in the regulation of its activity, which is associated with increased membrane expression of GLUT1 (Zhou *et al.*, 2008). GLUT1 membrane trafficking was also dependant on increased phosphorylation of m-TOR and P70-S6 kinase downstream of Akt phosphorylation as

denoted by sensitivity to the m-TOR inhibitor rapamycin. Akt-induced GLUT membrane trafficking was also observed to enhance insulin-mediated de-oxy glucose uptake in 3T3-L1 mouse adipocytes (Zhou *et al.*, 2008). It should also be noted that Akt can phosphorylate WNK1 (Cheng and Huang, 2011), which adds another possible explanation for its pro- GLUT1 membrane trafficking action.

GLUT1 expression is essential for glucose uptake and, consequently, utilisation by mast cells. Both allergic (FcεRI-mediated) and pseudo allergic (MRGPRX2-mediated) mast cell pro-inflammatory responses (Immediate degranulation and late phase cytokine release) are dependent on glucose internalisation and metabolism, which can be either anaerobic glycolysis or aerobic mitochondrial oxidation, resulting in ATP production, as shown in figure 5 (Kitahata *et al.*, 2010; Caslin *et al.*, 2018; Mendoza, Fudge and Brown, 2021).

As noted previously Akt and WNK1 phosphorylation are known to enhance the membrane trafficking of GLUT1 resulting in increased intracellular glucose uptake and the subsequent metabolic changes, which enhance mast cell activation and pro-inflammatory mediator release. At the same time, STAT3 enhances mitochondrial oxidative respiration enhancing mast cell activation (Bernier *et al.*, 2011b; Erlich *et al.*, 2014), and the phosphorylation of the transcription factor STAT3 at the serine 727 position has been reported to be an essential event for mast cell degranulation (Qin *et al.*, 2016). Such evidence might provide a possible explanation for the mast cell stabilising effects of MH through the inhibition of glucose uptake and subsequent metabolic activity in mast cells. However, this would require further studies investigating GLUT1 membrane trafficking levels, lactic acid and ATP production, and oxygen consumption during LAD-2 activation with or without MH.

Unlike other anti-inflammatory agents, honey is not a pure agent, but it has a unique natural composition consisting of a mixture of biologically active agents. This enables honey to produce several biological effects. For example, it possesses strong antimicrobial activity against a broad spectrum of bacterial strains including those that are resistant to antibiotics (Girma, Seo and She, 2019), and strongly inhibits bacterial biofilm formation (Emineke *et al.*, 2017). It promotes angiogenesis and tissue regeneration (Minden-Birkenmaier and Bowlin, 2018). It inhibits inflammation independent of its anti-microbial properties. In addition, MH was well tolerated by urothelial cells and protected them from harmful chemical injuries allowing for urothelial regeneration and restoration of the urothelial barrier in IC/BPS bladders models (Lwaleed *et al.*, 2014). Taken together, the above studies demonstrate that MH possesses three properties that could be exploited medically. Thus, it is anti-microbial, pro-angiogenic and anti-inflammatory. This “triple action” would appear ideally suited for the treatment and management of IC/BPS.

Conclusion and applications

Our findings demonstrate the potential anti-inflammatory properties of MH, through its ability to inhibit mast cell degranulation induced by the neuropeptide Sub P. Moreover,

the study provides the first scientific explanation for the stabilisation of mast cells by MH through the inhibition of intracellular Akt, WNK1 and STAT3 phosphorylation. These data support the potential use of MH as an anti-inflammatory in neurogenic inflammatory conditions including atopic dermatitis, chronic urticaria, and IC/BPS. Specifically, aqueous solutions of MH might be instilled intra-vesically (via a bladder catheter) to aid the management of patients with IC/BPS.

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Legends to Figures

Figure 1: The effect of MH on β-hexosaminidase from LAD2 cells

(A) Percentage release of β-hexosaminidase by LAD2 cells following one-hour incubation with Medihoney (MH) at 2, 4% and 6 % concentrations, 1µM substance P (Sub P) and 10µg/ml compound 48/80. (B) & (C) represent the percentage release of β-hexosaminidase induced by 40-minute incubation with 1µM substance P (Sub P) and 10 µg/ml Compound 48/80, respectively, with or without pre-incubation with Medihoney (MH) at 2, 4 and 6 % concentrations in LAD2 cells. The mean ± SEM for the spontaneous release was 6.7 % ± 0.09 % of the total value. The overall cellular content of β-hexosaminidase (total) was prepared by completely lysing the cells in 1% Triton-X in 1X PBS. Each bar represents mean ± SEM (** P value <0.01, **** P value <0.0001, n=12). The graph represent the results of 4 independent assays, and each assay was performed in triplicates. Analysis was performed by Kruskal-Wallis test using GraphPad Prism 9 software.

Figure 2: The effect of MH on the viability of the LAD2 cells

Lactate dehydrogenase activity as measured by specific detection of NADH in the cell culture supernatant after one hour incubation of the LAD2 cells with 1µM of substance P with or without pre-incubation with 2 and 4% MH. The experiment was performed 3 times (n=6).

Figure 3: The effect of MH on the substance P-induced mast cell signalling events

(A) Image of 4 sets of nitrocellulose membranes plated with monoclonal antibodies against various phosphorylated intracellular signalling protein kinases (Phospho MAPK array, Cat# ary003c, R&D systems, USA). The black, red, blue and green arrows point to duplicate dots plated with monoclonal antibodies against phosphorylated Lysine Deficient Protein Kinase 1 (WNK1), extracellular signal-regulated kinase (ERK1/2), protein kinase B/Akt (serine 473) and STAT 3 (serine 473), respectively. Graphs (B), (C), (D), (E) and (F) represent the level of phosphorylation of Akt at position serine 473, ERK1/2, WNK1, signal transducer and activator of transcription 3 (STAT3) at position serine 727, and GSK3α/β (Serine 21/9), respectively. The graphs represent the results of 4 independent assays and each assay was performed in duplicates. Differences between the levels of phosphorylated proteins were analysed by Friedman test using GraphPad Prism 9 software. Each bar represents mean ±

SEM (**** P value <0.0001, n=8). The differences between the means were analysed using 2-way ANOVA to normalise the LAD2 cell batch and passage differences.

Figure 4: The effect of MH on the substance P-induced Akt phosphorylation in LAD2 cells

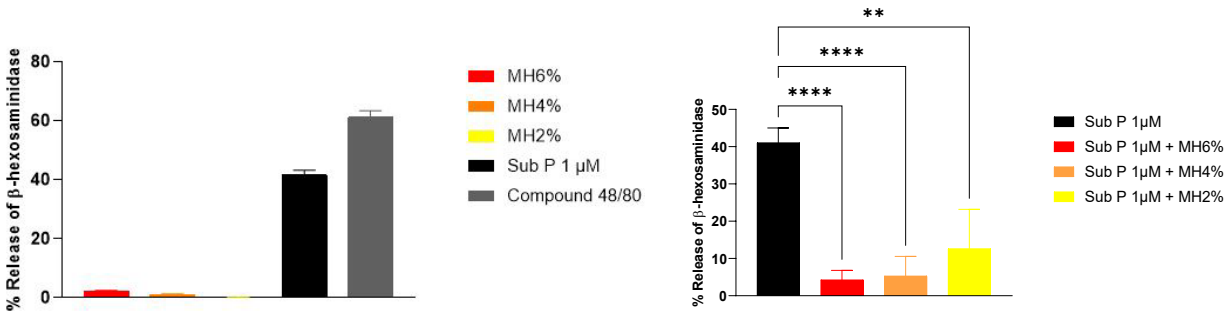
Percentage Phosphorylation of protein kinase B/Akt at serine 473 position in LAD2 cells as detected by the total/ser473 phospho Akt assay kit (MSD, USA). The graph represent the results of 3 independent assays and each assay was performed in duplicates. Statistical analysis one way ANOVA, and the differences between the means were analysed by the Bonferroni's multiple comparisons test (**** P value <0.0001, n=6).

Figure 5: Schematic representation of the potential modulatory effects of MH on the G protein-coupled receptor-signalling pathway underlying substance P-induced mast cell degranulation, P = phosphorylation and MH = inhibition of phosphorylation by MH.

Figures

Figure 1:

A



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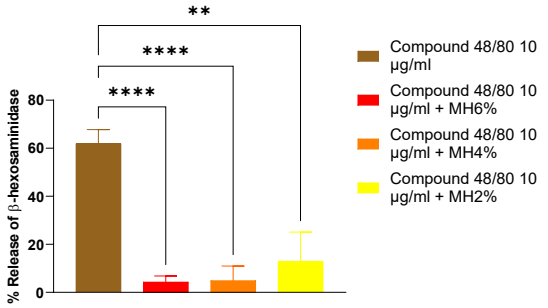

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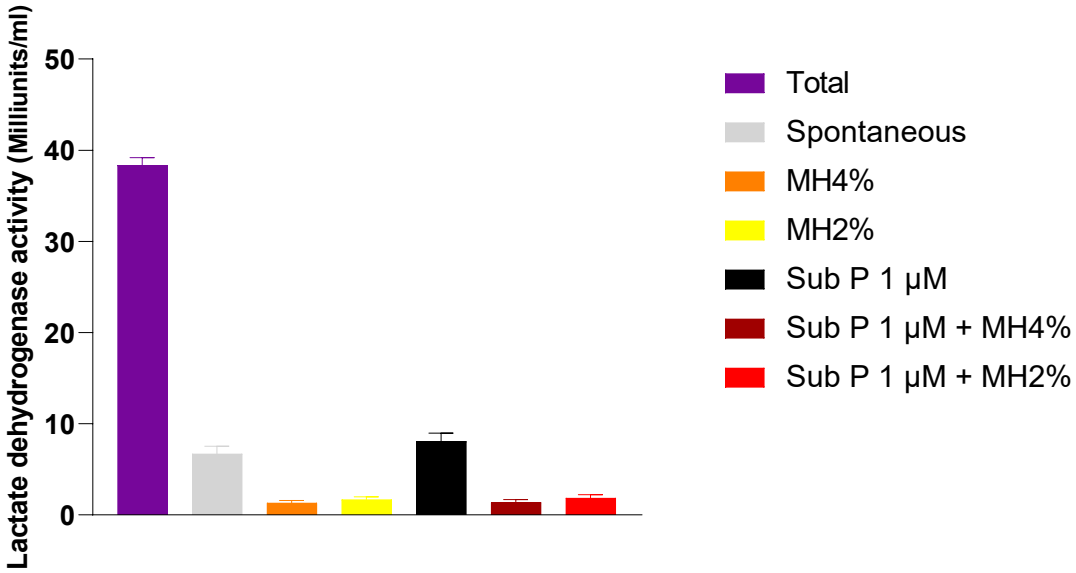
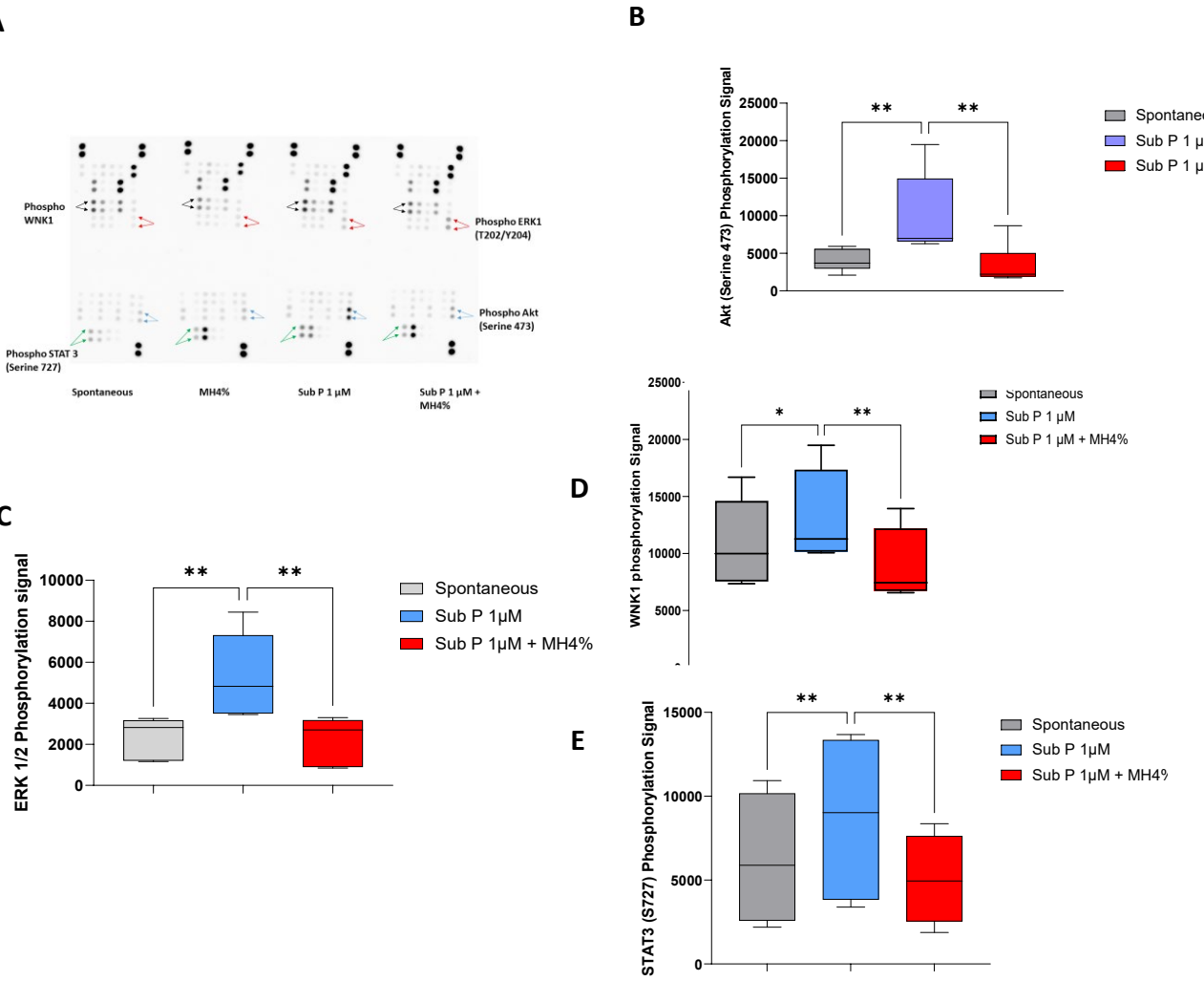

Lactate dehydrogenase activity (Milliunits/ml)
50
40
30
20
10
0
Total
Spontaneous
MH4%
MH2%
Sub P 1 µM
Sub P 1 µM + MH4%
Sub P 1 µM + MH2%

Figure 3:



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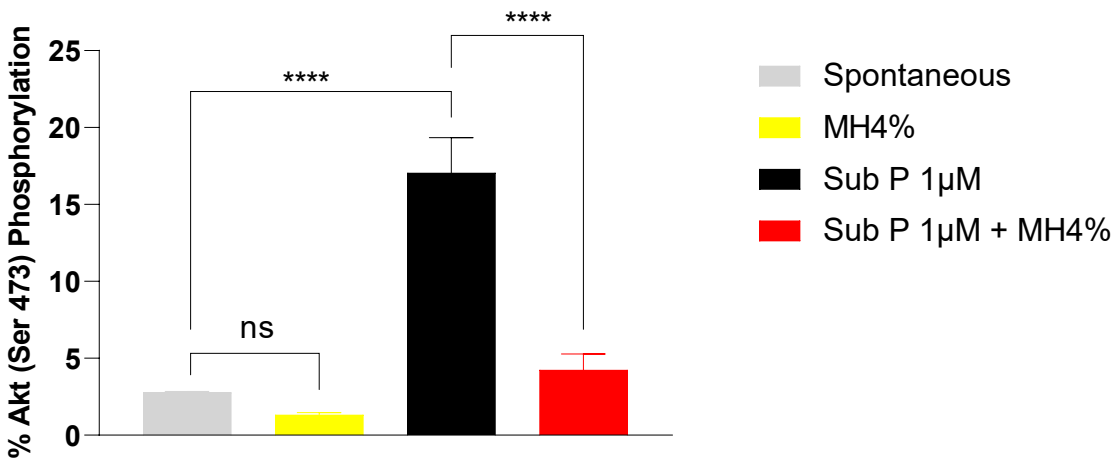
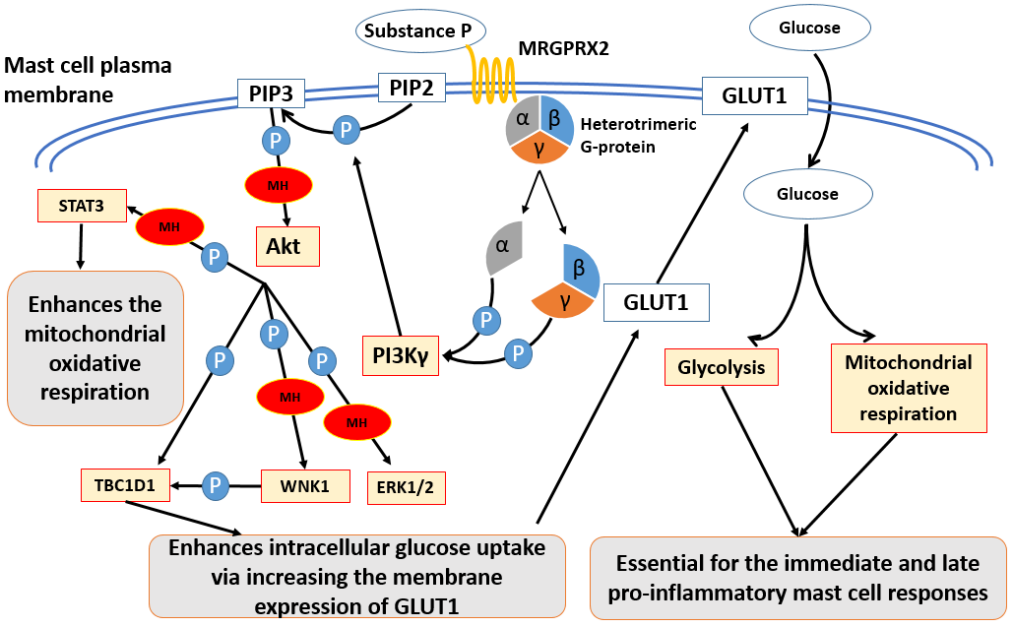

% Akt (Ser 473) Phosphorylation
25
20
15
10
5
0
****
****
ns
Spontaneous
MH4%
Sub P 1µM
Sub P 1µM + MH4%

Figure 5:


Substance P
MRGPRX2
Glucose
Mast cell plasma membrane
PIP3
PIP2
GLUT1
α
β
γ
Heterotrimeric G-protein
P
MH
STAT3
Akt
Glucose
Enhances the mitochondrial oxidative respiration
PI3Kγ
GLUT1
Glycolysis
Mitochondrial oxidative respiration
TBC1D1
WNK1
ERK1/2
Enhances intracellular glucose uptake via increasing the membrane expression of GLUT1
Essential for the immediate and late pro-inflammatory mast cell responses

12 Appendix 2: Paper submitted

Medical-Grade Mānuka Honey Inhibits Mast Cell Degranulation through inhibition of MRGPRX2 expression: Potential Intravesical Agent for the Management of Interstitial Cystitis/Bladder Pain Syndrome?

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Word counts: Abstract: 229 Text: 2,914

Keywords: Neurogenic Inflammation; Interstitial cystitis/Bladder pain syndrome (IC/BPS); Mast cells; Degranulation; MRGPRX2; Medihoney™

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Abstract

Aims: Medihoney™, a medical-grade Mānuka honey, has strong anti-microbial properties. We recently reported its effects on stabilising mast cell degranulation via inhibition of histamine release. Here, we investigated its inhibitory effect of chronic neurogenic inflammation, a common pathology in interstitial cystitis, via inhibition of substance P (Sub P)-induced mast cell degranulation and MRGPRX2 activation. In addition, we studied MRGPRX2 expression in IC/BPS bladder biopsies.

Methods: LAD2 human mast cells were activated by Sub P (1µM) for 40 minutes with or without 20-minute pre-incubation with Medihoney™. Degranulation was assessed by measuring the β-hexosaminidase enzyme release. MRGPRX2 activation was assessed by

Fluo-4 Ca^{++} imaging in MRGPRX2-expressing HEK-293 cells. Bladder biopsies from IC/BPS patients and controls were stained against mast cell tryptase, chymase and MRGPRX2.

Results: Medihoney™ (4% and 2%) strongly inhibited mast cell degranulation by approximately 90%, an effect that was replicated with a Mānuka honey extract This was accompanied by a dose dependent inhibition of the sub P-induced intracellular Ca^{++} signalling in the HEK-293 cells. Importantly, in bladder biposies from patients with interstitial cystitis ~66% of the tryptase+ve mast cells (identifies all mast cells) expressed MRGPRX2, which was significantly higher compared to the control group (25%).

Conclusion: Mast cells in IC/BPS bladders are potentially responsive to Sub P, suggesting a positive role for neurogenic inflammation in the pathophysiology of IC/BPS. Medihoney™ inhibits Sub P-induced LAD2 cell degranulation via inhibition of MRGPRX2 activation. Thus, Medihoney™ could potentially downregulate neurogenic bladder wall inflammation associated with IC/BPS. Human trials would be required to establish the safety and efficacy of Medihoney™ treatment of patients with IC/BPS.

Key message:

- Neurogenic MRGPRX2-mediated mast cell activation is implicated in chronic inflammatory conditions including IC/BPS.
- MedihoneyTM strongly inhibits the neurogenic mast cell degranulation induced by substance P in LAD2 mast cells via the modulation of intracellular signalling events.
- MedihoneyTM might have therapeutic value in reducing bladder inflammation in patients with IC/BPS.

Introduction

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic debilitating inflammatory condition associated with chronic bladder pain and urge to void as well as increased day- and night-time urination frequency (1). Recent reports show a progressive increase in the prevalence of IC/BPS (i.e. 52 to 500/100,000 in females and 8 to 41/100,000 in males), accompanied by an increase in the incidence rate across genders (2, 3). The incidence is estimated at 1.2/100,000. A recent report from the UK highlighted the significant burden that IC/BPS places on patients' quality of life, work productivity and how it drains healthcare reserves (4). These include a significantly impaired Health-Related Quality of Life (HRQOL) score, greater work productivity loss (41.7%), and, crucially, a higher number of IC/BPS patients attending GP surgeries, outpatient clinics and A&E Departments together with increased inpatient admissions (5). The above statistics are reflected in the finding that the number of patients with IC/BPS who commit suicide is four times that of healthy individuals (6).

The management of IC/BPS presents a notable burden on health services in the UK and worldwide. For example, in the USA, it has been calculated that the mean annual costs associated with the management of IC/BPS for confirmed cases is $14 billion per year; and if allowance is made for under diagnosed cases, this figure could rise up to $240 billion annually (7-10). The above places IC/BPS among the major health problems that merit research to bridge the wide gaps that exist between diagnosis and treatment. Several treatment regimens have been used to treat this complex condition (11). However, diagnosis is difficult and most treatments show variable, short-term improvements, which are not predictable for individual patients (12) making IC/BPS difficult to treat (13). Thus, there is an unmet need for more effective therapies. Whilst the pathophysiology of IC/BPS is elusive and multi-factorial (14), neurogenic inflammation

is believed to important underlying factor contributing to chronic bladder inflammation (14). Histological analysis has shown the urothelium of IC/BPS patients is thin and denuded with or without ulceration (15). This allows harmful urinary constituents to leak into the sub-urothelial layer and depolarize bladder sensory nerve endings (15, 16), resulting in the release of neuroactive substances including substance P (Sub P) and calcitonin gene-related peptide. In addition, the density of SP-positive nerve endings in IC/BPS is not only significantly higher compared to controls but the same nerves are also located in close proximity to mast cells (17).

Sub P activates mast cells resulting in degranulation and the release of both pre-stored and de-novo synthesized pro-inflammatory mediators, which promote chronic bladder wall inflammation (18). These include serine proteases, biogenic amines, arachidonic acid metabolites, cytokines and chemokines (19), which generate chronic inflammation and pain in IC/BPS bladders (20). Upon binding to its G protein-coupled receptors (MRGPRX2), Sub P triggers intracellular signalling events underlying mast cell activation and degranulation. These include phosphorylation of the enzymes phospholipase C-β (PLC-β), phosphoinositide-3 kinase (PI3K), protein kinase B/Akt, extracellular signal-regulated kinase (ERK1/2) and the signal transducer and activator of transcription 3 (STAT3) (21, 22).

However, the role of neurogenic inflammation in the pathophysiology of the IC/BPS is still contentious. This is due to the uncertainty regarding some factors essential for the development of neurogenic inflammation in the bladders of the patients. Many reports link BPS/IC with a characteristic increase in the density of mast cells in the detrusor layer of the bladders of the patients (23-30). However, other reports stated that there is no significant difference in the mast cell numbers between patients compared to controls (31, 32).

The neurogenic inflammation theory is based on positive responsiveness of mast cells to the neuroactive agents released by the desensitized sensory nerve endings, including the neuropeptide substance P. Such responsiveness is attributed to mast cell surface expression of the neuropeptide receptor MRGPRX2, which is responsible for substance P mediated mast cell activation in humans. The expression of MRGPRX2 is variable between different microenvironments (33), and its expression in urinary bladder mast cells has been assessed.

Mānuka honey is derived from the nectar of *Leptospermum scoparium* (Mānuka) trees in New Zealand, and is recognised for a wide range of bioactive properties including its unique non-peroxide antimicrobial activity and biofilm inhibition (34) with recent reports highlighting its potential anti-inflammatory and anti-oxidant effects (35). Interestingly, Medihoney^{TM}, a medical-grade Mānuka honey, has been shown to inhibit Ca^{++} ionophore-induced degranulation and histamine release in LAD2 human mast cells (36).

Therefore, the aim of the current study was to further explore the importance of the neurogenic inflammation in the pathophysiology of IC/BPS via assessment of mast cells numbers and their expression of MRGPRX2 in the urinary bladders of BPS/IC patients compared to non-IC/BPS controls using an immunohistochemistry approach. In addition, the second aim was to investigate the potential for Medihoney^{TM} to suppress Sub P-induced mast cell degranulation (as a model for neurogenic inflammation), and to explore its potential effect on the activation of MRGPRX2 receptor using representative human cell line models.

Materials and Methods

Reagents

100% Medihoney™ (#405, Comvita, New Zealand Ltd), Sub P (# S6883, Sigma), *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (CAS # 3459-18-5, Sigma) and Compound 48/80 (#C2313, Sigma Aldrich).

Cell culture of the human LAD-2 mast cells

The human mast cell line (LAD2), kindly provided by Professor Arnold Kirshenbaum NIH USA, is a well-established mast cell model system for in vitro neuro-inflammatory testing (37). As per the source instructions, LAD2 cells were cultured in Stem Pro 34-Serum free medium (Invitrogen) enriched with 100 ng /ml recombinant human stem cell factor (Peprotech), 2mM L-glutamine with penicillin/streptomycin (Sigma Aldrich), and 1 ml of Stem Pro growth supplement (Invitrogen) (37).

B-hexosaminidase (degranulation) assay using LAD-2 cells

Following two washes in 1x PBS, the LAD2 cells were re-suspended in Tyrode's buffer at a density of 50,000 cells per 160 µL of Tyrode's buffer/well. The cell suspensions were then transferred into a 96-well plate. The LAD2 cells were challenged with 1µM Sub P or 10µg/ml Compound 48/80 (both compounds being MRGPRX2-specific mast cell activators) for 40-minute with or without 20-minute pre-incubation with Medihoney™ at 2, 4 and 6% concentrations (W/V) or artificial honey (35% glucose and 35% fructose in ultrapure water). All agents were prepared in the reaction buffer (Tyrode's buffer) (38). After incubation, the plates were centrifuged at 693 g for 10 minutes at 4°C. Subsequently, 30µL of each well was transferred into the corresponding well of a 96 well assay plate. A 50 µL aliquot of beta-hexosaminidase substrate (*p*-nitrophenyl-N-acetyl-β-

D-glucosaminide (Sigma Aldrich) was added to each well, followed by one hour incubation at 37°C and 5% CO_2. Finally, 100 µL of the stop solution 0.2mM glycine was added into each well, and the plate read by a plate reader at Endpoint L1-L2, wavelength 410 to 595 nm.

The effects of substance P (1uM) and Compound 48/80 (10 ug/ml) on the intracellular Ca++ Flux in MRGPRX2-transfected HEK-293 cells (Creative Biogene)

HEK-293 cells transfected to stably express MRGPRX2 receptor (Creative Biogene) were cultured in high glucose full DMEM medium (Cat# 11965092, ThermoFisher) containing 10% foetal calf serum and 1 µg/mL Puromycin. Cells were cultured to confluence in poly lysine D-coated black/clear bottom 96 well plates. Cells were loaded with Fluo-4 Ca^{++} dye for one hour using Fluo-4 Direct Calcium Assay Kit (Cat# F10471, ThermoFisher), then activated with the MRGPRX2 agonists Sub P (1µm) or Compound 48/80 (10 µg/ml) with or Medihoney™ pre-incubation. The activation-induced rise in the cytoplasmic Ca^{++} levels were assessed using FlexStation II platform (Molecular Devices).

Immunohistochemistry in IC/BPS urinary bladder biopsies

The study commenced after obtaining ethical approval of the Research Ethics Committee (REC) (IRAS ID 246784). Formalin-fixed paraffin embedded (FFPE) urinary bladder biopsies from 17 IC/BPS patients and 16 non-IC/BPS controls were involved into the study. The included FFPE biopsies were taken for diagnostic purposes after patient consent. IC/BPS diagnosis was based according to the 2008 ESSIC diagnostic criteria. The control group include healthy bladder tissue from carcinoma in situ patients or patients with non-specific bladder wall inflammation. 4 serial sections, each of 4µm thickness, were cut from each bladder biopsy and stained with antibodies against mast cell-specific proteases

tryptase (AA1) and chymase (CC1), as unique mast cell markers, as well as anti-MRGPRX2 (Abcam, USA). All staining was performed using an automated auto Stainer platform. Following microscopic scanning, images from the serial sections were aligned and superimposed using Adobe Photoshop CS6 software. Mast cell quantification and co-expression patterns were studied using Image J software. Statistical differences between the IC/BPS and the control groups were analysed by Mann Whitney U test and unpaired t-tests using GraphPad Prism 9 software.

Results

The effect of Medihoney™ on LAD-2 cells

After one hour incubation of the LAD2 cells with 2, 4 or 6% concentrations of Medihoney™, β-hexosaminidase levels remained similar to the spontaneous levels, suggesting that Medihoney™ alone at all concentrations used did not induce LAD2 cell degranulation. On the other hand, the MRGPRX2 agonists, Substance P (Sub P) and compound 48/80, induced 42% and 61% release, respectively. The non-specific mast cell activator Ca++ ionophore was included as a positive control and induced 86% release above spontaneous. The overall cellular content of β-hexosaminidase (total) was prepared by completely lysing the cells in 1% Triton-X in 1X PBS (Figure 1 (A)).

The effect of Medihoney™ on Sub P and compound 48/80-induced LAD2 cell degranulation

Pre-incubation with Medihoney™ at 2%, 4% and 6% resulted in 79%, 97% and 94% inhibition of the β-hexosaminidase release induced by both Sub P or compound 48/80, respectively (Figure 1 (B) & (C)). Therefore, 2% and 4% where chosen for subsequent experiments. Importantly, pre-incubation with artificial honey at 2% and 4% concentrations failed to inhibit of the β-hexosaminidase release induced by Sub P (Figure 1 (D)). This finding excludes the sugar content from being responsible for the mast cell stabilizing effects of Medihoney™ .

The effect of Medihoney™ on MRGPRX2 activation in MRGPRX2-expressing HEK-293 cells

The effects of substance P (1μM) and Compound 48/80 (10 μg/ml) on the intracellular Ca++ Flux in MRGPRX2-transfected HEK-293 cells

Both substance P (1μM) and Compound 48/80 (10 μg/ml) induced a significant rise in the intracellular Ca^{++} levels in the MRGPRX2-transfected HEK-293 cells within 18 seconds of treatment. Both agents failed to induce any Ca^{++} signalling in the standard non-transfected HEK-293 cells. Typically, as a non-specific Ca^{++} ion carrier, Ca^{++} ionophore 1μM induced a rise in the intracellular Ca^{++} levels in both the transfected and non-transfected cell lines. The substance P vector (0.1M acetic acid) failed induce any rise in the intracellular Ca^{++} levels in HEK-293 cells or degranulation in LAD2 cells (Figure 2).

The effect of Medihoney™ on the substance P-induced rise in the cytoplasmic Ca^{++} levels in MRGPRX2-expressing HEK-293 cells

Twenty-minute pre-incubation of the HEK-293 cells with MH at 2 and 4% concentrations induced dose-dependent inhibition of the substance P (Figure 3 (A & D)) or the Compound 48/80-induced Ca^{++} flux (Figure 3 (C & F)), while artificial honey (AH) at 4 and 2% concentrations failed to inhibit the substance P-induced Ca^{++} flux (Figure 3 (B &E)).

Immunohistochemical analysis of mast cell density and MRGPRX2 expression in FFPE bladder biopsies from IC/BPS patients compared to controls

Mast cell density and subtypes

In the BPS/IC group, mast cells were abundant in both detrusor layer and the lamina propria (LP). The mean (± SEM) density of mast cells stained with tryptase (all mast cells) was 166.1 ± 21.02 and 215.0 ± 19.05 cells/mm^2 in the detrusor layer and the lamina propria, respectively. This was significantly higher compared to the control group (72.64 ±

9.150 and 84.88 ± 9.212, respectively; Figure 6 (A&B)). Similarly, the mean (± SEM) density of the Chymase^{+ve} mast cells (MC_{TC}) in the IC/BPS group was 155.7 ± 23.86 and 181.6 ± 17.09 cells/mm^2, in the detrusor layer and the LP, respectively. This was significantly higher compared to the control group (59.83 ± 9.153 and 65.27 ± 8.710, respectively; Figure 6 (C&D)). In the BPS/IC group, the mean (± SEM) percentage of chymase expression by the Tryptase^{+ve} mast cells was 70.95 ± 5.371 and 84.47 ± 4.209, in the detrusor and LP, respectively. This was significantly higher compared to the control group (54.45 ± 4.546 and 55.21 ± 4.332, respectively; Figure 72), indicating an IC/BPS-related class switch towards the MC_{TC} subtype.

Mast cell expression of MRGPRX2 in the patients vs controls

In the BPS/IC group, the mean (± SEM) percentage of tryptase+ve mast cells expressing MRGPRX2 was 67.99 ± 2.537 and 61.75 ± 3.366 in the detrusor and LP layers, respectively. This was significantly higher compared to the control group (28.38 ± 6.004 and 29.97 ± 6.352, n the detrusor and LP layers, respectively; Figure 6E-F)). Similarly, in the BPS/IC patient group, the mean (± SEM) percentage of Chymase^{+ve} mast cells expressing MRGPRX2 was 66.66 ± 2.629 and 61.64 ± 3.975 in the detrusor and LP, respectively. This was significantly higher compared to the control group (31.44 ± 6.046 and 24.99 ± 7.296, in the detrusor and LP, respectively; Figure 6 (G&H). This indicates an IC/BPS-related increase in MRGPRX2 expression by urinary bladder mast cells.

Discussion

Mast cells are key effectors in both the innate and adaptive immune responses. Their abnormal activation is believed to be involved in both the allergic and pseudo-allergic reactions implicated in many chronic inflammatory conditions (19). A mutual activation cycle between the neuropeptide Sub P releasing nerve endings and tissue resident mast cells is believed to maintain the chronic inflammatory state in interstitial IC/BPS. Such interaction has been implicated in the pathophysiology of many other chronic inflammatory diseases including atopic dermatitis, chronic urticaria, rheumatoid arthritis, and severe asthma (39). Mast cell responsiveness to the neuropeptide substance P in humans depends on their expression of MRGPRX2, a recently identified G protein coupled receptor (40). The expression of such a receptor on mast cells varies between different tissue micro-environments and its expression on urinary bladder mast cells has not been explored previously (33).

In the current study, the average density of mast cells stained with tryptase or chymase in both the detrusor or the LP layer of the BPS/IC group was significantly higher compared to the control tissue. Such evidence is consistent with previous reports highlighting the increased numbers of the tissue resident mast cells in the bladders of BPS/IC patients (29, 41-43). Regarding mast cell subtypes, about 70% and 84% of the Tryptase^{+ve} mast cells in the detrusor and the LP layer of the BPS/IC group co-expressed chymase, which was significantly higher than controls (only 50% co-expression). Such evidence suggests a shift of the mast cell subtype from MC_T to MC_{TC} in the BPS/IC group, which is consistent with other reports highlighting the shift of the MC subtype from MC_T to MC_{TC} in the pulmonary mast cells of severe asthma patients (44). Based on previous reports highlighting the pro-inflammatory and cell lysis effects of mast cell chymase (45, 46), the shift in the mast cell

subtype to MC_{TC} might, in part, explain the presence of the urothelial barrier defect seen in the bladders of the BPS/IC patients.

MRGPRX2 was expressed by around 60% and 70% of the mast cells in the LP and the detrusor layers of the IC/BPS bladders, which was significantly higher compared to controls (30% co-expression). To our knowledge, this is the first evidence confirming the expression of MRGPRX2 on the mast cells in a urinary bladder tissue and, specifically, in BPS/IC patients. Nevertheless, such evidence indicates the upregulation of MRGPRX2 receptor expression by mast cells in BPS/IC. In the current study, MC_{TC} was the dominant subtype (around 70% of all mast cells). This finding is supported by the MRGPRX2 expression, which is believed by many to be exclusively expressed by connective tissue mast cells (MC_{TC}) (44, 47).

Medihoney™ has been shown to inhibit mast cell degranulation and histamine release induced by Ca++ ionophore, a non-specific strong inducer of mast cell activation in vitro (20). In the current study, Medihoney™ inhibited mast cell degranulation (as assessed by β-hexosaminidase release) induced by the neuropeptide Sub P in a dose-dependent manner, suggesting a strong potential for Medihoney™ to supress neurogenic mast cell activation and tissue inflammation. Maximal inhibition (97%) was achieved using 4% Medihoney™ (Figure 1).

Such an effect was accompanied by dose-dependent inhibition of the substance P-induced Ca^{++} signalling in MRGPRX2-transfected HEK-293 cells, indicating inhibition of the receptor activation. Such evidence strongly supports an anti-inflammatory role of Medihoney™ against neurogenic inflammation.

Unlike other anti-inflammatory agents, honey is not a pure agent, it has a unique natural composition consisting of a mixture of biologically active agents. This enables honey to produce several biological effects. For example, it possesses strong antimicrobial activity against a broad spectrum of bacterial strains including those that are resistant to antibiotics (48), and strongly inhibits bacterial biofilm formation (34). Furthermore, it promotes angiogenesis and tissue regeneration (49) and inhibits inflammation independent of its anti-microbial properties. In addition, Medihoney™ was well tolerated by urothelial cells and protected them from cytotoxic insults while maintaining urothelial barrier integrity in IC/BPS bladder models (50). Taken together, the above studies demonstrate that Medihoney™ possesses three properties that could be exploited clinically. Thus, it is anti-microbial, pro-angiogenic and anti-inflammatory. This "triple action" would be ideally suited for the treatment and management of IC/BPS.

Conclusion and applications

Our current findings demonstrate the potential anti-inflammatory properties of Medihoney™, through its ability to inhibit mast cell degranulation induced by the neuropeptide Sub P. Moreover, the study provides the first evidence of the modulatory effects of Medihoney™ on the intracellular signalling events accompanying mast cell activation.

These data support the potential use of Medihoney™ as an anti-inflammatory agent in neurogenic inflammatory conditions such as IC/BPS and atopic dermatitis. Specifically, aqueous solutions of Medihoney™ could be used intravesically to aid the management of patients with IC/BPS.

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Legends to Figures

Figure 1: The effect of Medihoney™ on β-hexosaminidase from LAD2 cells

(A) Optical density for the release of β-hexosaminidase by LAD2 cells following one-hour incubation with Medihoney™ (MH) at 2, 4% and 6 % concentrations, 1µM substance P (Sub P),10µg/ml compound 48/80 (C48/80) and 1µM Ca^{++} ionophore (Positive control). (B) & (C) represent the percentage release of β-hexosaminidase induced by 40-minute incubation with 1µM substance P (Sub P) and 10 µg/ml Compound 48/80, respectively, with or without pre-incubation with Medihoney™ (MH) at 2, 4 and 6 % concentrations in LAD2 cells, while (D) represents the substance P (1µM)-induced percentage release with or without pre-incubation with artificial honey (MH) at 1, 2 and 4 % concentrations. The mean ± SEM for the spontaneous release was 6.7 % ± 0.09 % of the total value. The overall cellular content of β-hexosaminidase (total) was prepared by completely lysing the cells in 1% Triton-X in 1X PBS. Each bar represents mean ± SEM (** P value <0.01, *** P value <0.001 and **** P value <0.0001, n=12). The graph represents the results of 4 independent assays, and each assay was performed in triplicates. Analysis was performed by Kruskal-Wallis test using GraphPad Prism 9 software.

Figure 2: The effect of substance P and compound 48/80 on the intracellular Calcium signalling in MRGPRX2-transfected HEK-293 cells

Intracellular Calcium levels measured by Fluo-4 imaging in MRGPRX2-transfected HEK-293 cells (A) vs standard non-transfected HEK-293 cells (B) upon challenge with 1µM Sub P (A), 10µg/ml Compound 48/80 (B) and 1µM Ca ionophore (C).

Figure 3: The effect of Medihoney™ on the substance P and compound 48/80-induced intracellular Calcium signalling in MRGPRX2-transfected HEK-293 cells

Intracellular Calcium levels measured by Fluo-4 imaging in MRGPRX2-transfected HEK-293 cells upon challenge with Sub P 1µM with or without pre-incubation with Medihoney™ (MH) 4 and 2% (A) or artificial honey (AH) 4 and 2% (B), or challenge with compound 48/80 10 µg/ml with or without pre-incubation with Medihoney™ (MH) 4 and 2% (F). (D) & (E) represent maximum - minimum intracellular Calcium levels detected by Fluo-4 imaging in MRGPRX2-transfected HEK-293 cells upon challenge with Sub P 1 µM alone or after pre-incubation with 4 and 2 % of Medihoney™ (D) or artificial honey (E), or challenge with compound 48/80 10 µg/ml with or without pre-incubation with Medihoney™ (MH) 4 and 2% (F) (n=8). Statistical analysis were performed using GrapPad Prism 9 software. Differences between groups were assessed by 0ne-way ANOVA (** P<0.01 and **** P<0.0001).

Figure 4: Figure 4: Immunohistochemical analysis of the MRGPRX2 expression by the mast cells in the detrusor layer urinary bladders of IC/BPS vs controls

Serial sections of 2 urinary bladder biopsy from an BPS/IC patient (A, B and C), and healthy bladder tissue control (D, E and F) showing mast cells in the detrusor layer, some are indicated in arrows, stained with antibodies for mast cell chymase (A & D), MRGPRX2 (B & E) and mast cell tryptase (C& F).

Figure 5: **Immunohistochemical analysis of the MRGPRX2 expression by the mast cells in the lamina propria urinary bladders of IC/BPS vs controls**

Serial sections of 2 urinary bladder biopsy from an BPS/IC patient (A, B and C), and healthy bladder tissue control (D, E and F) showing mast cells in the lamina propria, some are indicated in arrows, stained with antibodies for mast cell chymase (A & D), MRGPRX2 (B & E) and mast cell tryptase (C& F).

Figure 6: Graphical representation of the mast cell densities and their MRGPRX2 expression in the urinary bladders of IC/BPS patients vs controls

(A) & (B) are scatter plots representing the density of mast cells stained with tryptase (MCT) in the urinary bladders of the IC/BPS patients vs controls in the detrusor vs the LP layer, respectively. (C) & (D) are scatter plots representing the density of mast cells stained with Chymase (MCTC) in the urinary bladders of the IC/BPS patients vs controls in the detrusor vs the LP layer, respectively. (E) & (F) Scatter plot representing the percentage of MRGPRX2 expression by the Tryptase^{+ve} mast cells (MCT) in the urinary bladders of IC/BPS patients vs controls in the detrusor and LP layers, respectively. (G) & (H) Scatter plot representing the percentage of MRGPRX2 expression by the Chymase^{+ve} mast cells (MCTC) in the urinary bladders of IC/BPS patients vs controls in the detrusor and LP layers, respectively. Each bar represents the mean. Statistical differences were analysed by Mann-Whitney U test using GraphPad Prism 9 software (**P<0.005, *** P value 0.002 & **** P value <0.0001).

Figure 1:

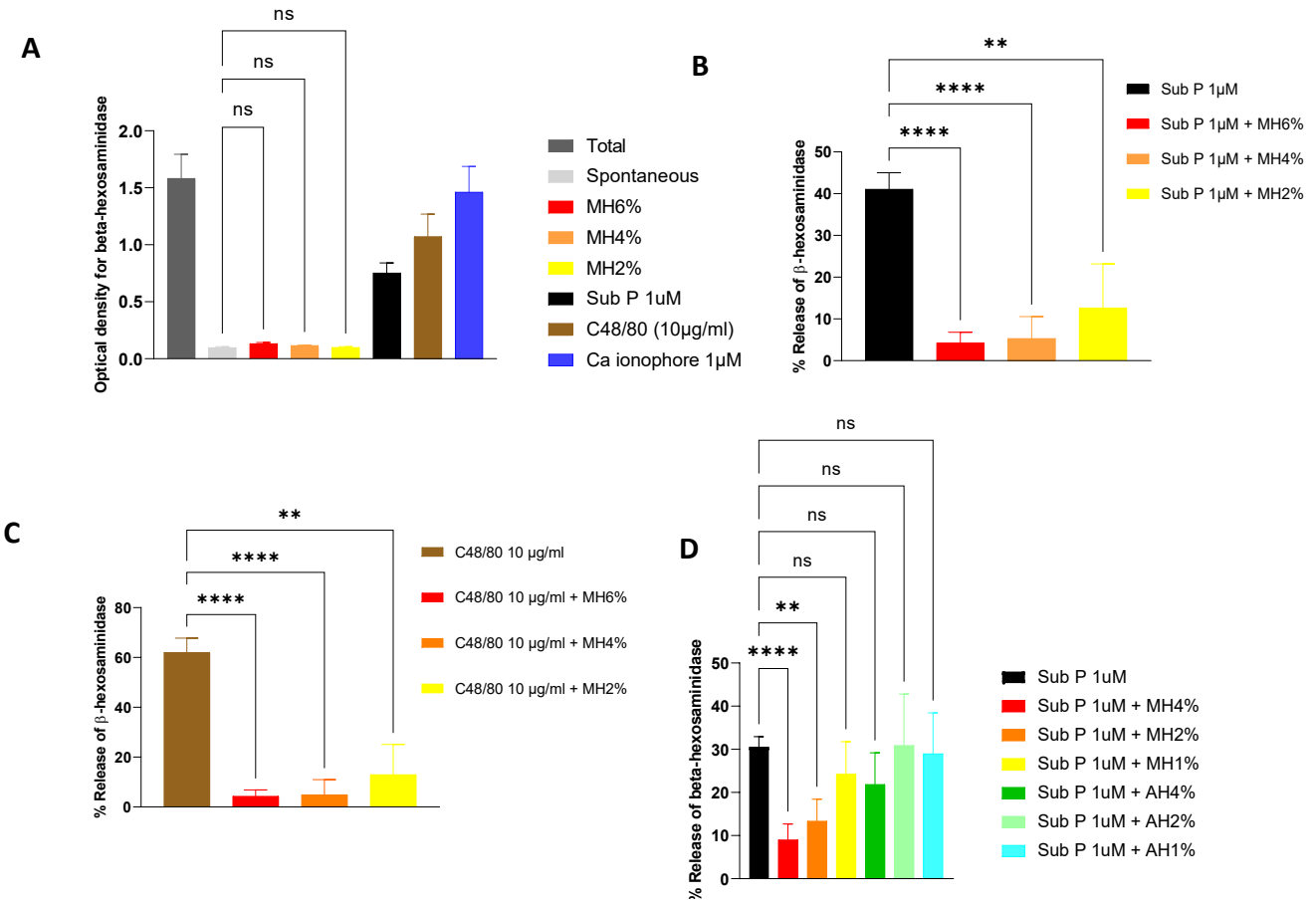
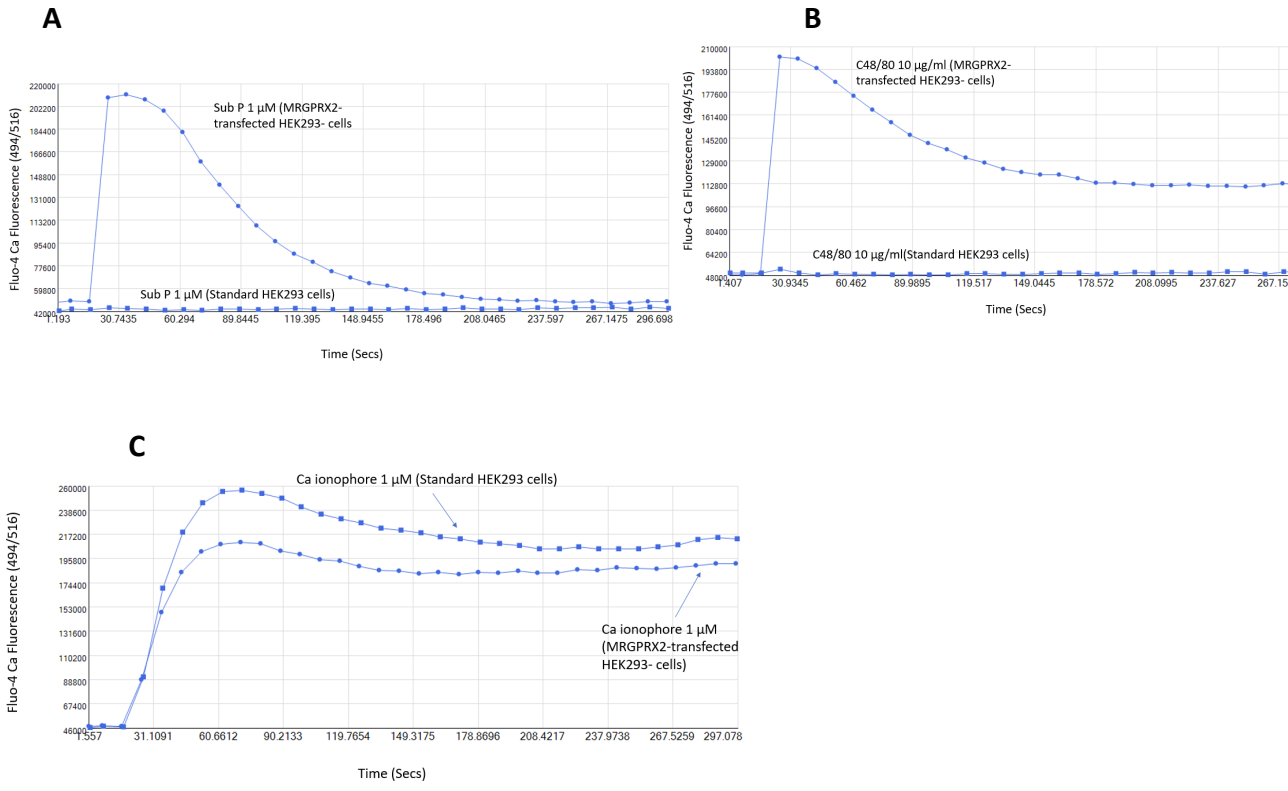


Figure 2:



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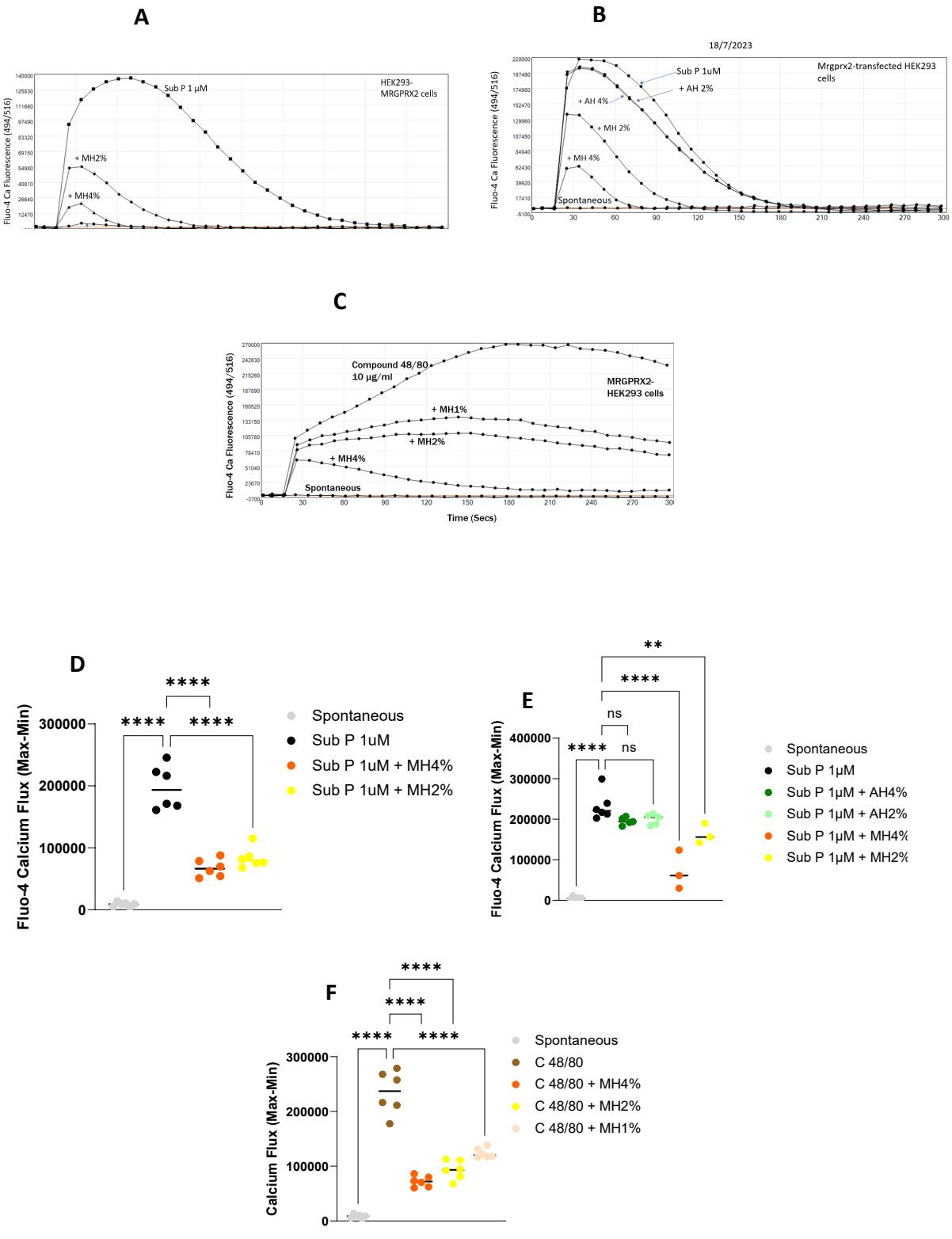

A
Sub P 1 µM
HEK293-MRGPRX2 cells
+ MH2%
+ MH4%
Fluo-4 Ca Fluorescence (494/516)
B
18/7/2023
Sub P 1uM
+ AH 2%
+ AH 4%
+ MH 2%
+ MH 4%
Spontaneous
Mrgprx2-transfected HEK293 cells
Fluo-4 Ca Fluorescence (494/516)
C
Compound 48/80 10 µg/ml
MRGPRX2-HEK293 cells
+ MH1%
+ MH2%
+ MH4%
Spontaneous
Fluo-4 Ca Fluorescence (494/516)
Time (Secs)
D
Fluo-4 Calcium Flux (Max-Min)
Spontaneous
Sub P 1uM
Sub P 1uM + MH4%
Sub P 1uM + MH2%
E
Fluo-4 Calcium Flux (Max-Min)
Spontaneous
Sub P 1µM
Sub P 1µM + AH4%
Sub P 1µM + AH2%
Sub P 1µM + MH4%
Sub P 1µM + MH2%
F
Calcium Flux (Max-Min)
Spontaneous
C 48/80
C 48/80 + MH4%
C 48/80 + MH2%
C 48/80 + MH1%

Figure 4:

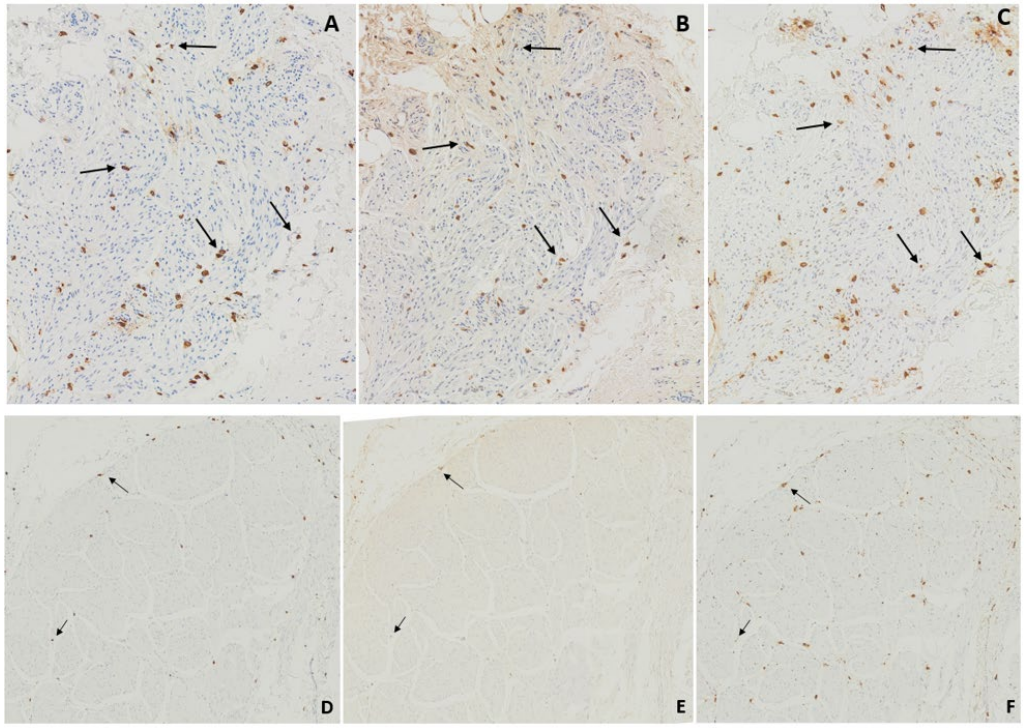


Figure 5:

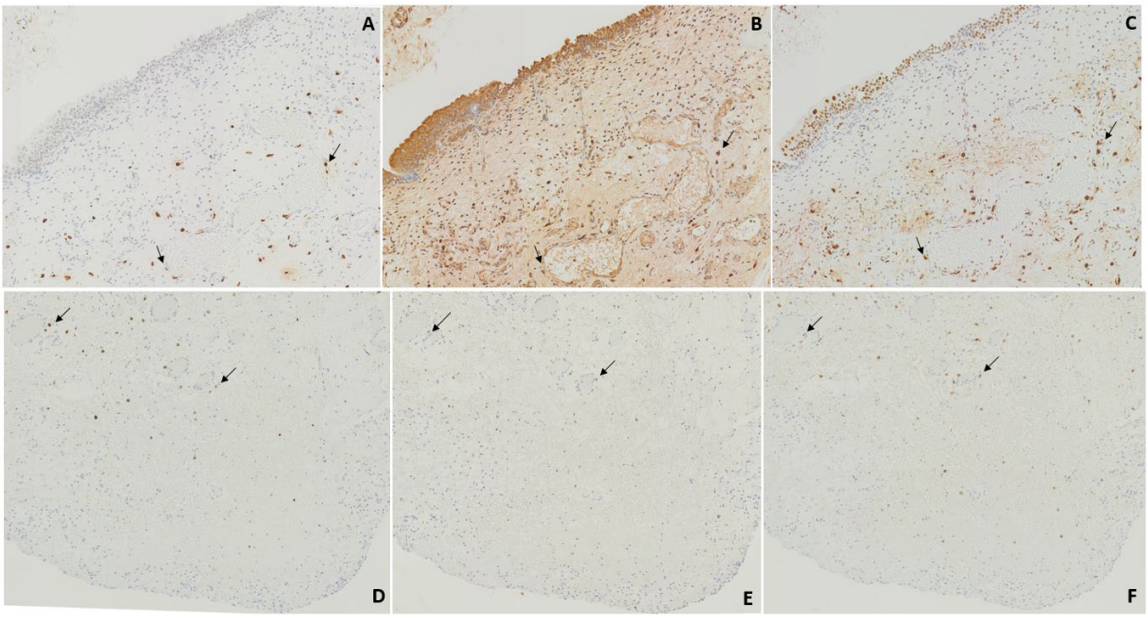
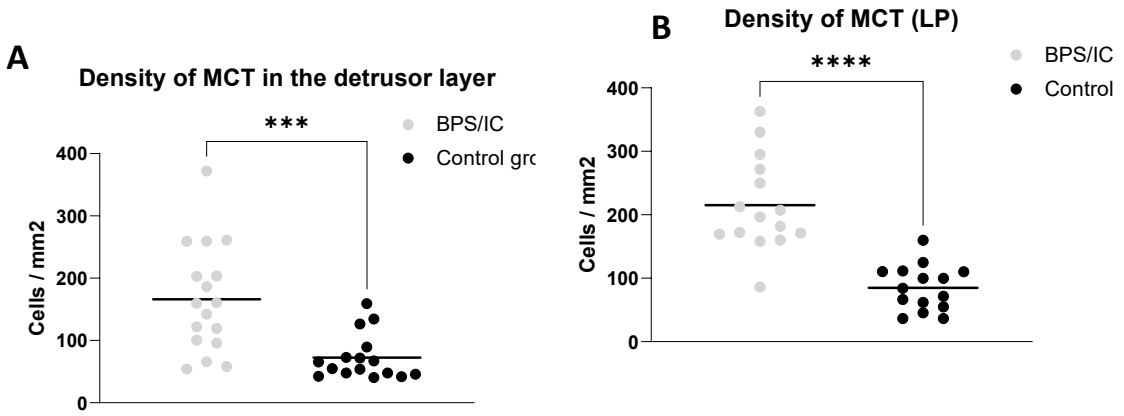
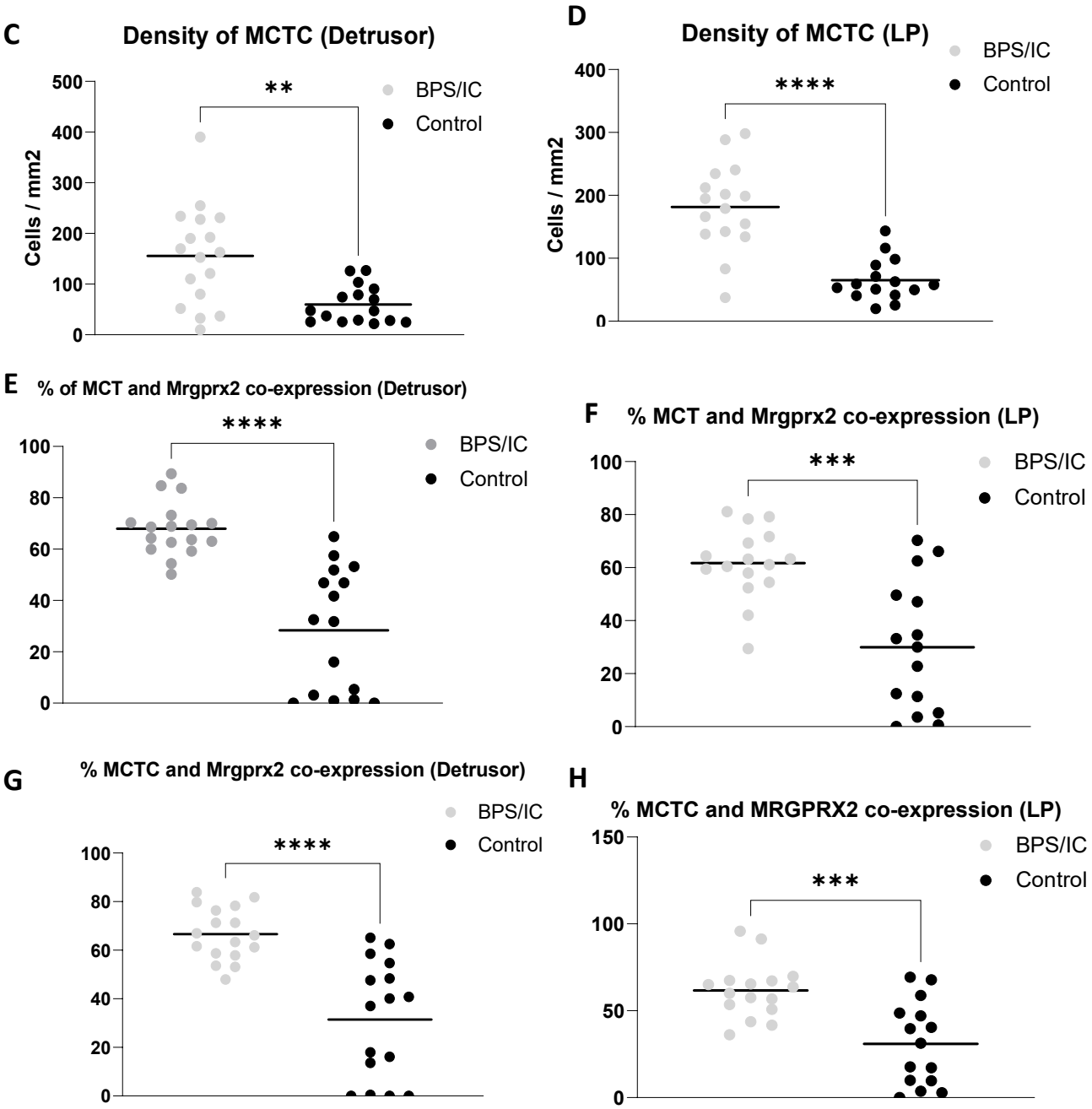

A
B
C
D
E
F

Figure 6:

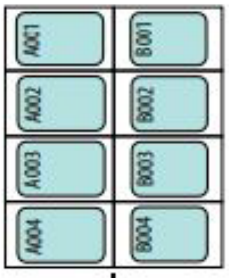

A
Density of MCT in the detrusor layer
***
BPS/IC
Control grc
Cells / mm2
400
300
200
100
0
B
Density of MCT (LP)
****
BPS/IC
Control
Cells / mm2
400
300
200
100
0


C
Density of MCTC (Detrusor)
Cells / mm2
500
400
300
200
100
0
**
BPS/IC
Control
D
Density of MCTC (LP)
Cells / mm2
400
300
200
100
0
****
BPS/IC
Control
E
% of MCT and Mrgprx2 co-expression (Detrusor)
100
80
60
40
20
0
****
BPS/IC
Control
F
% MCT and Mrgprx2 co-expression (LP)
100
80
60
40
20
0
***
BPS/IC
Control
G
% MCTC and Mrgprx2 co-expression (Detrusor)
100
80
60
40
20
0
****
BPS/IC
Control
H
% MCTC and MRGPRX2 co-expression (LP)
150
100
50
0
***
BPS/IC
Control

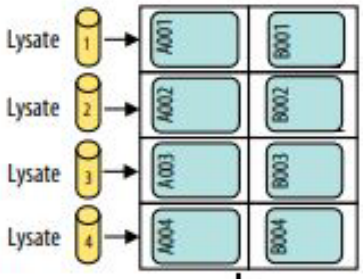
13 Appendix 3: Phospho MAPK Array Kit (ary003c, R&D Systems)

ARRAY PROCEDURE SUMMARY

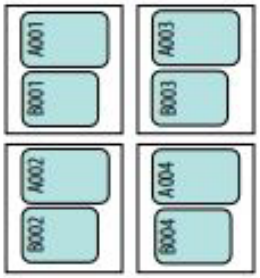
Step 1 (Blocking): Add 1.0 mL of Array Buffer 1 per well. Rock for 1 hour at room temperature.



Step 2 (Cell Lysates): Prepare 2.0 mL of diluted cell lysate. Remove Array Buffer 1. Add 1.0 mL of lysate to both Part A and Part B. Incubate overnight at 2-8 °C on a rocking platform shaker.



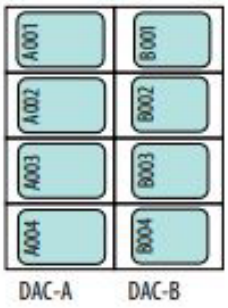
Step 3: (Wash 1) Wash in 1X Wash Buffer; 20 mL per dish. Wash a total of 3 times; 10 minutes on a rocking platform per wash. Wash corresponding parts (A and B) together.



Step 4 (Detection Antibody Cocktail): Pipette 1.0 mL of diluted Detection Antibody Cocktail A (DAC-A) into wells for Part A membranes.

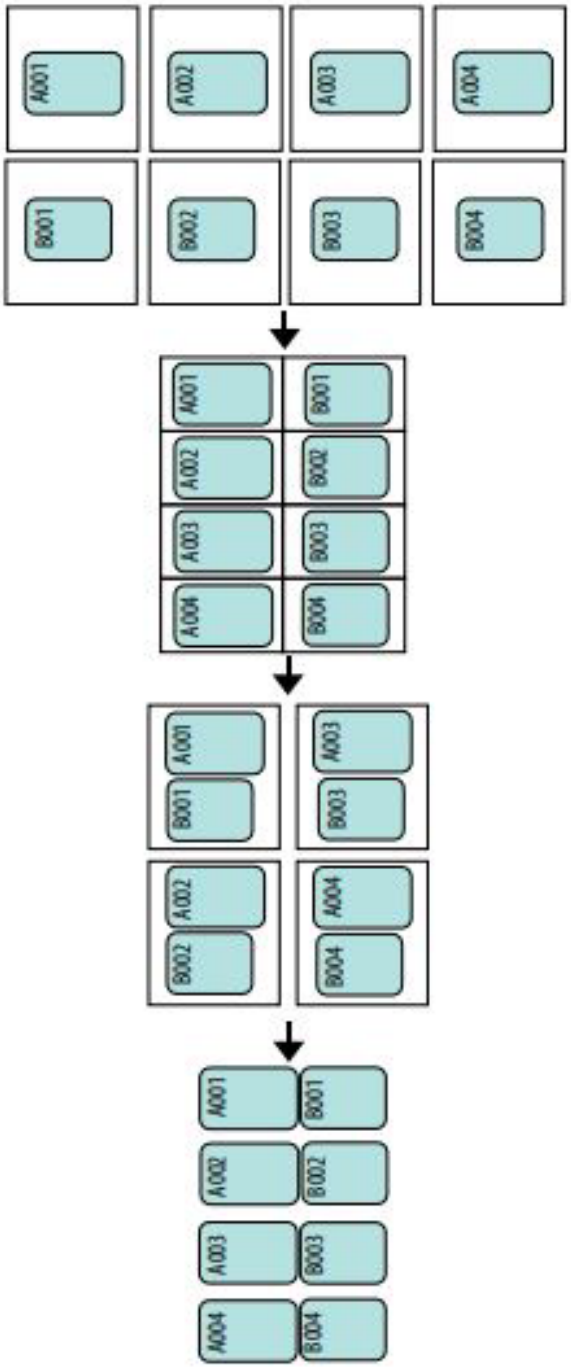
Pipette 1.0 mL of diluted Detection Antibody Cocktail B (DAC-B) into wells for Part B membranes.

Transfer the membranes to appropriate wells. Incubate for 2 hours at room temperature on a rocking platform.



ARRAY PROCEDURE SUMMARY *CONTINUED*

Step 5 (Wash 2): Wash in 1X Wash Buffer; 20 mL per dish. Wash a total of 3 times; 10 minutes on a rocking platform shaker per wash. **Wash all membranes separately.**



Step 6 (Streptavidin-HRP): Pipette 1.0 mL of diluted Streptavidin- HRP into each well. Transfer the membranes to the appropriate wells. Incubate for 30 minutes at room temperature on a rocking platform.

Step 7 (Wash 3): Wash in 1X Wash Buffer; 20 mL per dish. Wash a total of 3 times; 10 minutes per wash. Wash corresponding parts (A and B) together.

Step 8 (Signal Detection): Arrange the membranes on a sheet protector. Apply the Chemi Reagent Mix and expose to film.

For research use only. Not for use in diagnostic procedures.

DATA ANALYSIS

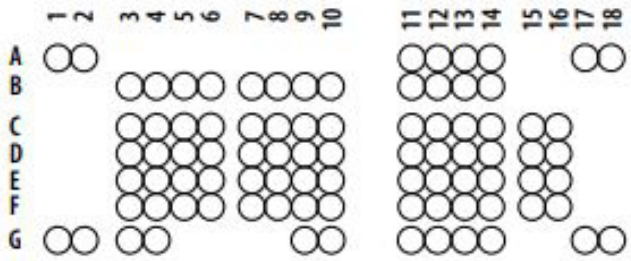
The positive signals seen on developed film can be quickly identified by placing the transparency overlay on the array image and aligning it with the pairs of reference spots in the corners of each membrane (two pairs on the left side of Part A and one pair on the right side of Part B). The stamped identification numbers on the membranes should be placed on the left hand side. The location of controls and capture antibodies is listed in the Appendix. It may be necessary to adjust the position of the transparency overlay template if the two parts of the membrane are not aligned.

Note: *Reference spots are included to align the transparency overlay template and to demonstrate that the array has been incubated with Streptavidin-HRP during the assay procedure.*

Pixel densities on developed X-ray film can be collected and analyzed using a transmission-mode scanner and image analysis software.

1. Create a template to analyze pixel density in each spot of the array.
2. Export signal values to a spreadsheet file for manipulation in a program such as Microsoft Excel.
3. Determine the average signal (pixel density) of the pair of duplicate spots representing each phosphorylated kinase protein.
4. Subtract an averaged background signal from each spot. Use a signal from a clear area of the array or negative control spots as a background value.
5. Compare corresponding signals on different arrays to determine the relative change in phosphorylated kinase proteins between samples.

Human Phospho-Kinase Array Coordinates



This image is not to scale. It is for coordinate reference only.
Please use the transparency overlay for analyte identification.

APPENDIX

Refer to the table below for the Human Phospho-Kinase Array coordinates.

Membrane/ Coordinate	Target/Control	Phosphorylation Site
A-A1, A2	Reference Spot	—
B-A11, A12	Akt 1/2/3	T308
B-A13, A14	Akt 1/2/3	S473
B-A17, A18	Reference Spot	—
A-B3, B4	CREB	S133
A-B5, B6	EGF R	Y1086
A-B7, B8	eNOS	S1177
A-B9, B10	ERK1/2	T202/Y204, T185/Y187
B-B11, B12	Chk-2	T68
B-B13, B14	c-Jun	S63
A-C3, C4	Fgr	Y412
A-C5, C6	GSK-3α/β	S21/S9
A-C7, C8	GSK-3β	S9
A-C9, C10	HSP27	S78/S82
B-C11, C12	p53	S15
B-C13, C14	p53	S46
B-C15, C16	p53	S392
A-D3, D4	JNK 1/2/3	T183/Y185, T221/Y223
A-D5, D6	Lck	Y394
A-D7, D8	Lyn	Y397
A-D9, D10	MSK1/2	S376/S360
B-D11, D12	p70 S6 Kinase	T389
B-D13, D14	p70 S6 Kinase	T421/S424
B-D15, D16	PRAS40	T246

Membrane/ Coordinate	Target/Control	Phosphorylation Site
A-E3, E4	p38α	T180/Y182
A-E5, E6	PDGF Rβ	Y751
A-E7, E8	PLC-γ1	Y783
A-E9, E10	Src	Y419
B-E11, E12	PYK2	Y402
B-E13, E14	RSK1/2	S221/S227
B-E15, E16	RSK1/2/3	S380/S386/S377
A-F3, F4	STAT2	Y689
A-F5, F6	STAT5a/b	Y694/Y699
A-F7, F8	WNK1	T60
A-F9, F10	Yes	Y426
B-F11, F12	STAT1	Y701
B-F13, F14	STAT3	Y705
B-F15, F16	STAT3	S727
A-G1, G2	Reference Spot	—
A-G3, G4	β-Catenin	—
A-G9, G10	PBS (Negative Control)	—
B-G11, G12	STAT6	Y641
B-G13, G14	HSP60	—
B-G17, G18	PBS (Negative Control)	—

For research use only. Not for use in diagnostic procedures.

A0117
A0118
A0119
A0120
B0117
B0118
B0119
B0120

14 Appendix 3: Human Phopho (Ser473)/Total Akt kit (K15100A-3, MSD, USA)

Principle of the Assay

MSD phosphoprotein assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. The assays are available in both singleplex and multiplex formats. In a singleplex assay, an antibody for a specific protein target is coated on one electrode (or "spot") per well. In a multiplex assay, an array of capture antibodies against different targets is patterned on distinct spots in the same well. The Phospho(Ser473)/Total Akt Assay is a sandwich immunoassay (Figure 1). MSD provides a plate that has been pre-coated with capture antibodies for Phospho-Akt (Ser473) and total Akt. The user adds the sample and a solution containing the detection antibody—anti-total Akt conjugated with an electrochemiluminescent compound, MSD SULFO-TAG label—over the course of one or more incubation periods. Analytes in the sample bind to the capture antibodies immobilized on the working electrode surface; recruitment of the conjugated detection antibody by bound analytes complete the sandwich. The user adds an MSD read buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into an MSD SECTOR® Imager for analysis. Inside the SECTOR Imager, a voltage applied to the plate electrodes causes the labels bound to the electrode surface to emit light. The instrument measures intensity of emitted light to provide a quantitative measure of phosphorylated and total Akt present in the sample.

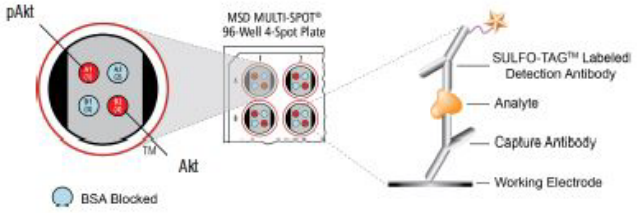


Figure 1. Spot diagram showing placement of analyte capture antibody. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing records.

Sample Preparation and Storage

This cell lysis protocol is provided as a reference. Specific cell types or targets may benefit from alternative buffer components or techniques, depending upon the particular research application. Most lysis buffers are compatible with MSD MULTI-SPOT plates, although high concentrations of denaturing detergents (>0.1%) and reducing agents (DTT >1mM) should be avoided. Please contact MSD Scientific Support with any questions regarding lysate preparation options.

All manipulations should be performed on ice. The amount of complete lysis buffer required will vary depending on scale of preparation and type of cells. Larger cells (e.g. NIH3T3, HeLa) should be lysed at concentrations of 1-5 x 10^6 cells per mL of lysis buffer. Smaller cells (e.g. Jurkat) should be lysed at concentrations of 1-5 x 10^7 cells per mL of lysis buffer.

Analysis of proteins in their activated state (i.e. phosphorylated) usually requires stimulation prior to cell lysis. Verification of cell stimulation and sample preparation should be performed prior to using this kit. Phosphate Buffered Saline (PBS) should be ice-cold prior to use.

Suspension Cells

Pellet cells by centrifugation at 500 x g for 3 minutes at 2-8°C. Discard supernatant and wash the pellet once with cold PBS. Pellet the cells again, discard supernatant and resuspend in complete lysis buffer at 1 - 5 x 10^7 cells per mL. Incubate on ice for 30 minutes. A shorter incubation time of 15 minutes may be adequate for many targets. Clear cellular debris from the lysate by centrifugation greater than or equal to 10000 x g, at 2-8°C for 10 minutes. Discard the pellet and determine protein concentration in the lysate using a detergent compatible protein assay such as BCA. Unused lysates should be aliquoted and quickly frozen in a dry ice-ethanol bath and stored at ≤-70°C.

Adherent Cells

All volumes are determined for cells plated in 15 cm dishes. Remove media from the plates and wash cells one time with 5 mL cold PBS. Add 2 mL PBS to the plates and scrape the cells from the surface of the dish and transfer into 15 mL conical tubes. Pellet the cells by centrifugation at 500 x g for 3 minutes at 2-8°C. Discard supernatant and resuspend cells in 0.5 – 2 mL of complete lysis buffer per dish. Incubate on ice for 30 minutes. A shorter incubation time of 15 minutes may be adequate for many targets. Clear cellular debris from the lysate by centrifugation greater than or equal to 10000 x g, at 2-8°C for 10 minutes. Discard the pellet and determine protein concentration in the lysate using a detergent compatible protein assay such as BCA. Unused lysates should be aliquoted and quickly frozen in a dry ice-ethanol bath and stored at ≤-70°C.

Refer to Appendix I for cell lysate preparation protocol modifications that accommodate the use of 96-well culture plates.

Assay Protocol

The following protocol describes the most conservative approach to achieving optimal results with the MULTI-SPOT Phospho(Ser473)/Total Akt Assay. The entire assay, including plate analysis on the MSD reader, can be completed in 3.5 hours. Once desired results are achieved, the protocol can be streamlined to eliminate multiple incubations and wash steps. Samples may be prepared for testing in the manner outlined in the Sample Preparation and Storage section.

1. **Block Plate and Prepare Samples:**
   a. Add 150 µL of blocking solution into each well. Seal the plate with an adhesive plate seal, and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.
   b. Prepare complete lysis buffer just prior to sample dilution.
      Note: Samples, including cell lysates, etc., may be used neat or after dilution.
      - MSD plates are compatible with most sample matrices. Avoid reagents that will denature the capture antibodies (e.g. high concentrations of reducing agents such as DTT should be avoided, and also SDS and other ionic detergents should be 0.1% or less in the sample applied to the well).
      - Depending on the stability of the target in the matrix, additional protease and phosphatase inhibitors may be required in the matrix or diluent.
      - If working with purified protein, only a few nanograms per well will generally provide a strong assay signal. Purified recombinant proteins may exhibit differences in both signal and background as compared to native proteins in cell lysates.
      - Keep diluted samples on ice until use
   c. Prepare positive and negative cell lysates:
      (if purchased separately).
      - Thaw cell lysate samples on ice, and dilute them immediately before use. Keep on ice during all manipulations, and discard all remaining thawed, unused material.
      - Dilute cell lysate in complete lysis buffer to a final concentration of 0.8 µg/µL. This will deliver 20 µg/well in 25 µL. A dilution series may also be prepared if desired.

Notes

Read entire protocol prior to beginning the assay.

Solutions containing MSD Blocker A should be stored at 2-8°C and discarded after 14 days.

Complete lysis buffer should be kept ice-cold during all experimental manipulations.

The sensitivity of MSD immunoassays rivals that of ELISAs and Western blots. The amount of sample required for a given assay will depend on the abundance of the analyte in the matrix and the affinities of the antibodies used.

Samples and standards cannot be serially diluted in the MSD plate. Use microcentrifuge tubes or a separate 96-well polypropylene plate to prepare dilutions.

2. **Wash and Add Samples:** Wash the plate 3 times with 300 µL/well of Tris Wash Buffer. Add 25 µL of samples per well. Seal the plate with an adhesive plate seal, and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.

   Prepare detection antibody solution during this time.

3. **Wash and Add Detection Antibody:** Wash the plate 3 times with 300 µL/well of Tris Wash Buffer. Add 25 µL of detection antibody solution to each well of the MSD plate. Seal the plate with an adhesive plate seal, and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.

   Prepare 1X Read Buffer T during this time.

4. **Wash and Read:** Wash the plate 3 times with 300 µL/well of Tris Wash Buffer. Add 150 µL of 1X Read Buffer T to each well of the MSD plate.

   Analyze the plate on the SECTOR Imager:

   a. Double click on DISCOVERY WORKBENCH® icon on computer desktop (if not already open).
   b. Click the SECTOR Imager icon in upper left corner of screen (if not already open to plate reading screen).
   c. From the pull down menu select "Read From Barcode."
   d. If only reading one plate check "Return Plate to Input Stack." Then check "Read Plate(s)" checkbox and enter 1.
   e. If reading multiple plates, check the "Read Plate(s)" checkbox and enter number of plates to be read in the text field. For example, if five plates need to be read, type in "5."
   f. Click the "Run" button. The "Run Options" window will be displayed
   g. If the data from each microplate is to be exported as individual files, select "Separate Files" in the "Export" area of the "Run Options" window. Select "Appended File" if all data from the entire stack run is to be exported to one file. Select "Default" in the "Export Format" area. Check the box to export default data file.
   h. If desired, make selections to export a custom data file.
   i. Browse and select the location to export data files.
   j. Click OK to initiate the run.
   k. Data will be automatically saved in the software database. Text versions of the requested data files will be exported to the designated folder.

Notes

Shaking a 96-well MSD MULTI-ARRAY or MULTI-SPOT plate during an incubation step will typically accelerate capture at the working electrode.
The lysate sample incubation time provided is optimized for the use of MSD cell lysates. Samples from other sources may require a longer incubation.

Excess diluted read buffer may be kept in a tightly sealed container at room temperature for later use.

Bubbles introduced during the read buffer addition will interfere with imaging of the plate and produce unreliable data.

Plate should be imaged within 5 minutes following the addition of read buffer. Due to the varying nature of each research application, assay stability should be investigated prior to allowing plates to sit with read buffer for extended periods.

An all-inclusive indelible copy of the data and associated instrument information will be saved on the internal database, regardless of data file export selection. Additional copies of the data can be exported in any layout at a later time using this database. Consult the instrument user manual for more information.

Analysis of Results

The percent phosphoprotein in a sample can be calculated using independent MSD phosphoprotein and total protein singleplex assays or MSD phospho-/total multiplex phosphoprotein assays.

INDEPENDENT ASSAY FORMAT: Anti-Total Singleplex and Anti-Phospho-Singleplex Assays
% Phosphoprotein = (Phospho-signal / Total signal) x 100

MULTIPLEX ASSAY FORMAT: Anti-Total and Anti-Phospho-Assay in the same well
% Phosphoprotein = ((2 x Phospho-signal) / (Phospho-signal + Total signal)) x 100

Note:

1. The above calculation assumes that the capture antibodies on the anti-phospho and anti-total spots have very similar binding affinities.
2. The numerator in the equation contains a distribution factor of 2 based on the assumption that the phosphorylated isoform of the protein binds with a similar affinity to the phospho-specific and total capture antibodies. Given equivalent binding of the phosphorylated isoform to both capture antibodies, half of the phosphorylated species will be captured by the phospho-specific and the other half will be captured by the phosphorylation-independent (total) antibody. Therefore, the phospho-specific signal can be referred to as 2X of the phospho spot.
3. The denominator is "phospho + total" because this represents the total of all the analyte captured on both of the spots.
4. If the % phosphorylation is > 100%, then the distribution factor in the numerator may be adjusted to less than 2X such that the % phosphorylation with the control lysates is 100%.

Example:

Phosphoprotein Assay							
Lysates (µg)	Positive Control Lysate			Negative Control Lysate			P/N
	Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
0	245	4	1.4	242	6	26.0	
5.0	19235	2342	12.2	461	3	0.6	42

Total Protein Assay							
Lysates (µg)	Positive Control Lysate			Negative Control Lysate			P/N
	Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
0	561	18	3.2	569	19	3.4	
5.0	7304	1227	16.8	14530	585	4.0	0.5

% Phosphoprotein = [(2 x Phospho signal) / (Phospho signal + Total signal)] x 100

Therefore, % phosphoprotein with 5 µg of positive lysate will be:

[(2 x 19235) / (19235 + 7304)] x 100 = 144% phosphorylation

In this case, the constant in the numerator may be adjusted using the control lysates as follows:

[(1.38 x 19235) / (19235 + 7304)] x 100 = 100% phosphorylation

1.38 should be used as the numerator for further calculations in the same experiment.

A

B

Figure 86: Medihoney (A) and the sugar-free Manuka extract (B).