Characterisation of $\alpha$-1 adrenergic receptors in peripheral blood mononuclear cells of complex regional pain syndrome

This thesis is presented for the degree of Bachelor of Science Honours

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BSc Biomedical Science and Molecular Biology
Declaration

I declare this thesis is my own account of my research and contains as its main content work which has not been previously submitted for a degree at any tertiary education institution.

Mollie Walker
29/05/2017
Abstract

Complex regional pain syndrome (CRPS) is a chronic pain condition that may occur after injury or trauma to a limb. The underlying pathophysiology of CRPS is largely undetermined, although CRPS patients commonly present with a persisting inflammatory response in the early stage of the condition and over-express the α-1 adrenergic receptor (α-1AR) on nociceptors and keratinocytes in the affected limb. In other chronic inflammatory conditions, increased α-1AR mRNA expression in peripheral blood mononuclear cells (PBMCs) has been shown, and is thought to contribute to persisting inflammation. The α-1AR are expressed on a range of cells, including nerve, smooth muscle, skin and immune cells, and bind to adrenaline and noradrenaline released after activation of the sympathetic nervous system. The aims of this project were to examine the mRNA expression of α-1AR subtypes (α-1A, α-1B and α-1D) by qPCR in PBMCs isolated from fractionated blood of CRPS patients, and to quantify the percentages of various PBMC subpopulations compared to healthy controls. Subpopulations of PBMCs were determined by flow cytometry using a panel of fluorescent antibodies that identified CD4⁺ T cells, CD8⁺ T cells, CD4⁺ CD8⁺ T cells, CD4⁺ CD25⁺ T cells, CD8⁺ CD25⁺ T cells, B cells, natural killer (NK) cells, NKT cells, and subsets of monocytes. No differences in expression of α-1AR subtypes in PBMCs of CRPS patients were found when compared to healthy controls. However, a significant increase in the concentration of total PBMCs isolated per mL of blood in CRPS patients and a shift from CD16⁻ to CD16⁺ monocyte subpopulations was identified when compared to healthy
controls. These results show there is an inflammatory component to CRPS and provide preliminary evidence that the persisting inflammation in CRPS patients may originate from over-proliferation of PBMC progenitors in the bone marrow, which is sympathetically modulated by α-1AR, and/or an expansion of other PBMC cell types that were not examined in this project.
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## List of Abbreviations

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<th>Full Form</th>
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<tbody>
<tr>
<td>7AAD</td>
<td>7-Amino-Actinomycin D</td>
</tr>
<tr>
<td>α-1AR</td>
<td>α-1 Adrenergic Receptor</td>
</tr>
<tr>
<td>α-2AR</td>
<td>α-2 Adrenergic Receptor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>β-AR</td>
<td>β Adrenergic Receptor</td>
</tr>
<tr>
<td>B cell</td>
<td>Bone-marrow derived Cell</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CD4</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>CD8</td>
<td>Cytotoxic T cell</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calciton Gene Related Peptide</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle Threshold</td>
</tr>
<tr>
<td>CRPS</td>
<td>Complex Regional Pain Syndrome</td>
</tr>
<tr>
<td>CSS</td>
<td>CRPS Severity Score</td>
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<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescently Activated Cell Sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>IASP</td>
<td>International Association for the Study of Pain</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRAM</td>
<td>Injury-triggered, Regionally-restricted Autoantibody-Mediated</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescent Intensity</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RSD</td>
<td>Reflex Sympathetic Dystrophy</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic Nervous System</td>
</tr>
<tr>
<td>T Cell</td>
<td>Thymus-derived Cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TH</td>
<td>T Helper Cell</td>
</tr>
<tr>
<td>T reg</td>
<td>T Regulatory Cell</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>UBE</td>
<td>Ubiquitin converting Enzyme</td>
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Acknowledgements

I cannot begin to comprehend how much I’ve learnt and grown as a scientist over the past year. This thesis has pushed me to limits I didn’t know I was capable of reaching, yet has definitely given me positive insight into the life of a researcher. The list of people I’d like to acknowledge is far beyond who is mentioned on this page, which is why a personal extended thank-you will be given in due time, but for now a special mention is needed for the following people:

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1. Literature Review

1.1. Introduction

Complex regional pain syndrome (CRPS) describes a group of painful disorders that may develop after limb trauma or nerve lesion. Although heavily defined by severe pain which may be either spontaneous or stimulus-induced, patients experience a range of inflammation, sensory-motor disturbances and autonomic signs and symptoms involving abnormal blood flow, sweating, oedema and trophic changes of the affected extremity (Geertzen et al., 1998). These symptoms are representative of an impaired sympathetic nervous system (SNS) and an inflammatory response that can persist for as long as eighteen months (Marchand et al., 2005). In an attempt to accommodate the varying symptoms, patients are categorised into one of two types. Type I CRPS, formerly known as reflex sympathetic dystrophy (RSD), describes disease states without apparent nerve injury, whereas type II CRPS (formerly known as causalgia) groups patients with damage to a peripheral nerve trunk (Merksey and Bogduk, 1994; Borchers and Gerschwin, 2014).

Studies have suggested a link between the SNS and inflammation in the pathogenesis of CRPS, with several observations showing an increase in density, or responsiveness, of α-1 adrenergic receptors (α-1AR) in sensory nerve fibres and skin cells of CRPS patients (Dawson et al., 2011; Drummond et al., 2014a; Drummond et al., 2014b). Increased α-1AR on pain-signalling neurons (nociceptors) are subject to sensitisation by circulating catecholamines, likely contributing to SNS malfunction, and a network of pro-inflammatory mediators.
during the inflammatory response that likely stimulate the release of neuropeptides (Borchers and Gerschwin, 2014; Birklein and Schlereth, 2015). These neuropeptides evoke vasodilation and protein extravasation, facilitating neurogenic and local inflammation (Birklein et al., 2001a; Lei et al., 2003; Schinkel et al., 2006). This suggests α-1AR may facilitate the link between the malfunctioning SNS and the inflammatory response of CRPS.

Furthermore, α-1AR mRNA is detectable in human peripheral leukocytes of other chronic inflammatory disease states such as asthma and rheumatoid arthritis. In this case, α-1AR is functionally linked to the release of pro-inflammatory cytokines from these cells when stimulated with adrenergic ligands such as the catecholamine noradrenaline, or the α-1AR agonist phenylephrine (Ricci et al., 1999; Szentivanyi et al., 1979; Wahle et al., 1999; Heijnen et al., 1996). As α-1AR is abnormally expressed on keratinocytes and nociceptors in CRPS, and due to the evidence of an aberrant inflammatory response, evidence that CRPS leukocytes express α-1AR could provide further evidence of an SNS association with persisting inflammation in CRPS (Figure 1.1).

Therefore, the aims of this project were to compare the expression of α-1AR mRNA in leukocytes of CRPS patients to that of healthy non-CRPS controls, and to compare the composition of different leukocytes in CRPS to non-CRPS individuals, as a shift in distribution or numbers of leukocytes could provide further evidence for a role of inflammation in CRPS.
This chapter will provide an introduction to the α-1AR inflammatory pathogenesis in CRPS that involves the following concepts: i) the clinical features of CRPS including symptoms and difficulties in diagnosis; ii) an overview of α-1AR and their main physiological functions; iii) an overview of the inflammatory response describing the roles of different cell types and mediators in inflammation; iv) the role of α-1AR signalling in inflammation, describing their potential roles in regulating the immune system and inflammatory disease states, and; v) the role of inflammation in the pathogenesis of CRPS and how this could be regulated by α-1AR signalling.
Figure 1.1: Concept diagram describing the role of α-1AR in CRPS. After injury, an inflammatory response is initiated to restore homeostasis by eliminating any infectious or noxious agents and promoting healing and repair. This is achieved through production of inflammatory mediators and migration of leukocytes to the site of injury. Stimulation of α-1AR on leukocytes by catecholamines and sensitisation by cytokines in CRPS may facilitate persisting inflammation, whereas non-CRPS healthy leukocytes do not express α-1AR and can regain homeostasis.
1.2. The clinical presentation of complex regional pain syndrome

1.2.1. Signs and symptoms

The symptoms of CRPS are multifarious and vary substantially between patients, with some patients experiencing heightened levels of one symptom but not of another that may be elaborated in another patient. Often, patients also experience a change in symptoms throughout the course of disease. These symptoms can be grouped into five categories: i) pain and sensitivity; ii) motor disorders; iii) autonomic symptoms; iv) trophic changes and; v) body perception disturbances (GalveVilla et al., 2016). Symptoms are mostly confined to the extremities, however; in severe cases these symptoms can spread from the affected nerve region to an unaffected region, proximally or contra-laterally (GalveVilla et al., 2016). There have been few cases where symptoms develop proximally (i.e. in the shoulder or the knee) without any other symptoms distally (van Bussel et al., 2015). In addition, symptoms may also spread to the head and neck (Allen et al., 1999; Schwartzman et al., 2009).

Pain and sensitivity symptoms include causalgia, allodynia, hyperalgesia and hypoalgesia. Causalgia is the term used to describe burning pain in a limb due to peripheral nerve damage (Todorova et al., 2012). Most CRPS patients describe their pain as a burning or causalgic sensation (Hassantash et al., 2003). Type I patients also describe this as a deep tissue localisation such as a dull ache or tear (Birklein et al., 2000; Birklein et al., 2001b). Allodynia is a term used to describe the provocation of pain by stimuli that would not usually cause pain, whereas
hyperalgesia and hypoalgesia are heightened and reduced sensitivity to pain, respectively (Gierthmuhlen et al., 2012).

Motor disturbances include reduced movement due to inhibitory influences of pain and oedema, as well as fibrotic contractions that can limit further movement during the chronic stages of CRPS (GalveVilla et al., 2016). Some patients experience central motor symptoms such as tremors, irregular muscle spasms (myoclonus) and/or fixed dystonia-like postures, seen more commonly in CRPS type I patients (de Mos et al., 2009b; de Boer et al., 2011). Inappropriate pain behavior such as fear-avoidance can lead to prolonged immobilisation of the affected limb, resulting in joint stiffness and muscle atrophy (GalveVilla et al., 2016).

Autonomic symptoms are due to overcompensated vasodilation and vasoconstriction. During the acute stages of CRPS, the affected limb usually presents as warm, red and swollen (“warm type” CRPS) but may begin to feel colder during the course of the disease and into the chronic stages (“cold type” CRPS) (Wasner et al., 1999) (Figure 1.2). Skin temperatures may also fluctuate over time dependent upon the environment (Veldman et al., 1993; Birklein et al., 2000). This may cause patients to develop hyper- or hypohidrosis (increased or decreased sweating) and skin colour changes to red or blue when compared to the contralateral side (Figure 1.2). Patients often experience oedema and swollen limbs during the acute phases of CRPS (GalveVilla et al., 2016).
Trophic changes include skin that may appear thin and shiny (Figure 1.2A) with decreased or increased hair and nail growth, and the nails may become brittle, ridged, curved or dull (Gierthmuhlen et al., 2012; Birklein et al., 2000). Body perception disturbances are a less specific symptom of CRPS. They resemble typically neglect-like phenomena, with abnormal limb perception in relation to size and altered proprioception (GalveVilla et al., 2016).

1.2.2. Diagnosis

Complex regional pain syndrome is a heterogeneous disease with a challenging diagnosis. Not only are the signs and symptoms extremely variable over time and differ between individuals, CRPS can also mimic a localised response to infection or trauma. Thus, CRPS diagnosis rests entirely on clinical assessment.
and is a diagnosis of exclusion. The diagnostic criteria have been carefully reviewed and developed by a subgroup of the International Association for the Study of Pain (IASP) now known as the CRPS Budapest diagnostic criteria as shown in Tables 1.1 and 1.2 (Harden and Bruehl, 2005).

Dichotomous (yes/no) CRPS diagnoses are necessary clinically but do not adequately capture the severity, change in condition or variability among patients. The continuous CRPS Severity Score (CSS) addresses these issues, evolving from the original Budapest criteria, including all 17 diagnostic CRPS features shown in Table 1.3. “Self-reported symptoms” are noticed and reported by the patient, whereas “signs observed on examination” are reported by the physician or health expert examining the patient. Allodynia and hyperpathia (exaggerated level of pain to stimuli) are measured by the patients’ response to a pinprick and temperature asymmetry is measured with a temperature gauge (GalveVilla et al., 2016). The remaining symptoms are examined by observation (Harden et al., 2010). The CSS provides a quantitative index of the signs and symptoms of CRPS, with a score of 1 given to the presence of each symptom. With the CSS, not only are patients dichotomously diagnosed with CRPS, but also the severity of the condition can be monitored and defined accordingly (Harden et al., 2010).
Table 1.1: IASP Diagnostic Criteria for CRPS (Merskey and Bogduk, 1994).

<table>
<thead>
<tr>
<th>CRPS type I</th>
<th>CRPS type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The presence of an initiating noxious event, or a cause of immobilisation.</td>
<td>1. Type II is syndrome that develops after nerve injury. Spontaneous pain or alldynia/hyperalgesia occurs and is not necessarily limited to the territory of the injured nerve.</td>
</tr>
<tr>
<td>2. Continuing pain, alldynia, or hyperalgesia in which the pain is disproportionate to any known inciting event.</td>
<td>2. There is or has been evidence of oedema, skin blood flow abnormality, or abnormal sudomotor activity in the region of the pain since the inciting event.</td>
</tr>
<tr>
<td>3. Evidence at some time of oedema, changes in skin blood flow, of abnormal sudomotor activity in the region of pain.</td>
<td>3. This diagnosis is excluded by the existence of conditions that would otherwise account for the degree of pain and dysfunction.</td>
</tr>
<tr>
<td>4. This diagnosis is excluded by the existence of other conditions that would otherwise account for the degree of pain and dysfunction.</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.2: The Harden/Bruehl Criteria, which became The Budapest Research Criteria with minor modifications (Harden and Bruehl, 2005)

<table>
<thead>
<tr>
<th>1. Continuing pain which is disproportionate to any inciting event.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Must report at least one symptom in each of the four following categories:</td>
</tr>
<tr>
<td>a) Sensory: report of hyperaesthesia</td>
</tr>
<tr>
<td>b) Vasomotor: reports of temperature asymmetry and/or skin colour changes and/or skin colour asymmetry</td>
</tr>
<tr>
<td>c) Sudomotor/oedema: reports of oedema and/or sweating changes and/or sweating asymmetry</td>
</tr>
<tr>
<td>d) Motor/trophic: reports of decreased range of motion and/or motor dysfunction (weakness, tremor, dystonia) and/or trophic changes (hair, nail, skin)</td>
</tr>
<tr>
<td>3. Must display at least one sign in two or more of the following categories:</td>
</tr>
<tr>
<td>a) Sensory: evidence of hyperalgesia (to pinprick) and/or allodynia (to light touch)</td>
</tr>
<tr>
<td>b) Vasomotor: evidence of temperature asymmetry and/or skin colour changes and/or asymmetry</td>
</tr>
<tr>
<td>c) Sudomotor/oedema: evidence of oedema and/or sweating changes and/or sweating asymmetry</td>
</tr>
<tr>
<td>d) Motor/trophic: evidence of decreased range of motion and/or motor dysfunction (weakness, tremor, dystonia) and/or trophic changes (hair, nail, skin)</td>
</tr>
<tr>
<td>4. There must be no other diagnosis that better explains the signs and symptoms.</td>
</tr>
</tbody>
</table>

Table 1.3: Diagnostic signs and symptoms included in the CRPS Severity Score by subgroup (Harden et al., 2010)

<table>
<thead>
<tr>
<th>Self reported Symptoms</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Allodynia, Hyperpathia</td>
<td>Asymmetric oedema</td>
</tr>
<tr>
<td>Temperature asymmetry</td>
<td>Trophic changes</td>
</tr>
<tr>
<td>Skin colour asymmetry</td>
<td>Motor changes</td>
</tr>
<tr>
<td>Sweating asymmetry</td>
<td>Decreased active range of motion</td>
</tr>
<tr>
<td>Signs observed on examination</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>-----------------------------------------------------------------</td>
</tr>
<tr>
<td>Hyperpathia to pinprick</td>
<td>Asymmetric oedema</td>
</tr>
<tr>
<td>Allodynia</td>
<td>Trophic changes</td>
</tr>
<tr>
<td>Temperature asymmetry by palpation</td>
<td>Motor changes</td>
</tr>
<tr>
<td>Skin colour asymmetry</td>
<td>Decreased active range of motion</td>
</tr>
<tr>
<td>Sweating asymmetry</td>
<td></td>
</tr>
</tbody>
</table>
1.2.3. Precipitating events

Fractures, sprains and surgery of a limb are the most likely events provoking CRPS onset. Less commonly, injections, local infections, burns, frostbite, pregnancy and myocardial infarction may also lead to CRPS (Veldman et al., 1993; Harden et al., 2010). The exact nature and severity of the trauma is unrelated to the combination of symptoms and their severity, with more than 10% of patients not recalling any predisposing injury or event (de Boer et al., 2011). Onset of CRPS type I is more often a result of trivial injuries, and the medical procedure or surgery in the form of treatment may make an important contribution to CRPS onset (Oaklander et al., 2006). Conversely, CRPS type II is often due (in more than 70% of documented cases in the USA) to high velocity missiles, or bullets, although it may also materialise after nerve injury during surgical procedures and improperly placed injections (Hassantash et al., 2003; Birch, 2009). In more than 90% of cases the nerve is only partially transected (Horowitz, 1984). Symptoms generally begin within a few days to a month after the predisposing injury in both CRPS types I and II, but 85% of type I patients experience clinical signs more or less immediately after injury (de Boer et al., 2011).

1.2.4. Epidemiology

Data on the incidence of CRPS are scarce and mostly hospital based and as such, the extent of the problem is largely unknown. Of the two population-based studies that have been performed, different results were reported: 5.5 cases per 100,000 person years were reported in a USA-based study, while 26.2 cases per
100,000 person years were reported in the Netherlands (Sandroni et al., 2003; de Mos et al., 2007). These differences could be due to ethnicity, socioeconomic status, incidence of injury and screening methods for CRPS. However, as a general observation, European middle-aged females (~37 – 52 years) have been shown to be at least 3-4 times more at risk than males, although both genders at any age can develop CRPS (Sandroni et al., 2003; de Mos et al., 2007). Childhood and adolescent CRPS is extremely rare, with paediatric patients constituting <10% of sufferers (Wilder et al., 1992). Family members and siblings of young-onset patients have increased risk of developing the syndrome, suggesting a possible genetic predisposition or familial adoption of inappropriate pain-coping strategies (Shirani et al., 2010; de Rooij et al., 2009). The upper extremity is affected more frequently than the lower extremity (60% to 40% of cases), with fracture being the most prevalent cause (45%), followed by sprains (18%) and elective surgery (12%) (de Mos et al., 2009b). Ankle fractures are also observed to lead to type I more often than type II CRPS (de Rooij et al., 2010). Many patients involved in these epidemiological studies described CRPS as having been associated with substantial disability, loss of quality of life, and a personal and economic burden (Subbarao and Stillwell, 1981).

In summary, CRPS is a rare chronic pain disease of the limb that can develop after trauma. Its multifarious symptoms prove a challenge for diagnosis and complicate the understanding of its pathophysiology. However, an increase in expression of α-1AR in nociceptors and keratinocytes of CRPS patients has been described and may contribute to disease pathogenesis (Dawson et al., 2011; Drummond et al., 2014a; Drummond et al., 2014b). As α-1AR are thought to be
involved in other chronic inflammatory conditions through expression on immune cells, and as CRPS patients often present with persistent inflammation, α-1AR expression in immune cells of CRPS may provide a link between the malfunctioning SNS of CRPS and its persisting inflammatory response (Szentivanyi et al., 1979; Wahle et al., 1999; Heijnen et al., 1996; Marchand et al., 2005; Geertzen et al., 1998).

1.3. α-1 adrenergic receptors

While α-1 adrenergic receptors (α-1AR) may play a pathogenic role in CRPS, they also have many physiological roles in the maintenance of health. The α-1AR belongs to one of three major functional classes of adrenergic receptors, which also include the α-2AR and the β-AR (Piascik and Perez, 2001). Through coupling with GTP-binding proteins, the adrenergic receptors are effector receptors of the SNS (Strosberg, 1993; Hein and Kobilka, 1997). Their physiological roles include mediation of sympathetic neurotransmission and control of vascular tone, as well as modulation of hepatic metabolism, cardiac contraction and the regulation of smooth muscle activity in the genitourinary system (Calzada and de Artinano, 2001; Koshimizu et al., 2003). The α-1ARs respond to a number of peptidergic and nonpeptidergic hormones, drugs and neurotransmitters, but are largely activated by the catecholamines adrenaline and noradrenaline (Piascik and Perez, 2001).
1.3.1. Subtypes

Progression in cloning, sequencing and pharmacologic analysis has identified three different subclasses of α-1AR: α-1A, α-1B and α-1D (Chen and Minneman, 2005). These three classes are transcribed from separate genes located on different chromosomes (Table 1.4). Despite this, they are extremely homologous and mediate similar functions, respond to the same ligands and stimulate the same G-protein pathway (Koshimizu et al., 2003). The primary polypeptide structure of the three subtypes ranges from 466 to 572 amino acids that fold into a highly conserved structure of seven transmembrane domains (Strosberg, 1993; Michelotti et al., 2000). These domains create a highly specific hydrophilic ligand-binding pocket surrounded by a hydrophobic core. Slight changes in the primary sequence among these three subtypes alters the secondary structure, resulting in distinct changes in this ligand-binding pocket (Michelotti et al., 2000). The effect is differing affinities for circulating ligands among subtypes (Docherty, 1998). The α-1AR subtypes are often co-expressed in tissues and cells; however, the expression-ratio appears to be species- and tissue-dependent (Michelotti et al., 2000; Koshimizu et al., 2003). Additionally, distinct isoforms of α-1A cDNA generated by alternative splicing have been identified. These receptors differ in length and sequence at the carboxyl terminal region of the gene and are named α-1A-1, α-1A-2, α-1A-3 and α-1A-4 (Huh et al., 2010).

1.3.2. Physiological role

The study and distribution of α-1AR subtypes in various organs and tissues has been difficult to establish due to the low abundance of mRNA and the detection of false positives. Little research on human α-1AR subtype function has been
published, but rather a variety of different functions have been described amongst animal species. These studies suggest that α-1A is more implicated in the maintenance of vascular tone and of arterial blood pressure in conscious animals, whereas α-1B participates more in response to exogenous agonists (Garcia-Sainz et al., 1999). Expression of α-1B can be modified in pathological situations such as cardiac hypertrophy and hypertension (Garcia-Sainz et al., 2000). The α-1D subtype expression is scarce in comparison to α-1A and α-1B but is involved in cardiac function and vascular tone (Calzada and de Artinano et al., 2001).

In human studies, α-1AR exhibits a wide range of physiological roles in both health and disease, with particular importance in the cardiovascular system where it mediates cardiac smooth muscle contraction, in the skin, and also in nervous tissue and the liver (mediation of glycogenesis and gluconeogenesis) (Michelotti et al., 2000). In the vasculature, α-1A is predominantly expressed in arteries, whereas all three subtypes are present in veins (Rudner et al., 1999). The expression of α-1AR in the vasculature is modulated by age; in particular, the overall α-1AR expression doubles with age (>65 years compared to <55 years) (Michelotti et al., 2000). In the nervous system, α-1AR are expressed on peripheral nociceptive neurons and are responsible for sensory-sympathetic coupling (Dawson et al., 2011). Although there is slight variation in the function of each α-1AR, most cells and tissues that express α-1AR will express all three subtypes, thus exhibit a range of functions (Perez et al. 1994).
1.3.3. Ligands

All three α-1AR subtypes have a high affinity for the biological agonists adrenaline and noradrenaline, the pharmacological agonist phenylephrine and the pharmacological antagonist prazosin (Calzada and de Artinano et al., 2001; Waugh et al., 1999; Marrow and Crease, 1986). Noradrenaline seemingly has a higher affinity than adrenaline for α-1AAR, but these two ligands share the same binding affinity for α-1BAR and α-1DAR. There are select few agonists that show selectivity for each subtype: for example A61603, a potent pharmacological agonist, shows specificity for α-1AAR but not α-1BAR or α-1DAR (Calzada and de Artinano et al., 2001; Knepper et al., 1995). However, subtype selectivity is seen more commonly in antagonist binding (Table 1.4).

1.3.4. Cellular localisation

Classically, G-protein binding receptors are expressed on the cell membrane where they are accessible to water-soluble ligands. However, reports suggest this may not entirely be the case for α-1AR, with studies showing major differences in the subcellular distribution of these receptors. Immunohistochemistry using a peptidergic antibody against the C-terminal region of α-1B show that α-1B is expressed on the surface cells (Fonseca et al., 1995). However, α-1A is expressed both intracellularly and on the cell surface with approximately 40% of the receptor expressed intracellularly (Hirasawa et al., 1997; McGrath et al., 1999). The α-1D receptor is very hard to detect on the cell surface, but is detectable intracellularly, suggesting that α-1D may be an entirely intracellular receptor (McGrath et al., 1999; Mackenzie et al., 2000).
Table 1.4. Summary of α1AR. (Koshmizu et al., 2003; Calzada and de Artinano, 2001)

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>α-1A</th>
<th>α-1B</th>
<th>α-1D</th>
</tr>
</thead>
<tbody>
<tr>
<td>G- proteins</td>
<td>$G_q/11$</td>
<td>$G_q/11$</td>
<td>$G_q/11$</td>
</tr>
<tr>
<td>Potency order</td>
<td>NA≥A</td>
<td>A=NA</td>
<td>A=NA</td>
</tr>
<tr>
<td>Selective agonist</td>
<td>A61603</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selective antagonist</td>
<td>KMD3213 (+)</td>
<td>AH11110A</td>
<td>BMY7378</td>
</tr>
<tr>
<td></td>
<td>Niglugipine</td>
<td>Chlorehytcilonidine</td>
<td>SKF 105854</td>
</tr>
<tr>
<td></td>
<td>5-Methyluradipil</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SNAP5089</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WM4101</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RS17053</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other agonists</td>
<td>PHE</td>
<td>PHE</td>
<td>PHE</td>
</tr>
<tr>
<td>Other antagonists</td>
<td>Prazosin</td>
<td>Prazosin</td>
<td>Prazosin</td>
</tr>
<tr>
<td>Amino acid residues</td>
<td>466</td>
<td>519</td>
<td>572</td>
</tr>
</tbody>
</table>
Chapter 1. Literature Review

<table>
<thead>
<tr>
<th>Gene</th>
<th>ADRA1C</th>
<th>ADRA1B</th>
<th>ADRA1D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human chromosome</td>
<td>8</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Cellular localisation</td>
<td>Cell surface and intracellular</td>
<td>Cell surface</td>
<td>Mostly intracellular</td>
</tr>
<tr>
<td></td>
<td>NA = noradrenaline; A = adrenaline; PHE = phenylephrine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.4. Cells and regulation of the inflammatory response

The inflammatory response aims to eliminate the initial cause of cell injury, remove necrotic cells and tissues damaged from the original insult and inflammatory process, and to initiate healing and repair (Medzhitov, 2008). As the majority of CRPS patients experience nerve and/or tissue trauma, an inflammatory response is expected to be evident at the time of injury. This inflammation is commonly described among the symptoms of CRPS, with limbs presenting as warm, swollen, and red; and being described as having a loss in function and increased pain. These symptoms are a result of the inflammatory response coordinating the delivery of leukocytes, cytokines and inflammatory mediators to the site of insult, regulated by a highly complex molecular system (Vigano et al., 2012). Under normal circumstances inflammation would resolve after the insult has been cleared, however this inflammation commonly persists in CRPS patients for reasons not fully understood. Neurogenic inflammation (inflammation arising from the release of neuropeptides such as substance P and calcitonin-gene-related-peptide) is also observed in CRPS patients, however, as it is beyond the scope of this project, will not be discussed in detail (Birklein et al., 2001a; Lei et al., 2003; Schinkel et al., 2006). The following section will describe in detail the cells and mediators of the inflammatory response, and which cell types and mediators may indicate persisting inflammation.

1.4.1. Cells and mediators of the inflammatory response

Leukocytes involved in the inflammatory response include granulocytes, lymphocytes and monocytes (Chaplin, 2010). Granulocytes are the most
abundant leukocyte and include neutrophils, basophils and eosinophils involved in innate immunity (Vigano et al., 2012). Lymphocytes are cells of the adaptive immune system and include B cells, T cells, T regulatory cells, Natural Killer (NK) cells and NKT cells, whereas monocytes are the blood-borne precursor for phagocytic tissue macrophages and some forms of specialised antigen-presenting cells (APC), such as dendritic cells (Chaplin, 2010). An intact immune system and the inflammatory response require contributions from many subsets of leukocytes (Medzhidov, 2008). Different leukocytes with varying functions can be identified by expression of differentiation proteins on their cell membrane that are assigned a cluster of differentiation (CD) number (Engel et al., 2015). Monoclonal antibodies specific for these cell surface molecules can bind to the CD antigen and be used for phenotyping of leukocyte subsets (Engel et al., 2015). There are currently more than 350 defined CD antigens (Chaplin, 2010). One of these, the CD45 marker, is a pan-marker for all leukocytes and is a receptor-linked protein tyrosine-phosphatase involved in the function of all leukocytes (Engel et al., 2015). A variety of other CD markers can be used to identify subsets of CD45⁺ leukocytes in the peripheral blood of humans: Table 1.5 provides a summary of the CD markers (and the cells they identify) to be discussed in this section, some of which were used in this study.
Table 1.5: CD markers for each of the different types of leukocytes discussed in Section 1.4.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>CD marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>CD45</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>CD6, CD11b, CD66</td>
</tr>
<tr>
<td>Monocytes</td>
<td>CD14, CD16</td>
</tr>
<tr>
<td>T cells</td>
<td>CD3</td>
</tr>
<tr>
<td>T helper cells</td>
<td>CD3, CD4</td>
</tr>
<tr>
<td>T cytotoxic cells</td>
<td>CD3, CD8</td>
</tr>
<tr>
<td>T regulatory cells</td>
<td>CD3, CD4, CD25, FoxP3</td>
</tr>
<tr>
<td>B cells</td>
<td>CD19</td>
</tr>
<tr>
<td>NK cells</td>
<td>CD16, CD56</td>
</tr>
<tr>
<td>NKT cells</td>
<td>CD3, CD56, CD16</td>
</tr>
</tbody>
</table>

1.4.1.1. Granulocytes

Amongst the granulocytes, neutrophils are blood-borne non-specific phagocytic cells of the innate immune system of mammals that engulf microbes and release cytoplasmic granules (Medzhitov, 2008). They are the first cells of defence, migrating within ninety minutes of the initial insult. Their granules contain enzymes and other antibacterial substances that are used to destroy and degrade engulfed microbes and dead tissue (Vigano et al., 2012). Neutrophils also have oxygen-dependent metabolic pathways that generate toxic oxygen and nitrogen products that aid in the destruction of engulfed pathogens (Chaplin, 2010). Phenotypic surface markers used for identification of human neutrophils typically include CD6, an Fc receptor for immunoglobulin (Ig); CD11b, the protein of which is involved in cellular adhesion during phagocytosis; and CD66, also involved in adhesion and activation of neutrophils (Lakschevitz et al., 2016).
Eosinophils and basophils produce lipid mediators and cytokines that induce inflammation. Eosinophils are also blood-borne granulocytes and are involved during allergic reactions and parasite infections. The granules of eosinophils contain a protein that is highly toxic to parasitic worms too large to be phagocytosed. Basophils bind an antibody, IgE, secreted by plasma cells through receptors on their cell surface. Binding to IgE triggers release of histamine and vasoactive agents from the basophil granules. Mast cells are tissue-resident basophils (Chaplin, 2010).

1.4.1.2. Monocytes

Monocytes are blood-borne mononuclear cells constituting about 3 to 8% of the white blood cell count (Medzhitov, 2008; Vigano et al., 2012). They migrate into tissues at the site of injury approximately 24 to 48 hours after the initial insult, and differentiate into macrophages, which are tissue-resident monocytes, and dendritic cells, which are specialised antigen-presenting cells (APCs) involved in stimulating the immune response. Monocytes and macrophages produce potent vasoactive mediators that promote regeneration of tissues (Chaplin, 2010). The circulating life span of the monocyte is three to four times higher than that of the granulocytes. These longer-lived phagocytes help to destroy the causative agent, aid in the signalling process of immunity, serve to resolve the inflammatory process and contribute to initiation of the healing processes. They also play an important role in chronic inflammation, where they can surround and wall off foreign material that cannot be digested (Medzhitov, 2008). Monocytes express CD14 and CD16 on their cell surface that can be used to identify three subpopulations: CD14$^+$ CD16$^-$ (classical monocyte), CD14$^{low}$ CD16$^+$
(intermediate monocyte) and CD14\textsuperscript{high} CD16\textsuperscript{+} (non-classical monocyte). Non-classical monocytes display higher levels of CD14 expression than the intermediate CD14\textsuperscript{+} CD16\textsuperscript{+} monocyte. Classical monocytes constitute 85% of monocytes, whereas intermediate and non-classical monocytes make up 5% and 10%, respectively (Wong et al., 2012). Classical monocytes are involved in phagocytosis whereas intermediate and non-classical monocytes are thought to be involved in T cell migration and proliferation. The CD14 molecule is a pattern-recognition receptor, aiding in the recognition of bacterial lipopolysaccharide (LPS), whereas CD16 is an Fc receptor, binding to the Fc portion of IgG antibodies (Wong et al., 2012).

1.4.1.3. Lymphocytes

As will be discussed below, there are two distinct types of lymphocytes (B cells and T cells) that differ in their maturation pathways and functions, but are morphologically similar. Additionally, Natural Killer (NK) cells and NKT cells are not specifically identified as lymphocytes, but share some markers and functional properties (Brennan et al., 2013). Most lymphocytes specifically recognise and respond to foreign proteins and antigens, although populations of self-responsive lymphocytes are generated in some individuals. Antigen presenting cells (APC) such as dendritic cells, and to a lesser extent, macrophages, “present” antigens to lymphocytes: this occurs after the APC has phagocytosed the antigen and processed it into fragments, which are then expressed on their cell surface in order to specifically activate lymphocytes (Chaplin, 2010).
1.4.1.3.1. T cells

The T lymphocytes (or T cells) arise from bone marrow stem cells, but mature in the thymus, where they undergo rearrangement of genes needed for expression of a unique T-cell antigen receptor (Vigano et al., 2012). The T-cell receptor (TCR) is composed of two polypeptides that fold to form a groove that recognises processed antigens from antigen-presenting cells (APC) via the major histocompatibility complex (MHC), a cell surface protein that binds to and displays foreign antigens to the immune system (Masopust and Schenkel, 2013). The TCR is associated with other surface molecules, known as the CD3 complex, that aid in cell signalling. The CD3 surface receptor can be used as a phenotypic marker for all T lymphocytes. Subpopulations of T cells can be divided by contributions of other cell surface markers associated with the TCR complex, such as CD4+ (helper) T cells and CD8+ (cytotoxic) T cell (Masopust and Schenkel, 2013).

1.4.1.3.1.1. CD4+ Helper T cells

The CD4+ helper T cell (T\textsubscript{H}) serves as a master regulator for the immune system. Activation of T\textsubscript{H} cells depends upon the recognition of antigen associated with MHC Class II molecules on APCs such as dendritic cells. Once activated, T\textsubscript{H} cells release a variety of cytokines that influence the function of all other inflammatory cells. Different types of cytokines released by T\textsubscript{H} cells induce different kinds of immune responses: for example, they can regulate antibody production by B cells, and activate and regulate other immune cells such as APC, CD8+ cytotoxic T cells, NK cells and monocytes (Baranovski et al., 2015).
The cytokine interleukin-2 (IL-2) is one of the first cytokines to be produced during activation of lymphocytes, and is necessary for the proliferation and function of T\textsubscript{H} cells, cytotoxic T cells and NK cells. IL-2 interacts by binding to IL-2 receptors on these cells (Baranovski \textit{et al.}, 2015). The activated T\textsubscript{H} cell can further differentiate into T\textsubscript{H}1 or T\textsubscript{H}2 cells based on the cytokines secreted by the APC during activation: the cytokine IL-12 produced by macrophages and dendritic cells stimulates maturation into T\textsubscript{H}1 type cells, whereas IL-4 produced by basophils and T cells induces differentiation towards T\textsubscript{H}2 type cells. The type of T\textsubscript{H} cell activated drives the type of inflammatory response produced. Activated T\textsubscript{H}1 cells characteristically produce the cytokines IL-2 and interferon (IFN)-\textgreek{gamma}, whereas T\textsubscript{H}2 cells produce IL-4 and IL-5. Some cytokines produced by T\textsubscript{H}2 cells (e.g. IL-4 and IL-10) inhibit macrophage activation and suppress T\textsubscript{H}1 responses, and are considered anti-inflammatory (Noelle and Snow, 1992). In most immune responses, a balanced response of T\textsubscript{H}1 and T\textsubscript{H}2 is needed to maintain homeostasis. However, injury or exposure to different types of antigens (e.g. pathogens or allergens) can skew the response to one or the other subset and in some cases, lead to pathogenic inflammation (Masopust and Schenkel, 2013). The T\textsubscript{H}17 type cell is a less common type of helper T cell that produces the cytokines IL-17, IL-21 and IL-22 (Ouyang \textit{et al.}, 2008). The cytokines released by T\textsubscript{H}1, T\textsubscript{H}2 and T\textsubscript{H}17 cells play a number of important roles in inflammation, including amplification of the response through recruitment and activation of other inflammatory cells (Baranovski \textit{et al.}, 2015)
1.4.1.3.1.2. Regulatory T cells

In contrast to T<sub>H</sub> cells, regulatory T cells (T<sub>reg</sub>) suppress inflammatory responses by inhibiting the proliferation of other potentially harmful self-reactive lymphocytes. They express the CD4 marker, but additionally express CD25, an activation marker that is the receptor for IL-2; FoxP3 and Helios, which are both transcription factors involved in the development and function of T<sub>reg</sub> cells (Takatori <i>et al.</i>, 2015; Sakaguchi <i>et al.</i> 2010). There are two types of T<sub>reg</sub> cells: naturally occurring T<sub>reg</sub> cells that suppress immune response and, although express the CD4 marker, are different from T helper cells. Induced T<sub>reg</sub> cells on the other hand, derive from mature CD4<sup>+</sup> T helper cells (Takatori <i>et al.</i>, 2015). The regulation by these cells is antigen-specific and controlled through activation of the T cell receptor (TCR) by the antigen and subsequent secretion of IL-10 and TGF-β. These cytokines inhibit the proliferation and activation of various lymphocytes and macrophages (Beissert <i>et al.</i>, 2006).

1.4.1.3.1.3. CD8<sup>+</sup> Cytotoxic T cells

Cytotoxic CD8<sup>+</sup> T Cells are activated through binding to MHC class I/viral antigen complexes on the surface of any cell type after infection with virus. They destroy infected cells by releasing cytolytic enzymes and toxic cytokines, or program cell death by triggering surface molecules to initiate apoptosis (Chaplin, 2010). Cytotoxic T cell responses are amplified by cytokines, including IFN-γ, produced by T<sub>H1</sub> cells that also become activated during viral infections (Baranovski <i>et al.</i>, 2015).
1.4.1.3.2. B cells

B lymphocytes (B cells) contribute to the inflammatory response by producing antibodies specific for target antigens. They are identified by the presence of CD19, a membrane immunoglobulin (Ig) that functions as the antigen receptor for the production and release of antibodies. During maturation in the bone marrow, B cell progenitors develop into mature or naïve B cells. Naïve B cells express membrane-bound IgD and IgM that function as receptors for antibody but do not secrete antibody. Mature B cells leave the bone marrow, enter the circulation, and migrate to the various peripheral lymphoid tissues, where the B cell is stimulated to respond to a specific antigen. Each stage of B cell development is indicated by the pattern and expression of Ig on their cell surface, that serve as phenotypic markers of these maturational steps (Masopust and Schenkel, 2013). For most antibody responses, B cells require help from T\(_H\) cells in order to become activated and to make antibodies of different isotypes (eg. IgG, IgE, IgA) (Baranovski \textit{et al.}, 2015).

1.4.1.3.3. NK cells

Natural killer (NK) cells comprise approximately 10% to 15% of peripheral blood lymphocytes and do not bear T-cell receptors or cell surface immunoglobulins (Brennan \textit{et al.}, 2013). Morphologically, they are somewhat smaller than T and B cells and contain abundant cytoplasmic granules. NK cells are a part of the innate immune system, and may be the first line of defence against viral infections (Brennan \textit{et al.}, 2013). They also have the ability to recognise and kill tumour cells, abnormal body cells, and virally infected cells. Two cell surface molecules, CD16 and CD56 are widely used to identify NK
cells. CD16 serves as a receptor for the IgG molecule, which provides NK cells with the ability to lyse IgG-coated target cells (Trincheri, 1989). CD56, or the neural cell adhesion molecule (NCAM) is a glycoprotein involved in cellular adhesion (Brennan et al., 2013).

1.4.1.3.4. NKT cells

NKT cells are an additional subgroup of T cells that share NK cell properties (Brennan et al., 2013). They express a unique TCR, which recognises glycolipid antigens that are only presented by CD1d, although is still identified as CD3. They combine both innate and adaptive immune properties by promoting cell-mediated immunity to tumours and infectious orgasms yet can also suppress the cell-mediated immunity associated with autoimmune responses and allograft rejection. The precise means by which these cells carry out these functions is unclear. NKT cells additionally express the CD56 marker (Brennan et al., 2013).

1.4.2. Chronic inflammation

A successful inflammatory response results in the elimination of the initiating stimulus, followed by a healing and repair phase to reestablish tissue homeostasis. A switch from pro-inflammatory mediators to anti-inflammatory mediators (eg. IL-10, TGF-β and lipoxins) mediates the resolution response, as well as recruitment of cells involved in healing and repair such as macrophages and fibroblasts. Ideally this should only span a few days; however, inflammation can become chronic when the inflammatory stimuli cannot be removed (Chaplin, 2010).
Chronic inflammatory diseases are a group of clinical disorders that are characterised by an aberrant, non-resolving inflammatory response. Chronic inflammation is pathogenic and can last for many years. The acute response (neutrophils and activated T cells) is replaced with macrophages and lymphocytes (Vigano et al., 2012). It impedes injury resolution and rather than repairing the tissue, the persisting inflammation results in tissue destruction and scarring may result in organ dysfunction (Gabay, 2006). The process may be localised, but it often progresses to disabling diseases such as asthma, rheumatoid arthritis and atherosclerosis (Shaacter and Weitzman, 2002). As CRPS patients present with persisting inflammation, it has been proposed that CRPS is a chronic inflammatory disease. Interestingly, as discussed further below, some chronic inflammatory diseases have shown α-1AR expression in leukocytes, and that this may play a significant role in this persisting inflammation, with little or no expression of α-1AR in immune cells of a healthy immune state.

### 1.5. α-1 adrenergic receptors in inflammation

While the most well-described influences of α-1AR are on the vasculature, sensory nerves and keratinocytes, there is growing evidence to show that α-1AR may also be differentially expressed by cells of the immune system, influencing inflammatory cytokine production and promoting chronic inflammation. Characterisation of α-1AR expression has been difficult, since many commercially available antibodies have been shown to be non-specific for α-1AR, meaning that most investigations rely on RT-PCR to analyse mRNA rather than protein expression. However, RT-PCR is also prone to contamination and
lack of specificity. This could be the reason for the contradictory literature surrounding $\alpha$-1AR expression on leukocytes, with numerous reports documenting an absence of $\alpha$-1AR while others report expression following phytohemagglutinin (PHA) or lipopolysaccharide (LPS) stimulation (Casale and Kaliner, 1984; Kavelaars, 2002; Rouppe van der Voort et al., 2000). The majority of these studies were performed on peripheral blood mononuclear cell (PBMC) preparations, which include a mixture of several blood cell types such as T cells, B cells, NK cells, NKT cells and monocytes. Nevertheless, in situ hybridisation techniques show that PBMCs can express $\alpha$-1bAR and $\alpha$-1aAR, with $\alpha$-1dAR found to a lesser extent (Tayebati et al., 2000). Immunocytochemistry analyses in the same study showed that the majority of PBMCs expressed the mature $\alpha$-1bAR protein with fewer cells expressing $\alpha$-1aAR and $\alpha$-1dAR.

More specifically, the human monocytic cell line (THP-1) endogenously expresses $\alpha$-1bAR and $\alpha$-1dAR mRNA, whereas genomic $\alpha$-1aAR expression can be induced following treatment with TNF-$\alpha$ or IL-1$\beta$ (Heijnen, 2002). Functional $\alpha$-1AR expression has also been described on primary monocytes isolated from human blood (Takahashi, 2005). Conversely, other studies have documented no detectable $\alpha$-1AR mRNA from human monocytes unless they were cultured in the presence of glucocorticoid, dexamethasone or the $\beta$-2AR agonist terbutaline, which induced $\alpha$-1bAR and $\alpha$-1dAR mRNA (Rouppe van der Voort, 1999). There are suggestions that inhibition of $\alpha$-1AR on monocytes regulates their migration (Kintscher, 2001). Other reports suggest that $\alpha$-1AR
may be involved in complement synthesis through stimulation by adrenaline or noradrenaline (Lappin and Whaley, 1982). More recently, activation of α-1AR by phenylephrine was found to influence the modulation of cytokines such as IL-1β, TNF-α, IL-6, IL-8 and IL-10 from monocytes stimulated with LPS, supporting the concept that α-1AR influences the production of and also responses to cytokines (Grisanti et al., 2011).

Under normal conditions, T cells do not appear to express α-1AR, although some studies suggest that α-1AR expression may be regulated in certain lymphoid compartments or under certain pathological conditions. For example, lymphocytes from rat mesenteric lymph nodes transcriptionally express α-1AR, while α-1ΑAR and α-1ΔAR expression was observed in rat lymphocyte populations from the thymus, spleen and peripheral blood (Bao et al., 2007). Peripheral blood T cells do, however, show an increase in α-1AR mRNA activation with a T cell mitogen, suggesting a possible maturation expression with earlier studies demonstrating α-1AR activation to inhibit proliferative T cell responses (Schaunestein et al., 2000; Heilig et al., 1993). To date, specific α-1AR expression on B cells has not been reported (Casale and Kaliner, 1984; Rouppe van der Voort et al., 2000; Taybati et al., 2000).

In summary, it is unclear whether leukocytes typically express α-1AR in a healthy immune state although it appears α-1AR can be induced following stimulation with inflammatory mediators. Studies on cell lines that do express α-
1AR suggest that the receptor may be involved in cell migration and complement synthesis, or may be indicative of maturation.

### 1.5.1 α-1 adrenergic receptors in chronic inflammatory diseases

Juvenile rheumatoid arthritis (JRA) is a chronic inflammatory joint disease associated with an increase in the level of noradrenaline, a ligand that binds to both β- and α- adrenergic receptors. Activation of β-2 and α-2 on macrophages leads to an upregulation of LPS-induced TNF-α production in vivo, promoting an inflammatory response (Heijnen et al., 1996). The same study found that PBMCs of JRA patients respond to α-1AR activation with increased production of the pro-inflammatory cytokine IL-6. Healthy PBMCs from this study did not functionally express α-1AR therefore the production of IL-6 from PBMCs in this case likely exacerbates disease (Heijnen et al., 1996). Further analysis revealed that in JRA, PBMCs expressed mRNA that encoded the α-1DAR subtype, and did not produce IL-8, an anti-inflammatory mediator (Rouper van der Voort et al., 2000). Moreover, another study suggests that it may not be a total increase in α-1AR that exacerbates disease but rather a shift in the ratio of β to α-1AR that responds to catecholamine effects in rheumatoid arthritis (Wahle et al., 1996; Goebel et al., 2010). Allergic asthma is another chronic inflammatory disease that results in an obstructed airway. Lymphocytes in lung tissue from asthmatic patients show the same adrenergic receptor shift from β-AR to α-1AR that is thought to exacerbate inflammation in chronic disease (Szentivayi et al., 1979). In summary, it appears that α-1AR is only expressed in immune cells following an inflammatory stimulus or during chronic inflammation. Therefore, whether
CRPS could pose as a chronic inflammatory disease is an area worth investigating.

1.6 Inflammatory and $\alpha$-1AR pathophysiology of CRPS

1.6.1. Inflammation in CRPS

Inflammation is a core component of the key CRPS symptoms: skin reddening, warmth, oedema, trophic changes, loss of function and pain. CRPS patients commonly experience inflammation that may persist twelve to eighteen months after the initial injury. As the type of cellular infiltrate during an inflammatory response reflects the type of insult present, examining leukocyte distributions relative to that of a healthy immune state may provide insight into why CRPS patients commonly present with persisting inflammation. The same applies for inflammatory mediators, as they are produced selectively by different insults, act on different cell types and can mediate the symptoms of pain.

Monocytes and macrophages are phagocytic inflammatory cells, and along with dendritic cells, are involved in processing and presenting foreign proteins (antigens) for activation of the adaptive immune system (see Section 1.4.1.2 above). Although total monocyte counts in CRPS patients have been shown to be unaltered compared to normal individuals, the ratio of the CD14$^+$ CD16$^+$ to CD14$^+$ CD16$^-$ monocyte subgroup was raised in comparison to that of normal individuals (Ritz et al., 2011). Individuals with this heightened CD14$^+$ CD16$^+$ ratio also have decreased serum levels of the anti-inflammatory cytokine IL-10, suggesting a pro-inflammatory response (Uceyler et al., 2007). It is unclear
whether these immune changes are present before the onset of CRPS (in which case they may contribute to the onset of CRPS) or after developing CRPS (in which case they might play a role in its maintenance).

After neuronal lesions, microglia (CNS macrophages) in the spinal cord change their immunophenotype, proliferate and migrate such that they exert a regulatory influence on synaptic transmission (Banati, 2002). In a rat model of CRPS, T cells infiltrate the spinal cord in response to the release of pro-inflammatory cytokines (possibly from microglia) with a change in the T\(_{\text{H}}\)1/T\(_{\text{H}}\)2 pro-inflammatory cell ratio that results in neurogenic inflammation (Moalem et al., 2004). A pro-inflammatory T\(_{\text{H}}\)1 response is more likely to amplify hyperalgesia after a nerve lesion is repaired with an increase in the T\(_{\text{H}}\)2 response more likely to prevent hyperalgesia (Cao and Deleo, 2008; Moalem et al., 2004). Additionally, FoxP3\(^{+}\) knockout mice develop neuropathic pain after nerve lesions, suggesting that neuropathic pain can be controlled by FoxP3\(^{+}\) regulatory T cells (Austin et al., 2012). Mice without B cells and T cells have less pain behaviour after nerve lesions than wild-type mice, indicating the importance of lymphocytes for the development of pain (Costigan et al., 2009). Interestingly, as CRPS develops most often after fractures, fracture healing improves in animals without an adaptive immune system (Toben et al., 2011).

Skin biopsies taken from CRPS patients show an increase in expression of the inflammatory mediators TNF-\(\alpha\) and IL-6 when compared to unaffected individuals (Birklein and Schlereth, 2015). Blister fluid taken from the skin of affected limbs in CRPS patients has also been shown to have increased levels of
TNF-α and IL-6 compared to that of the unaffected limb (Kramer et al., 2011). Correspondingly, serum concentrations of soluble TNF-receptors, as well as pro-inflammatory cytokines TNF-α, IL-1β and IL-8 were increased, whereas anti-inflammatory cytokines IL-4, IL-10 and transforming growth factor β1 (TGF-β1) were decreased in CRPS patients (Schinkel et al., 2006; Uceyler et al., 2007). In addition to their immune cell regulatory effects, inflammatory cytokines in CRPS also act on peptidergic nociceptors to enhance the release of neuropeptides substance P and calcitonin-gene-related-peptide (CGRP) (Birklein et al., 2001a; Lei et al., 2003; Schinkel et al., 2006). Increased levels of these neuropeptides have been found in the affected skin of CRPS individuals, and are thought to evoke vasodilation and protein extravasation in this tissue during inflammation. The resulting visible symptoms (reddening, warmth and oedema) are termed neurogenic inflammation (Dallos et al., 2006; Weber et al., 2001; Hou et al., 2011). Substance P induces plasma extravasation in the CRPS-affected limb, and may be involved in mast cell degranulation and further release of inflammatory mediators (Oyen et al., 1993; Weber et al., 2001). Downstream this could further upregulate its own release from peptidergic nerves, potentially resulting in persisting inflammation (Woolf et al., 1994; Wei et al., 2009). Neurogenic inflammation is also observed in areas other than the affected region, suggesting a systemic response (Birklein and Schmelz, 2008). Additionally, the mRNA of metalloproteinase-9, which cleaves cytokines and neuropeptides, is upregulated four-fold in CRPS patients, and many microRNAs known to control the inflammatory process are downregulated, which may act to keep the inflammatory process switched on (Orlova et al., 2011). Physiologically, these
microRNAs travel with exosomes released from inflammatory cells in the blood and systemically regulate inflammation in target cells (McDonald et al., 2014). Although it is hypothesised that an increase in neuropeptides from sensory nerve fibres may be the cause of this persisting inflammation, how this is mediated is unclear. As mentioned previously, research surrounding chronic inflammatory diseases suggest that α-1AR are upregulated on leukocytes in an inflammatory state, and are functionally linked to the release of pro-inflammatory mediators. Whether α-1AR drives the release of mediators from leukocytes in CRPS, and ultimately the release of neuropeptides from sensory nerve fibres and the infiltration of leukocytes during inflammation, is a question that remains to be answered.

1.6.2. Autoimmunity in CRPS

Additional to chronic inflammatory characteristics of CRPS, there is some evidence that there may also be autoimmune characteristics to CRPS. Autoimmune disorders occur where leukocytes are unable to distinguish between self-antigens and foreign antigens, producing autoantibodies. Immune self-tolerance is the ability of the immune system to recognise self-proteins from non-self proteins, where self-reactive T and B cells are eliminated or regulated during maturation (Kamradt and Mitchison. 2001). As such, the breakdown of immune self-tolerance can result in autoimmune disorders (Davidson and Diamond, 2001). Approximately 40% of a small cohort of CRPS patients were found to have autoantibodies for β-2 adrenergic receptors and muscarinic-2 receptors (Blaes et al., 2004; Kohr et al., 2011). These findings led to the formation of the
IRAM hypothesis: Injury-triggered, Regionally-restricted Autoantibody-Mediated autoimmune disorder with minimally-destructive course, which describes pre-existing circulating autoantibodies becoming pathogenic after the individual experiences trauma (Goebel and Blaes, 2013). Goebel et al. (2010) proposed there may be a relative shift in the mediation of adrenergic effects from β to α adrenergic receptors contributing to CRPS onset, although how this occurs is unclear. More recently, autoantibodies specific for α-1AR were detected in patient sera, and it has been suggested that anti-α-1AR antibodies isolated from CRPS patients could enhance pain via activation of α-1AR on dorsal root ganglion (DRG) neurons (Dubuis et al., 2014; Reilly et al., 2016). Other signs indicative of an autoimmune contribution to CRPS include the improvement after intravenous immunoglobulin (a treatment for autoimmune inflammation) of α-1AR (Goebel and Blaes, 2013). Furthermore, α-1AR autoantibodies were found in the serum of patients who have had previous infections with chlamydia, parvovirus and campylobacter (Goebel and Blaes, 2013). An increased frequency of expression of the human leukocyte antigens (HLA) DR-2, A3 and HLA-B7, associated with other autoimmune diseases such as multiple sclerosis, were also observed in CRPS patients (Mailis and Wade, 1994; Weiner et al., 1993). These HLA antigens are more likely to present self-antigens to self-reactive lymphocytes of the immune system (Goodnow et al, 2005). However, as there are limited data on the role of autoimmunity in the pathogenesis of CRPS and with only a small number of CRPS patients expressing autoantibodies, more investigations into the nature of this observation need to be conducted.
1.6.3. Biological susceptibility to CRPS

While attempts to find a psychological susceptibility to CRPS are ongoing, a strong correlation has yet to be found (Bruehl and Carlson, 1992; Field and Gardner, 1997; Bruehl, 2001; Harden et al., 2003; Puchalski and Zyluk, 2005; Beerthuizen et al., 2011). Several observations suggest that genetic determinants may play a role in the predisposition to develop CRPS. In particular, the distribution of polymorphisms in various cytokine, neurotransmitter and adrenergic receptor genes has been examined. A significant association with an $\alpha_{1A}$-AR polymorphism has been detected (Herlyn et al., 2010). However, the relevance of this finding is unclear since the polymorphic variant does not differ pharmacologically (Herlyn et al., 2010). Particular HLA genotypes show a clear pattern of inheritance among CRPS patients, with the HLA-DQ1 and HLA-DR2 alleles significantly more frequent in CRPS I patients (Mailis and Wade, 1994; Kemler et al., 1999). Moreover, a polymorphism in the TNF-$\alpha$ promoter gene has been associated with the occurrence of the disease, with homozygosity for this allele increasing the risk of having more than one extremity involved (Vaneker et al., 2002).

1.6.4. $\alpha$-1 adrenergic receptors in CRPS

Additional to evidence suggesting $\alpha$-1AR is a target for autoantibodies in CRPS and/or subject to genetic polymorphism leading to disease onset, atypical $\alpha$-1AR expression has been described in CRPS throughout the vasculature, nervous system and within the skin. Skin biopsies taken from CRPS type I individuals also show an increase in expression of $\alpha$-1AR on keratinocytes and cutaneous
nociceptors (Drummond et al., 1996; Drummond et al., 2014a; Drummond et al., 2014b). Previous studies have suggested that decreased noradrenaline levels and/or pro-inflammatory mediator influence may account for this increased α1-AR expression (Drummond et al., 1991; Kurvers et al., 1995; Heijnen et al., 2002). As shown in Figure 1.3, a decrease in noradrenaline levels in the blood may result in a compensatory increase in α-1AR expression in an attempt to increase ligand-receptor binding affinity. Whilst this initially has no apparent effect, when noradrenaline levels finally return to normal, this increase in ligand-receptor binding affinity results in supersensitivity to circulating noradrenaline and catecholamines (Wasner et al., 2001; Wasner et al., 1999). Clinically, the supersensitivity to noradrenaline results in vasoconstriction, causing decreased blood flow and a cold limb develops. Decreased catecholamine levels in CRPS patients support this model, with hyperalgesia increasing when patients were exposed to the α-1AR agonist noradrenaline or phenylephrine (Wasner et al., 1999; Harden et al., 1994). Unfortunately, treating patients with sympathetic blockade and blocking the effect of noradrenaline on adrenergic receptors is unsatisfactory as an all-round treatment for CRPS as only 30% of patients are relieved of their symptoms (Cepeda et al., 2002; van Eijs et al., 2012).

Nerve fibres surviving after injury become more sensitive to the α-1AR agonists phenylephrine and noradrenaline (Ali et al., 2000; Torebjork et al., 1995). Blocking α-1AR with a potent antagonist (prazosin) in human patients inhibits dynamic allodynia and hyperalgesia in the CRPS-affected limb as well as axon-reflex vasodilation induced by the iontophoresis of phenylephrine in healthy controls (Drummond et al., 2016). Continual noradrenaline released by the
sympathetic nerves might activate or sensitise the affected afferent neurons (Gibbs et al., 2008). This sympathetic coupling forms the basis of the sympathetically maintained pain theory, and is supported by observations of patients reporting pain relief after sympathetic blockade (Marinus et al., 2011).

<table>
<thead>
<tr>
<th>SNS dysfunction</th>
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<tbody>
<tr>
<td>Decreased noradrenaline</td>
</tr>
<tr>
<td>Increased $\alpha_1$-AR density/ responsiveness in vasculature</td>
</tr>
<tr>
<td>Supersensitivity to circulating catecholamines</td>
</tr>
<tr>
<td>Vasoconstriction in chronic stages of CRPS and cold limb</td>
</tr>
</tbody>
</table>

**Figure 1.3: $\alpha_1$-AR involvement in CRPS.** An impaired sympathetic nervous system produces a decreased level of noradrenaline. A compensatory increase of $\alpha_1$-AR in the vasculature results in supersensitivity to circulating catecholamines. When the SNS function finally restores, this supersensitivity clinically results in vasoconstriction of blood vessels and a cold limb develops.

In summary, the role of $\alpha_1$-AR in CRPS pathogenesis is potentially extensive, with influences among vascular tone and decreased blood flow, as well as on keratinocytes and nerve fibres at the site of injury. With a small but significant number of CRPS patients reporting pain relief when $\alpha$-1AR is antagonistically blocked, the evidence of sensitisation of nociceptors by cytokines, and evidence
that α-1AR is expressed in the immune system especially in chronic inflammatory diseases, the pathogenic role of α-1AR may not solely be confined to the vasculature, skin and nerve cells, but may also extend to the immune system and the inflammatory responses.

1.7. Summary

Complex regional pain syndrome is a debilitating disorder characterised by chronic causalgic pain, inflammation and autonomic disturbances in response to injury (Geertzen et al., 1998; Veldman et al., 1993; Harden et al., 201). Its multifarious symptoms prove a challenge for diagnosis, thus diagnosis relies on clinical evaluation and exclusion by the IASP criteria (Harden and Bruehl, 2005). These criteria can be assembled into a severity score, which is a quantitative index of the number of symptoms the patient experiences, that allows clinicians and researchers to more accurately understand the extent of disease (Harden et al., 2010).

The majority of CRPS patients experience persisting inflammation localised to the site of injury (Borchers and Gerschwin, 2014; Birklein and Schlereth, 2015). Assessing the types of cellular infiltrate can contribute to an understanding of why this inflammation persists. Previous studies show an increase in the CD14+CD16+ monocyte ratio that likely resembles a pro-inflammatory response (Ritz et al., 2011). Additionally, an increase in production of pro-inflammatory cytokines was also shown in skin biopsies and serum levels of CRPS patients when compared to that of a non-CRPS healthy control, as well as a decrease of anti-inflammatory cytokines in patient sera (Kramer et al., 2011; Schinkel et al.,
2006; Uceuler et al., 2007). Increased levels of pro-inflammatory cytokines likely stimulate sensory nerve fibres to produce neuropeptides, which in turn evoke vasodilation, protein extravasation and mast cell degranulation to release further inflammatory mediators, all contributing to persisting inflammation (Birklein et al., 2001; Lei et al., 2003; Schinkel et al., 2006; Guo et al., 2012).

The α-1AR are receptors of the SNS expressed throughout the nervous, vascular and hepatic systems (Hague et al., 2003; Koshimizu et al., 2003). They respond mainly to the catecholamines noradrenaline and adrenaline, however the three α-1AR subtypes (α-1A, α-1B and α-1D) differ in their affinities to other circulating ligands, and particularly for their antagonists (Piascik and Perez, 2001; Marrow and Crease, 1986; Michelotti et al., 2000; Docherty, 1998). Increased expression of α-1AR on sensory nerve fibres, keratinocytes and smooth muscle cells in the vasculature of CRPS patients indicates that its atypical expression may contribute to disease pathogenesis (Kurvers et al., 1995; Arnold et al., 1993; Drummond et al., 1996; Drummond et al., 2014a; Drummond et al., 2014b). Other studies also show α1-AR may be a target for autoantibodies during the breakdown of immunological tolerance following injury, with an association of polymorphism within the α-1AAR subtype, and also certain HLA genotypes, also suggesting a genetic susceptibility (Dubuis et al., 2014; Heryln et al., 2010). With contradictory reports of their expression throughout the immune system in health, confirmed expression in chronic inflammatory diseases such as JRA and asthma suggest their role in the immune system may be confined to a pathologic state (Heijnen et al., 1996; Rouper van der Voort et al., 2000; Wahle et al., 2006; Goebel et al., 2010).
2. Hypothesis and Aims

As CRPS patients commonly present with persisting inflammation and are already known to overexpress α-1AR in nociceptors and keratinocytes, this study investigated whether α-1AR is also abnormally expressed in the immune system of CRPS patients, by analysing α-1AR expression by leukocytes isolated from the peripheral blood. The leukocyte profile in CRPS and non-CRPS individuals was also assessed, as a shift in distribution or numbers of leukocytes could provide further evidence for a role of inflammation in CRPS.

2.1. Project hypothesis

The basic hypothesis for this project is that CRPS patients will have altered α-1AR subset expression in their PBMCs, and show an altered or more inflammatory PBMC subset distribution, compared to non-CRPS healthy individuals.

2.2. Project aims

To determine a potential link between α-1AR expression on leukocytes and the development of CRPS, the overall aim of this project was to examine the expression of α-1AR on peripheral blood mononuclear cells (PBMC) of patients with CRPS, and to examine the distribution of sub-populations of PBMCs in these patients.
The specific aims of this project were:

1. To determine the expression of \( \alpha_{1A}AR, \alpha_{1B}AR \) and \( \alpha_{1D}AR \) mRNA using quantitative polymerase chain reaction (qPCR) in total PBMCs from a range of healthy individuals.

2. To compare the expression levels of \( \alpha_{1A}AR, \alpha_{1B}AR \) and \( \alpha_{1D}AR \) mRNA in total PBMCs from healthy individuals with those of patients diagnosed with CRPS.

3. To compare the distribution of PBMC subsets in healthy individuals with those of CRPS patients using multi-parameter flow cytometry.
3. Methods and Materials

3.1. Participants

Subject participation was entirely voluntary and was approved by the Murdoch University Human Research Ethics Committee (Approval 2014 27).

3.1.1. CRPS patients

Study participants included twenty CRPS patients who presented for clinical evaluation and treatment at the practice of Dr. Phil Finch (MB BS, DRCOG, FFPMANZCA) and/or the Centre for Research on Chronic Pain and Inflammatory Diseases at Murdoch University. All CRPS patients met published International Association for the Study of Pain (IASP) diagnostic criteria for the disorder (Harden and Bruehl, 2005). Patients were allocated a CRPS severity score (CSS) based on their clinical evaluation (Harden et al., 2010). The CSS was determined by appointing a score of 1 for the presence or a score of 0 for the absence for the symptoms listed in Table 3.1, using the clinical evaluation sheet and questionnaire documented in Appendix 1.

3.1.2. Healthy controls

A group of twenty non-CRPS healthy volunteers from around the Perth metropolitan region were age- and sex-matched to the CRPS patients. Controls were considered healthy where there was no previous history of chronic pain disorders, chronic inflammatory disorders and/or autoimmune disorders, the participant was not currently taking anti-inflammatory medication, antibiotics,
corticosteroids and/or immunosuppressive medication, had not had any vaccinations within the week prior to blood collection and were not having any flu-like symptoms or feeling ill on the day of blood collection.

<table>
<thead>
<tr>
<th>Self reported symptoms (Recorded by questionnaire)</th>
<th>Signs observed by examination (Recorded by clinical examination)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Allodynia</td>
<td>8. Allodynia</td>
</tr>
<tr>
<td>-Patient asked if increased pain to light and heavy stimulus</td>
<td>-Brush device, pressure/pain threshold</td>
</tr>
<tr>
<td>2. Temperature asymmetry</td>
<td>9. Temperature asymmetry</td>
</tr>
<tr>
<td>-Patient asked if affected limb feels colder or warmer than unaffected</td>
<td>-Temperature gauge</td>
</tr>
<tr>
<td>3. Skin colour asymmetry</td>
<td>10. Skin colour asymmetry</td>
</tr>
<tr>
<td>-Patient’s observation</td>
<td>-Examiner’s observation</td>
</tr>
<tr>
<td>4. Asymmetric oedema</td>
<td>11. Sweating asymmetry</td>
</tr>
<tr>
<td>-Patient’s observation</td>
<td>-Examiner’s observation</td>
</tr>
<tr>
<td>5. Trophic changes</td>
<td>12. Asymmetric oedema</td>
</tr>
<tr>
<td>-Patient’s observation</td>
<td>-Examiner’s observation</td>
</tr>
<tr>
<td>6. Motor changes</td>
<td>13. Trophic changes</td>
</tr>
<tr>
<td>-Patient asked if they experience dystonia, tremor or weakness in limb</td>
<td>-Examiner’s observation</td>
</tr>
<tr>
<td>7. Decreased range of motion</td>
<td></td>
</tr>
<tr>
<td>-In joints/ soft tissue/muscle</td>
<td></td>
</tr>
<tr>
<td><strong>Total score:</strong> 13</td>
<td></td>
</tr>
</tbody>
</table>

3.2. Blood collection

Subjects provided written informed consent before venipuncture. A sample of 12 mL of venous blood was obtained by a 21-gauge needle into lithium heparin anticoagulant vacutainers by a licensed phlebotomist either at Dr. Finch’s clinic or at Murdoch University in the School of Psychology and Exercise Science or School of Veterinary and Life Sciences. Blood was taken from the median cubital vein of the non-symptomatic limb of CRPS patients and the volunteers’ non-dominant limb. Lithium heparin was chosen as the optimal anticoagulant.
for both flow cytometry and RNA extraction. Blood was obtained in a sterile manner and subjects were rested and observed for a period of ten minutes after venipuncture. The blood sample was stored at room temperature until processing.

### 3.3. PBMC isolation from whole blood

Blood samples were stored for a maximum of 2 hours before processing. Whole blood was diluted 1:1 with phosphate-buffered saline (PBS) (Sigma-Aldrich, Castle Hill, NSW, Australia) and carefully layered, to prevent mixing of solutions, on ficoll separation solution Lymphoprep™ (STEMCELL Technologies Pty Ltd, Tullamarine, VIC, Australia) at a 3:1 ratio. The Lymphoprep™/diluted blood solution was centrifuged at 800 x g for 20 minutes at 21 °C with no break. Centrifugation produced a layered media based on the density of blood components as demonstrated in Figure 3.1. The buffy coat was extracted from the separated solution with a glass Pasteur pipette and transferred to a new Falcon™ tube. The extracted buffy coat was diluted 10:1 with PBS and centrifuged for 10 minutes at 250 x g at room temperature with a high break to wash and pellet the cells. After centrifugation, the supernatant was discarded and the cells were resuspended in 1 mL PBS for cell counting by haemocytometer.

![Figure 3.1: Separating diluted blood with Ficoll solution produces a density gradient of the components of blood. Plasma is the least dense and rises to the surface of the media, erythrocytes are the most dense and sink to the bottom. PBMCs form a layer just under plasma and were separated from the media using a glass Pasteur pipette.](image-url)
3.3.1. PBMC cell counts and viability analysis

To count viable cells, 10 µL of resuspended PBMCs were diluted with 10 µL of 0.4% Trypan Blue (Sigma-Aldrich, Castle Hill, NSW, Australia) solution and 10 µL of the PBMC/Trypan Blue solution was loaded into a haemocytometer chamber and examined immediately at 40 x magnification under a light microscope. Viable (live) cells excluded the Trypan Blue dye and appeared clear, whereas dead cells accumulated Trypan Blue in the cytoplasm and appeared blue under the microscope. An average of total cell count (live + dead) and live count was taken from the counts of the top left corner of the haemocytometer grid and the bottom right corner (see Figure 3.2) and multiplied x 10\(^4\) to account for the initial dilution factor and again x 2 for the Trypan Blue dilution factor. Cell viability was determined by live cell count divided by total cell count.

![Figure 3.2: Method of counting cells using haemocytometer.](image)

Figure 3.2: Method of counting cells using haemocytometer. Cells loaded onto the haemocytometer disperse throughout the grid. An average of the total count of all cells (live and dead) counted from the top left and bottom right grids was taken, as well as the total live cell count (live only). The counts were then multiplied by 10\(^4\) and 2. Viability was determined by dividing live cell count by total cell count.
3.3.2. PBMC lysis for RNA extraction and qPCR

Based on the total live cell count, one million cells were removed and centrifuged at 250 x g at room temperature for 10 minutes, resuspended and lysed in 350µL of Isolate II RNA Mini Kit Lysis Buffer RLY™ (Bioline Pty Ltd, Alexandria, NSW, Australia; see Appendix 2) + 3.5 µL 2-Mercaptoethanol (βME). Lysed cells were vigorously vortexed and stored in Lysis Buffer RLY at -80°C for later RNA extraction.

3.3.3. PBMC cryopreservation for flow cytometry analysis

After cell removal for RNA extraction, the remaining live PBMCs were centrifuged at 250 x g at room temperature for 10 minutes and resuspended in freezing medium at a concentration of 1 x 10^6 live cells/ mL for cryopreservation. Freezing medium contained 10:1 heat inactivated Fetal Bovine Serum (FBS) (Serana, Bunbury, WA, Australia): dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Castle Hill, NSW, Australia). One mL aliquots (1 x 10^6 PBMCs) were added to 1.5 mL cryovials tubes, which were then stored overnight in Nalgene® Mr. Frosty™ (Thermo Fisher Scientific Pty Ltd, Scoresby, VIC, Australia) that contained 100% isopropyl alcohol at room temperature, at -80°C. The following day the vials were removed from Nalgene® Mr. Frosty™ and stored in liquid nitrogen.
3.4. α-1AR analysis of PBMCs by qPCR

3.4.1. RNA extraction and analysis

RNA was extracted from PBMCs following the Isolate II RNA Mini Kit protocol (Bioline Pty Ltd, Alexandria, NSW, Australia) (Figure 3.3). Reagents used in this protocol are listed in Appendix 2. Lysed cells that had been stored at -80°C in Lysis Buffer RLY (see Section 2.3.2) were thawed and filtered through an ISOLATE II collection tube filter to clear any cellular debris before the RNA binding conditions of the lysate were adjusted with 350 µL 70% ethanol. RNA was bound to a silica membrane by centrifugation at 11,000 x g for 30 seconds and desalted with membrane desalting buffer (MEM). The RNA was incubated with 10% DNase 90% DNase reaction buffer I for fifteen minutes at room temperature to digest genomic DNA, then washed once with wash buffer RW1 and twice with wash buffer RW2 by centrifugation before elution in 40 µL of RNase/DNase free water (Figure 2.3).

Eluted RNA was stored on ice until analysis by spectrophotometer NanoDrop™ 2000 (Thermo Fisher Scientific Pty Ltd, Scoresby, VIC, Australia) to measure nucleic acid concentration and blanked with 1 µL of RNase free water before, after and in-between samples. Two x 1 µL volumes of each sample were measured, recording the RNA concentration (ng/µL), A260/A280 and A260/A230 ratios, taking an average. If the difference between the samples were greater than 10%, a third sample was measured. The A260/A280 ratio is used to determine protein contamination of a nucleic acid sample whereas the A260/A230 ratio indicates the presence of organic contaminants such as
chaotropic salts. Pure RNA should have an A260/A280 ratio around 2.1 with an A260/A230 ratio above 1.8.

After RNA extraction and analysis, samples were stored in -80°C until cDNA synthesis.

Figure 3.3: RNA extraction protocol following Bioline Isolate II RNA mini kit protocol.
3.4.2. cDNA synthesis

As a first step for PCR analysis, complementary DNA (cDNA) was synthesised from extracted RNA using the SensiFAST cDNA Synthesis Kit™ (Bioline Pty Ltd, Alexandria, NSW, Australia). The kit contains 5 x concentrated TransAmp Buffer and Reverse Transcriptase. The 5 x TransAmp Buffer contains a series of anchored oligo dT that anneal to the poly-A tail found on the 3’ end of eukaryotic mRNA and random hexamer primers to cover other segments of the RNA 3’ to 5’, randomly (see Appendix 3). This gives a cDNA pool representative of the transcriptome. The TransAmp Buffer also contains reverse transcription enhancers to assist the reverse transcriptase by reducing complex RNA secondary structure. The reactions were prepared to produce a total volume of 20 µL per cDNA sample (Table 3.2).

<table>
<thead>
<tr>
<th>Table 3.2: cDNA synthesis preparation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA</td>
</tr>
<tr>
<td>5 x TransAmp Buffer</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
</tr>
<tr>
<td>DNase/RNase free water</td>
</tr>
<tr>
<td>Total =</td>
</tr>
</tbody>
</table>

The amount of RNA used for cDNA synthesis was the same across all samples and was determined by the smallest concentration of RNA obtained from 15 µL (Total volume =20 - 4 µL Buffer - 1 µL RT) of the 40 (20 CRPS and 20 control) lysed PBMC subject samples using the following formula:

\[
n \text{ (µL)} = \frac{\text{Conc. of sample A (ng/µL)} \times 15 \text{uL}}{\text{Conc. of sample } n \text{ (ng/µL)}}
\]
Where sample A refers to the smallest RNA concentration obtained from all CRPS and healthy participants and sample \( n \) refers to the volume of RNA in question. For example, if the smallest concentration of RNA extracted was 20 ng/µL (i.e. sample A) and the concentration from sample \( n \) was 50 ng/µL:

\[
\frac{n \text{ (µL)}}{20 \text{ (ng/µL)}} = \frac{15 \text{ µL}}{50 \text{ (ng/µL)}}
\]

Then the volume of RNA needed from sample \( n \) for cDNA synthesis would be 6 µL. The remaining volume for cDNA preparation was topped up to 20 µL with RNase free/ DNase free water (i.e. 9 µL).

RNA extractions stored at -80 °C were thawed on ice. To ensure there was no DNA contamination from the RNA extractions, a negative reverse transcriptase control was included (- RT control). Where there was no reverse transcriptase in this sample, an extra 1 µL of water was added. The samples were run through the program showed in Table 3.3 through a T100™ Thermocycler (Bio-Rad, Gladesville, NSW, Australia). When finished, the 20 µL cDNA samples were stored at -20 °C for later qPCR analysis.
### Table 3.3: Thermocycler program for cDNA synthesis.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C</td>
<td>10 minutes</td>
<td>Primer annealing</td>
</tr>
<tr>
<td>42 °C</td>
<td>15 minutes</td>
<td>Reverse Transcription</td>
</tr>
<tr>
<td>85 °C</td>
<td>5 minutes</td>
<td>Inactivation</td>
</tr>
<tr>
<td>4 °C</td>
<td>Infinite</td>
<td>Hold</td>
</tr>
</tbody>
</table>

#### 3.4.3. qPCR analysis of α1-AR in PBMCs

Quantitative-PCR (qPCR) analysis followed the SensiFAST™ Probe No-ROX (Bioline Pty Ltd, Alexandria, NSW, Australia) kit protocol. The kit contained 2 x Sensifast No-ROX master mix that included dNTPs, stabilisers and enhancers. External pre-made TaqMan® Gene Expression Assays were purchased from In Vitro Life Technologies (Noble Park North, VIC, Australia) for α-1AAR (cat #Hs00169124_m1), α-1BAR (cat #Hs00171263_m1) α-1DAR (cat #Hs00169865_m1) and UBE2D2 (Hs00366152_m1). UBE2D2 was used as an internal control and house-keeping gene for each of the samples tested. It encodes the gene for the ubiquitin-conjugating enzyme E2 D2 that functions in the ubiquitination of the tumour-suppressor protein p53 and is widely used as a house-keeping gene for human PBMC qPCR analysis (Hollams et al., 2009). In Vitro TaqMan assays contain a probe, forward and reverse primers at 20 x concentration, thus for a 20 µL sample, only 1 µL of assay was used. cDNA samples were diluted 1:10 with RNase/DNase free water. The qPCR samples were set up in duplicates as shown in Table 3.4 and run Rotor-Gene® Q (Qiagen, Chaldstone Centre, VIC, Australia) with the program shown in Table 3.5.
Table 3.4: qPCR reaction preparation.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x Sensifast No-ROX mix</td>
<td>10 µL</td>
</tr>
<tr>
<td>20 x Taqman Primer</td>
<td>1 µL</td>
</tr>
<tr>
<td>Diluted cDNA template</td>
<td>4 µL</td>
</tr>
<tr>
<td>RNase/DNase free water</td>
<td>5 µL</td>
</tr>
</tbody>
</table>

Table 3.5: qPCR program for Rotor-Gene Q.

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95 °C</td>
<td>2 minutes</td>
<td>Polymerase activation</td>
</tr>
<tr>
<td>40</td>
<td>95 °C</td>
<td>10 seconds</td>
<td>Denaturation</td>
</tr>
<tr>
<td></td>
<td>60 °C</td>
<td>30 seconds</td>
<td>Annealing/extension (acquired at end of step)</td>
</tr>
</tbody>
</table>

3.4.4. Optimisation of TaqMan Assays

To ensure that TaqMan assays were functional, the fluorescent signal was measured from confirmed positive and negative controls for each of the assays.

3.4.4.1. α-1A AR optimisation using the CHO-α-1AAR cell line.

The CHO-α-1AAR cell line is an epithelial cell line derived from Chinese Hamster Ovary, transfected with the human α-1AAR gene. These cells express high levels of human α-1AAR protein on their cell surface (a gift from Prof. Roger Summer at Monash University). Non-transfected CHO cells were used as a negative control, although non-transfected CHO cells are known to express low levels of endogenous hamster α-1AAR.
3.4.4.2. \(\alpha_{1B}AR\) and \(\alpha_{1D}AR\) optimisation in HaCat cells

The HaCat cell line (DKFZ-German Cancer Research Centre) is a spontaneously immortalised human keratinocyte cell line that expresses the human \(\alpha_{1B}AR\) and \(\alpha_{1D}AR\) subtypes after exposure to TNF alpha and were kindly provided by Linda Wijaya. Non-template controls were used as the negative control.

3.4.4.3. \(UBE2D2\) optimisation in PBMCs

\(UBE2D2\) is widely used as a house-keeping gene particularly in PBMCs (Hollams et al., 2009). cDNA converted from RNA isolated from PBMCs from one CRPS patient and one healthy control were used in optimisation of \(UBE2D2\). A non-template control was used as a negative control.

3.4.5. qPCR and \(\alpha_{1}AR\) analysis

\(\alpha_{1}AR\) expression was determined by cycle threshold (Ct) values that represent the number of cycles needed for a fluorescent signal to cross a threshold. This was produced using Rotor-Gene® Q software. Rotor-Gene® Q software output were analysed by GraphPad prism (version 7.0a).

3.5. Analysis of PBMC cellular composition by flow cytometry

3.5.1. Thawing of cryopreserved PBMC

Prior to analysis, at least \(1 \times 10^6\) cells (~1 vial) were thawed from liquid nitrogen by immediate exposure to a 37°C water bath. Once liquefied, they were immediately diluted 1:10 in 9:1 PBS:FBS fluorescence activated cell sorter
(FACS) buffer. Diluted PBMCs were then centrifuged for 10 minutes at 250 x g at room temperature to pellet the cells. The supernatant was discarded and cells were resuspended in 1 mL FACS buffer. To determine cell viability, cells were counted using Trypan Blue as described in Section 3.3.1.

3.5.2. Identification of PBMC subsets using fluorochrome conjugated monoclonal antibodies and flow cytometry

Identification of PBMC subsets was based on the ability of specific monoclonal antibodies, conjugated to specific fluorophores, to bind to unique combinations of cell surface proteins expressed by each subpopulation of PBMCs. Activation of the fluorophores by appropriate lasers of different wavelengths (blue, red or violet) will cause them to emit fluorescence signals of different wavelengths, which can then be measured and converted to digital signals by light detectors within a flow cytometer. The flow cytometer used for this study was able to assess a combination of 10 different fluorophores per sample. PBMC subsets were analysed by staining cells with a cocktail of 8 monoclonal antibodies linked to different fluorophores contained in the Beckman Coulter Duraclone IM Phenotyping Basic Tube™ (Lane Cove, NSW, Australia), as well as the addition of BD Horizon™ BV421 Mouse Anti-Human CD25 (North Ryde, NSW, Australia) and viability dye 7-Amino-Actinomycin D (7AAD) (Beckman Coulter, Lane Cove, NSW, Australia). A summary of the fluorochromes and their markers is shown in Table 3.6.
Table 3.6: Monoclonal antibodies from Duraclone panel and their associated fluorochrome.

<table>
<thead>
<tr>
<th>Laser</th>
<th>Fluorochrome</th>
<th>Marker</th>
<th>Excitation</th>
<th>Emission peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>FITC</td>
<td>CD16</td>
<td>488 nm</td>
<td>525 nm</td>
</tr>
<tr>
<td>Blue</td>
<td>PE</td>
<td>CD56</td>
<td>488 nm</td>
<td>575 nm</td>
</tr>
<tr>
<td>Blue</td>
<td>ECD</td>
<td>CD19</td>
<td>488 nm</td>
<td>613 nm</td>
</tr>
<tr>
<td>Blue</td>
<td>7AAD</td>
<td>Viability</td>
<td>488 nm</td>
<td>650 nm</td>
</tr>
<tr>
<td>Blue</td>
<td>PE-Cy7</td>
<td>CD14</td>
<td>488 nm</td>
<td>720 nm</td>
</tr>
<tr>
<td>Red</td>
<td>APC</td>
<td>CD4</td>
<td>633 nm</td>
<td>660 nm</td>
</tr>
<tr>
<td>Red</td>
<td>A700</td>
<td>CD8</td>
<td>633 nm</td>
<td>720 nm</td>
</tr>
<tr>
<td>Red</td>
<td>APC-A750</td>
<td>CD3</td>
<td>633 nm</td>
<td>783 nm</td>
</tr>
<tr>
<td>Violet</td>
<td>BV421</td>
<td>CD25</td>
<td>405 nm</td>
<td>421 nm</td>
</tr>
<tr>
<td>Violet</td>
<td>Krome Orange</td>
<td>CD45</td>
<td>405 nm</td>
<td>528 nm</td>
</tr>
</tbody>
</table>

3.5.3. Monoclonal antibody staining of PBMCs

Each Duraclone tube contained a panel of 8 fluorochrome-conjugated monoclonal antibodies (CD3, CD4, CD8, CD14, CD16, CD19, CD45, CD56) that had been previously titrated and optimised by Beckman Coulter™. In addition, 5 µL of CD25-BV421 was added to the antibody cocktail prior to the addition of PBMCs. A single Duraclone + CD25 tube was used for each sample. Cells stained with single antibodies were also used for compensation setting and cytometer setup. For analysis, 100,000 live cells (~120 µL to 150 µL cells) were added to each of the Duraclone + CD25 tubes, then vortexed and incubated in the dark for 15 minutes. Samples were then washed with 1 mL FACS buffer and centrifuged at 300 x g for 5 minutes at 6 °C. The supernatant was discarded and labelled PBMCs were then resuspended in 500 µL FACS buffer. At this point, 10 µL of the 7AAD viability dye was added to the resuspended sample.
3.5.4. Single antibody staining for flow cytometer compensation settings

For each of the antibodies in the Duraclone panel, a single stain control tube was provided as part of the Duraclone kit, with 5 µl of each added to individual clean FACS tubes. For the CD25-BV421 single stain control, 5 µL was added to a clean FACS tube. For single staining, 100,000 live PBMCs were added to each of the tubes, vortexed and incubated in the dark for 15 minutes at room temperature. Samples were washed with 1 mL FACS buffer and centrifuged at 300 x g for 5 minutes at 6 °C. The supernatant was discarded and single-stained PBMCs were resuspended in 500 µL FACS buffer.

The 7AAD single stain tube involved 100,000 live cells incubated in a clean FACS tube at -20°C for 30 minutes to induce apoptosis. 10 µL 7AAD was then added to the sample.

3.5.5. Analysis of PBMC subsets by flow cytometry

Resuspended PBMC (either labelled with the full Duraclone panel+CD25, or as single stain controls) were collected and analysed using a Beckman Coulter Gallios™ 10-colour flow cytometer equipped with a blue, red and violet laser, together with Kaluza™ acquisition software. Single stain samples were run first to adjust instrument and compensation settings prior to running cells stained with the full panel of markers. Cell surface fluorescence colour and mean fluorescence intensity (MFI) determine the type (i.e. which CD marker) and the amount (i.e. strength of expression) of antibody bound to the cell surface respectively. Percentage expression and MFI data was exported from the Kaluza
analysis software into Microsoft Excel and GraphPad Prism software for further analysis.

3.6. Statistics

Normality of the data was determined by a D’Agostino & Pearson test. For parametric values, statistical significance between groups was determined by unpaired t-test or analysis of variance (ANOVA). Correlation between variables was determined using Pearson’s correlation coefficient. For non-parametric values, statistical significance between groups was determined using a Mann-Whitney test. Two-tailed calculations were considered significant where \( p < 0.05 \). One-tailed calculations were used where there were directional hypotheses from previous studies and were considered significant where \( p < 0.05 \). Calculations were performed with the aid of statistical analysis software (GraphPad Prism version 7.0a).
4. Results

4.1. Participants

4.1.1. CRPS patients

Of the twenty CRPS patients studied in this project, 75% were female and 25% were male (Table 4.1). At the time of blood extraction, the average age was 51 years with the youngest aging 24 years and the oldest 70 years (standard deviation: 10.817 years; median age: 52). D’Agostino and Pearson tests of age and sex show this sample group was derived from a normal distribution. Surgery was the most common predisposing event (30%), followed by sprain (25%), fracture (20%), tendon damage (10%), hyperextension injury (5%), dislocation (5%) and crush injury (5%). The right side was affected in half of the cases, whereas upper extremity injuries were more common than the lower extremity (65% to 35%) (Table 4.1).

CRPS participants could not be categorised into CRPS type I or II due to several participants having inclusive nerve injury evaluation prior to this study. Patients were allocated a CRPS severity score (CSS) based on the number of symptoms they experienced (Appendix 1), following the International Association for the Study of Pain (IASP) diagnostic criteria (Harden and Bruehl, 2005; Harden et al., 2010). Symptoms were assessed, on a scale of 1 – 13, with the most severe CRPS case in this study scoring 12 and the least severe scoring 5. The duration of CRPS among patients was also documented, which ranged from as chronic as 21 years to as acute as 4 months.
4.1.2. Correlation analysis of variables

A D’Agostino and Pearson test showed the duration of disease, degree of severity and the age of CRPS patients were derived from a normal distribution and were assessed for correlation. This study showed no correlation between the duration of disease with the degree of severity \( (p = 0.100) \), the degree of severity and the age of the participant \( (p = 0.207) \) or the age of the participant with the duration of disease \( (p = 0.447) \).

4.1.3. Healthy controls

Healthy controls were age- and sex-matched with CRPS participants as summarised in Table 4.1. Age differences between CRPS patients and their matched healthy controls were no greater than five years, with the average age being 51.2 years (standard deviation: 11.674; median: 51.5) and a range of 21 to 74 years. The age of the healthy control group was derived from a normal distribution.
Table 4.1: Summary of patient demographics. Includes age and sex of patient, corresponding to the type of injury, extremity affected, severity score (CSS) and duration of disease. Patients were age-sex matched with healthy controls.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age</th>
<th>Sex</th>
<th>Extremity affected</th>
<th>Pre-disposing event</th>
<th>CRPS severity score</th>
<th>Duration (years)</th>
<th>Healthy control</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>50</td>
<td>F</td>
<td>R</td>
<td>Surgery</td>
<td>5</td>
<td>3.5</td>
<td>C1 47 F</td>
</tr>
<tr>
<td>P2</td>
<td>63</td>
<td>M</td>
<td>L</td>
<td>Fracture</td>
<td>7</td>
<td>16.5</td>
<td>C2 66 M</td>
</tr>
<tr>
<td>P3</td>
<td>62</td>
<td>F</td>
<td>L</td>
<td>Surgery</td>
<td>9</td>
<td>6</td>
<td>C3 61 M</td>
</tr>
<tr>
<td>P4</td>
<td>59</td>
<td>F</td>
<td>R</td>
<td>Sprain</td>
<td>8</td>
<td>7</td>
<td>C4 55 F</td>
</tr>
<tr>
<td>P5</td>
<td>53</td>
<td>F</td>
<td>R</td>
<td>Fracture</td>
<td>7</td>
<td>2</td>
<td>C5 52 F</td>
</tr>
<tr>
<td>P6</td>
<td>61</td>
<td>F</td>
<td>L</td>
<td>Sprain</td>
<td>9</td>
<td>20</td>
<td>C6 61 F</td>
</tr>
<tr>
<td>P7*</td>
<td>49</td>
<td>F</td>
<td>L</td>
<td>Sprain</td>
<td>*</td>
<td>19</td>
<td>C7 45 F</td>
</tr>
<tr>
<td>P8</td>
<td>42</td>
<td>F</td>
<td>R</td>
<td>Sprain</td>
<td>11</td>
<td>7</td>
<td>C8 46 F</td>
</tr>
<tr>
<td>P9</td>
<td>60</td>
<td>F</td>
<td>L</td>
<td>Surgery</td>
<td>7</td>
<td>9</td>
<td>C9 56 F</td>
</tr>
<tr>
<td>P10</td>
<td>33</td>
<td>F</td>
<td>R</td>
<td>Surgery</td>
<td>9</td>
<td>6</td>
<td>C10 33 F</td>
</tr>
<tr>
<td>P11</td>
<td>52</td>
<td>M</td>
<td>L</td>
<td>Crush injury</td>
<td>9</td>
<td>21</td>
<td>C11 52 M</td>
</tr>
<tr>
<td>P12</td>
<td>45</td>
<td>F</td>
<td>L</td>
<td>Hyper-extension</td>
<td>9</td>
<td>0.3</td>
<td>C12 47 F</td>
</tr>
<tr>
<td>P13</td>
<td>70</td>
<td>M</td>
<td>L</td>
<td>Dislocation</td>
<td>5</td>
<td>3.5</td>
<td>C13 74 M</td>
</tr>
<tr>
<td>P14</td>
<td>53</td>
<td>F</td>
<td>L</td>
<td>Sprain</td>
<td>9</td>
<td>8</td>
<td>C14 54 F</td>
</tr>
<tr>
<td>P15</td>
<td>24</td>
<td>F</td>
<td>R</td>
<td>Tendon damage</td>
<td>8</td>
<td>6</td>
<td>C15 21 F</td>
</tr>
<tr>
<td>P16</td>
<td>42</td>
<td>M</td>
<td>R</td>
<td>Surgery</td>
<td>10</td>
<td>17</td>
<td>C16 42 M</td>
</tr>
<tr>
<td>P17</td>
<td>52</td>
<td>M</td>
<td>R</td>
<td>Fracture</td>
<td>12</td>
<td>0.3</td>
<td>C17 50 M</td>
</tr>
<tr>
<td>P18</td>
<td>60</td>
<td>F</td>
<td>R</td>
<td>Surgery</td>
<td>10</td>
<td>19</td>
<td>C18 64 F</td>
</tr>
<tr>
<td>P19</td>
<td>48</td>
<td>F</td>
<td>L</td>
<td>Fracture</td>
<td>9</td>
<td>2</td>
<td>C19 47 F</td>
</tr>
<tr>
<td>P20</td>
<td>51</td>
<td>F</td>
<td>R</td>
<td>Tendon damage</td>
<td>10</td>
<td>2</td>
<td>C20 51 F</td>
</tr>
</tbody>
</table>

*Due to unforeseen circumstances, a CSS for this patient was not obtained.
4.2. PBMC isolation from whole blood

4.2.1. Cellular recovery

The cellular recovery (cells/ mL of whole blood) of total PBMCs isolated from the blood of CRPS and control participants is shown in Figure 4.1. As this sample group was derived from a normal distribution, an unpaired Student’s T-test confirmed there was a significant increase in the concentration of cells isolated from the blood of CRPS patients compared to healthy controls \( (p = 0.008) \). The average concentration of cells isolated from CRPS whole blood was \( 1.23 \times 10^6 \) (+/- SEM 0.058) cells/ mL of whole blood compared to \( 1.06 \times 10^6 \) (+/- 0.018) cells/ mL blood from healthy controls. CRPS participants P11 and P18 had the highest concentrations of PBMCs with \( 1.76 \) and \( 1.78 \times 10^6 \) cells/ mL, respectively. These two patients had a more chronic variation of CRPS, with duration of 21 and 19 years respectively.

4.2.2. PBMC viability by Trypan Blue exclusion

The viability of freshly isolated CRPS and healthy PBMCs, as measured by Trypan Blue exclusion, averaged 95.6% (+/- SEM 1.132) and 96.5% (+/- 0.374), respectively with no significant difference (Figure 4.2).

4.2.3. Relationship of cellular recovery with duration of disease, severity and age

Correlation analysis established there was no relationship between the PBMC concentration of CRPS with the duration of disease \( (p = 0.413) \); with the severity
of the condition \((p = 0.114)\) or with the age of the CRPS participant \((p = 0.439)\) in this study.

**PBMC Concentration of Whole Blood**

![Graph showing PBMC concentration of whole blood for CRPS and Healthy participants.](image)

**Figure 4.1:** Concentration of PBMCs (million per mL) of CRPS compared to healthy controls. CRPS PBMCs were isolated at a concentration of \(1.23 \times 10^6 \pm 0.058\) compared to health controls that was a concentration of \(1.059 \times 10^6 \pm 0.018\). P11 and P18 had the two highest PBMC concentrations.

**PBMC Viability Before Cryopreservation**

![Graph showing PBMC viability before cryopreservation for CRPS and Healthy participants.](image)

**Figure 4.2:** Viability of PBMCs once extracted from buffy coat of whole blood. Measured by Trypan Blue and the use of a haemocytometer. P15 had the lowest viability with 78% followed by P18 with a viability of 86%, although the majority of patient PBMC viability edged 95.6%. All control viability was consistent with 96.5%.
4.3. α-1AR expression analysis of PBMCs

4.3.1. RNA extraction and purity, and cDNA synthesis

The concentration (ng/µL) of total RNA extracted from 1 x 10^6 PBMCs varied substantially across CRPS participants and healthy controls, although overall there were no significant differences between CRPS and healthy controls (Figure 4.3). The average concentration of RNA extracted from CRPS PBMCs was 39.77 ng/µL (+/- SEM 3.899) compared to 34.66 ng/µL (+/- 2.591) from healthy control PBMCs.

The lowest concentration of RNA from any participant was 13.65 ng/µL extracted from healthy control C13. Therefore all samples were adjusted to 13.65 ng/µL of RNA, using the formulas shown in Section 3.4.2, prior to conversion to cDNA. As 15 µL of RNA was used for synthesis to cDNA, the amount of RNA needed for cDNA synthesis equated to 204.75 ng. The concentrations of RNA eluted from each CRPS and control participant, and the corresponding volumes needed for cDNA synthesis, are shown in Appendix 4.

In terms of RNA quality, the A260/A280 protein and DNA contamination ratio averaged at 2.124 (+/- SEM 0.023) for CRPS patients and 2.152 (+/- 0.029) for healthy controls, whereas the A260/A230 salt contamination ratio averaged at 1.394 (+/- 0.084) for CRPS and 1.311 (+/- 0.098) for controls (Figure 4.4).
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Total RNA Extracted from 1x10^6 PBMCs

- Figure 4.3: Total RNA extraction from one million PBMCs in CRPS (average 39.77 ng/µL (+/- SEM 3.899) and healthy individuals (average 34.66 ng/µL (+/- 2.591). There was no significant difference between the RNA concentrations from CRPS and healthy individuals \( p = 0.282 \), although the data is spread from 13.65 ng/µL and 80.55 ng/µL.

- Figure 4.4: RNA purity. A) A260/A280 ratio of RNA extractions representing protein/DNA contamination. RNA is considered pure above a ratio of 2.0. B) A260/230 ratio of RNA extractions representing salt contamination. RNA is considered pure above a ratio of 1.8.

4.3.2. Relationship between RNA concentration and age

Further analysis revealed that there was a significant correlation \( p = 0.042 \) between the concentrations of RNA extracted from 1 x 10^6 PBMCs to the
participants’ age, with a Pearson correlation coefficient of -0.323. The linear regression between the two variables is shown in Figure 4.5, using a regression model of RNA concentration of $-0.431 \times \text{age} + 59.35$, with a goodness of fit of $0.104$.

![Figure 4.5: Linear regression model of RNA concentration and the participants’ age shows a negative correlation. With a goodness of fit of 0.104, RNA concentration = 0.4313 x age + 59.35.](image)

### 4.3.3. $\alpha_1$-AR gene expression analysis by qPCR

For logistical reasons, analysis of $\alpha_1$-AR expression of all CRPS and control participants was divided into five separate qPCR runs over a three-day period. Each participant was tested for duplicate samples of $\alpha_1$AR$_A$, $\alpha_1$AR$_B$, $\alpha_1$AR$_D$ and UBE2D2 house-keeping gene expression. The qPCR runs were as listed in Table 4.2. Each run included a positive control for each of the $\alpha_1$AR TaqMan
assays (CHO-1A and HaCat cells – see Methods and Materials Section 3.4.4.), which had been optimised prior to participant analysis.

<table>
<thead>
<tr>
<th>Run</th>
<th>CRPS patient</th>
<th>Healthy Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td>P1 – P4</td>
<td>C1 – C4</td>
</tr>
<tr>
<td>Run 2</td>
<td>P5 – P8</td>
<td>C5 – P8</td>
</tr>
<tr>
<td>Run 3</td>
<td>P9, P11 – P13</td>
<td>C9, C11 – C13</td>
</tr>
<tr>
<td>Run 4</td>
<td>P14 – P17</td>
<td>C14 – C17</td>
</tr>
<tr>
<td>Run 5</td>
<td>P10, P18 – P20</td>
<td>C10, C18 – C20</td>
</tr>
</tbody>
</table>

4.3.3.1 TaqMan qPCR optimisation

To initially ensure that the commercial TaqMan assays were functional, fluorescent emission for each probe was measured by qPCR using confirmed positive and negative controls (CHO-1A cells for α-1\textsubscript{A}AR and HaCat cells for α-1\textsubscript{B}AR and α-1\textsubscript{D}AR). Fluorescence emissions of each of the positive and negative assay controls are shown on a linear scale (Figure 4.6A) and on a logarithmic scale (Figure 4.6B). The Ct value was calculated from the threshold positioned on the logarithmic graph above the negative control “background” fluorescence and during the exponential phase of amplification of the positive controls (Figure 4.6B). The threshold calculated was a value of 0.087 and was used consistently among all five experimental qPCR runs.

The following sections describe the separate fluorescent curves and Ct values for the α-1\textsubscript{A}AR, α-1\textsubscript{B}AR, α-1\textsubscript{D}AR and UBE2D2 house-keeping gene optimisations.
4.3.2.1.1. α-1AAR qPCR optimisation using CHO-1A cells

To confirm the use of CHO-1A cells as a positive control for α-1AAR, the Ct value for α-1AAR expression in transfected CHO-1A cells during optimisation was 22.31 with the Ct value for the non-transfected cell line (CHO) 32.02, confirming positive expression of human α-1AAR by the CHO-1A cells. A CHO-1A cDNA sample was subsequently included with all 5 of the qPCR runs as a positive control, where the average Ct value for α-1AAR expression across all 5 qPCR runs was 21.14 (+/- 0.740). Figure 4.6 shows the cycle curve for the positive CHO-1A compared to the non-transfected negative control CHO cells.

![Figure 4.6: Fluorescent signals for α-1AAR in positive CHO-1A cells and negative (non-transfected) CHO cells. Part A show the linear fluorescence of α-1AAR. Part B show the logarithmic fluorescence of α-1AAR in relation to the threshold. Ct values for α-1AAR in CHO-1A and CHO were 22.31 and 32.02, respectively.](image-url)
4.3.2.1.2. α-1\textsubscript{B}AR and α-1\textsubscript{D}AR qPCR optimisation using HaCat cells

To confirm the use of HaCat cells as a positive control for α-1\textsubscript{B}AR and α-1\textsubscript{D}AR, the cycle curves for α-1\textsubscript{B}AR and α-1\textsubscript{D}AR using HaCat cell cDNA are shown in Figures 4.7 and 4.8, respectively. The Ct value for α-1\textsubscript{B}AR and α-1\textsubscript{D}AR expression in HaCat cells during optimisation was 32.19 and 33.13 respectively calculated from the threshold value of 0.087, with no fluorescence detected using the negative non-template control. A positive α-1\textsubscript{B}AR and α-1\textsubscript{D}AR HaCat cDNA sample was included with every qPCR run, where the average Ct value for α-1\textsubscript{B}AR expression in HaCat cells across all 5 runs was 31.09 (+/- SEM 0.820) and 32.67 (+/- 0.750) for α-1\textsubscript{D}AR. As shown in Figures 4.7 and 4.8, the cycle curves for α-1\textsubscript{B}AR and α-1\textsubscript{D}AR confirmed no expression of these genes for the non-template control in either the linear or logarithmic scales.
Figure 4.7: Fluorescent signals for $\alpha_{1B}$AR in HaCat cells compared to the non-template control (NTC) HaCat cells which show no fluorescence. Part A show the linear fluorescence of $\alpha_{1B}$AR. Part B show the logarithmic fluorescence of $\alpha_{1B}$AR in relation to the threshold. The Ct value for $\alpha_{1B}$AR in HaCat cells was 32.19.
Figure 4.8: Fluorescent signals for $\alpha_{1D}$AR in HaCat cells compared to the non-template control (NTC) HaCat cells which show no fluorescence. Part A show the linear fluorescence of $\alpha_{1D}$AR. Part B show the logarithmic fluorescence of $\alpha_{1D}$AR in relation to the threshold. The Ct value for $\alpha_{1D}$AR in HaCat cells was 33.13.

4.3.2.1.3. UBE2D2 qPCR optimisation using PBMCs

The cDNA from PBMCs of CRPS participant P10 and control participant C10 was used for optimisation of the UBE2D2 house-keeping gene qPCR. The average Ct value for UBE2D2 expression in PBMCs was 28.21 for P10 and 28.88 for C10 calculated from the threshold as shown in Figure 4.9B. There was no expression of UBE2D2 in the non-template control (NTC) as shown in Figure 4.9A.
4.3.2.2. Analysis of UBE2D2 house-keeping gene expression by PBMCs

Expression of the UBE2D2 house-keeping gene was measured using duplicates of cDNA samples from all participants throughout the five qPCR assay runs. The Ct value was calculated as the cycle number of each sample at which the amplified signal crossed an arbitrary positive signal threshold that was consistent across all samples. The threshold line was determined from optimisation of the qPCR assays as described above and was set as a value of 0.087. The average Ct value for each of the qPCR runs is shown in Table 4.3, with a one-way ANOVA
test confirming no significant difference between each of the runs \( (p = 0.189) \).

An unpaired Student’s T test also established there was no significant difference in the Ct values of UBE2D2 between CRPS patients (28.63 +/- 0.135) and healthy controls (28.47 +/- SEM 0.127).

<table>
<thead>
<tr>
<th>Table 4.3 Average Ct values of UBE2D2 expression of PBMCs from both healthy controls and CRPS patients across the 5 qPCR runs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run</td>
</tr>
<tr>
<td>Average Ct value</td>
</tr>
</tbody>
</table>

### 4.3.2.3. **\( \alpha-1_A\)AR expression by PBMCs**

Of all the CRPS and control participants tested, only two samples (CRPS P15 and control C12) returned positive for \( \alpha-1_A\)AR expression. The P15 duplicate samples had Ct values of 33.85 and 34.4 whereas C12 had duplicate Ct values of 34.35 and 34.4. Figures 4.10 and 4.11 show the amplification curves, in both linear and logarithmic scales, of \( \alpha-1_A\)AR and UBE2D2 in C12 and P15, respectively. UBE2D2 Ct values for C12 were 28.08 and 28.07, and for P15 were 27.68 and 27.83.

### 4.3.2.4. **\( \alpha-1_B\)AR and \( \alpha-1_D\)AR expression by PBMCs**

No expression was observed for either \( \alpha-1_B\)AR or \( \alpha-1_D\)AR by PBMCs from either CRPS or healthy control participants.
Figure 4.10: α-1AAR amplification from PBMCs of C12. A) Shows the linear scale of fluorescent signal of α-1AAR compared to the house-keeping gene UBE2D2. B) Shows the logarithmic scale in relation to the threshold of 0.087. The Ct values of α-1AAR was 34.35 and 34.4 whereas UBE2D2 Ct values for C12 were 28.07 and 28.08.
Figure 4.11: α-1AAR amplification from PBMCs of P15. A) Shows the linear scale of fluorescent signal of α-1AAR compared to the house-keeping gene UBE2D2. B) Shows the logarithmic scale in relation to the threshold of 0.087. The Ct values of α-1AAR was 33.85 and 34.4 whereas UBE2D2 Ct values for P15 were 27.68 and 27.83.
4.4. PBMC subset analysis by flow cytometry

4.4.1. PBMC viability

4.4.1.1 *Thawed cryopreserved cell viability as assessed by Trypan Blue exclusion*

Initially, prior to flow cytometry analysis the viability of thawed, cryopreserved cells was determined by Trypan Blue exclusion. Using this method, the average viability of PBMCs isolated from CRPS patients once thawed after cryopreservation was 61.05% (+/- SEM 3.424), which was significantly lower than for PBMCs isolated from healthy controls after cryopreservation (70.45% +/- 2.427; p = 0.031) (Figure 4.12). Both groups were significantly less viable after cryopreservation when compared to viability before cryopreservation. Viability of CRPS PBMCs dropped from 95.6% (+/- 1.132) to 61.05% (+/-3.424) (p <0.0001). Viability of healthy control PBMCs dropped from 96.5% (+/- 0.3735) to 70.45% (+/- 2.427) (p <0.0001).
Figure 4.12: PBMC viability determined by Trypan Blue after cryopreservation from CRPS and healthy individuals. The average viability of CRPS PBMCs was 61.05%, which is significantly less than (p value: 0.0310) the viability of PBMCs from healthy controls (average: 70.45%).

4.4.1.2. Thawed cryopreserved cell viability as assessed by 7AAD staining and flow cytometry

As an alternative to Trypan Blue staining for assessing cell viability, the antibody panel design allowed a 7AAD viability dye to be included to assess cell viability during flow cytometry acquisition. Viability stained by 7AAD was significantly different ($p <0.0001$) to that stained by Trypan Blue and recorded by the haemocytometer as shown in Table 4.4. Using the 7AAD stain and flow cytometry, there was no significant difference in the viability between CRPS patient and control samples: the viability of CRPS PBMCs was 84.02% (+/- SEM 3.414) and healthy control PBMCs was 86.10% (+/- 1.548) (Figure 4.15). However, there was one obvious outlier; P18 (24.58% viable), which was subsequently excluded from further analysis. Figure 4.13 depicts the viability of PBMCs of the CRPS and healthy control groups once the outliers were removed. The average viability of PBMCs without outliers was 87.15% (+/- 1.442) for CRPS and 86.10% (+/- 1.548) for healthy controls.
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Figure 4.13: Viability of PBMCs after cryopreservation with and without outlier measured by 7AAD. P18 PBMCs after cryopreservation were only 24.58% viable and were removed from data analysis.

4.4.2. Optimisation of the flow cytometry multi-colour antibody panel

4.4.2.1. Fresh PBMCs vs. frozen PBMCs

For logistical reasons, ficoll-separated PBMCs were required to be stored frozen prior to analysis by flow cytometry. To assess the effect of freezing and thawing on the distribution of PBMC subsets, initially the forward scatter (FSC) and side scatter (SSC) of thawed cryopreserved PBMCs from one control (C10) were compared to that of PBMCs extracted from the same individual collected as fresh cells (Figure 4.14). This showed a slight increase in the proportion of monocytes from 6.25% to 10.40% after cryopreservation, with a slight decrease in the

<table>
<thead>
<tr>
<th></th>
<th>Viability after cryopreservation (%) (Trypan Blue)</th>
<th>Viability after cryopreservation (%) (7AAD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRPS</td>
<td>61.05 (+/-3.424)</td>
<td>84.02 (+/- 3.414)</td>
</tr>
<tr>
<td>Healthy control</td>
<td>70.45 (+/- 2.427)</td>
<td>86.10 (+/- 1.548)</td>
</tr>
</tbody>
</table>
proportion of lymphocytes from 82.80% to 81.10%. Thawed-cryopreserved monocytes became more tightly compacted around a lower SSC, with FSC remaining similar. More debris (low SSC/FSC) was evident in non-cryopreserved PBMCs compared to that of thawed-cryopreserved PBMCs, however there was a greater distinction between lymphocytes and debris in the sample that had not been cryopreserved compared to that of the thawed-cryopreserved sample (Figure 4.14).

![Figure 4.14: FSC and SSC of frozen and thawed PBMCs.](image)

Figure 4.14: FSC and SSC of frozen and thawed PBMCs. (A) in comparison to fresh (not frozen and thawed) PBMCs (B) of the same individual.

4.4.2.2. Adjusting compensation using single-stained controls

Prior to running the full antibody panel, cells stained with single antibodies were used to compensate for spectral overlap of fluorochromes, particularly those whose fluorescence was excited by the same laser. The fluorochromes FITC (CD16), PE (CD56), ECD (CD19), 7AAD (viability) and PE-Cy7 (CD14) all were excited by the blue laser; APC (CD4), A700 (CD8) and APC-A750 (CD3) by the red laser and BV421 (CD25) and Krome Orange (CD45) by the violet laser. Compensation values were set to remove spectral overlap for each antibody/fluorochrome into each fluorescent channel, as shown in Appendix 5.
4.4.3. Gating strategy for identification of PBMC populations

A schematic diagram showing the PBMC populations analysed and their surface markers is shown in Figure 4.15. Using this strategy, the electronic gating strategy for identification of these subpopulations in CRPS and control participant PBMC is shown in Figure 4.16. This gating strategy was applied to the PBMC isolated from all participants (excluding the one sample that was low viability as assessed by 7AAD staining as described above), although minor changes were applied to account for population shifts between acquisition runs. According to this strategy, cells were first gated for expression of CD45 (a pan-leukocyte marker) together with SSC to include granular cells (Fig. 4.16A), followed by gating for viable cells that were negative for 7AAD, which will only penetrate and label non-viable cells (Fig. 4.16B). Monocytes were then identified as CD14+ SSC\textsuperscript{high} (Fig. 4.16C) which were then separated into three populations (classical, intermediate and non-classical) based on CD16 and CD14 expression: CD14\textsuperscript{+} CD16\textsuperscript{-} monocytes represent “classical” monocytes, whereas CD14\textsuperscript{+} CD16\textsuperscript{+} monocytes were split into two populations – CD14\textsuperscript{high} “intermediate” monocytes and CD14\textsuperscript{low} “non-classical” monocytes (Fig. 4.16D) (Wong et al., 2012). The remaining PBMC populations were then gated as all cells that were CD14\textsuperscript{-} “non-monocytes” (Fig. 4.16C). As shown in Figure 4.16E, B cells were identified by the expression of CD19 and not the pan-T cell marker CD3 (CD19\textsuperscript{+} CD3\textsuperscript{-}). From this point, T cells were identified based on CD3 expression, but not CD56 (CD3\textsuperscript{+} CD56\textsuperscript{-}), NK cells were identified based on CD56 expression and not CD3 (CD56\textsuperscript{+} CD3\textsuperscript{-}) and NKT cells expressed both CD3 and CD56 (CD3\textsuperscript{+} CD56\textsuperscript{-}) (Fig. 4.16F). The CD3\textsuperscript{+} CD56\textsuperscript{-} T cells were then separated on the basis
of CD4 or CD8 expression into CD8$^+$ CD4$^-$ T cells (CD8$^+$ T cells), CD4$^+$ CD8$^+$ T cells (CD4$^+$ T cells) and CD4$^+$ CD8$^+$ T cells (double positive (DP) T cells) (Fig. 4.16I). For both CD4$^+$ and CD8$^+$ T cells, the activation marker CD25 was also examined, identifying CD25$^+$ activated T cells but also a potential population of “regulatory” T cells that express CD25 (CD4$^+$ T$_{reg}$ and CD8$^+$ T$_{reg}$) (Fig. 4.16H). However, accurate identification of T$_{reg}$ cells requires additional markers (e.g. FoxP3), which were not possible to incorporate in this panel due to limitations on the number of fluorochromes that could be used with the Gallios flow cytometer.

Finally, CD56$^+$ CD3$^-$ NK cells (Fig. 4.16F) were further separated based on levels of CD56 expression into CD56$^{bright}$ and CD56$^{dim}$ NK cells (Fig. 4.16G).
Figure 4.15: PBMC population sorting based on cell surface CD markers. CD45 is a standard leukocyte marker and is commonly used to separate leukocytes/PBMCs from whole blood. 7AAD negative cells isolated viable CD45 leukocytes. Monocytes express CD14 and varying levels of CD16 that further differentiates monocyte populations. CD14- cells account for lymphocytes, NK cells and NKT cells. CD19+ lymphocytes isolate B cells, where as CD19- separates all other cell types. CD19- CD3+ lymphocytes are T cells, which are further differentiated into CD4+ helper T cells, CD8+ cytotoxic T cells and CD4 CD8 double positive T cells. T regulatory cells stem off of either CD4 or CD8 and express CD25. CD19- CD3+ CD56+ marks NKT cells, whereas NK cells are CD19- cells that only express CD3. NK cells can further be differentiated dependent upon level of expression of CD56.
Figure 4.16. Flow cytometry gating strategy from Kaluza analysis software used to determine PBMC populations as following Figure 4.15.
### 4.4.4. Analysis of PBMC populations in CRPS and control participants

Table 4.5 shows an overview of the percentage composition of PBMCs, to be discussed throughout this section, in CRPS patients and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>CRPS (% of PBMCs)</th>
<th>Healthy (% of PBMCs)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n value</td>
<td>19</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Viable cells (with outliers)</td>
<td>84.02 (+/- 3.414)</td>
<td>86.1 (+/-1.548)</td>
<td>0.5834</td>
</tr>
<tr>
<td>Viable cells (minus outliers)</td>
<td>87.15 (+/- 1.442)</td>
<td>86.1 (+/- 1.548)</td>
<td>0.6220</td>
</tr>
<tr>
<td>Monocytes</td>
<td>13.52 (+/- 0.8819)</td>
<td>14.75 (+/- 1.102)</td>
<td>0.3951</td>
</tr>
<tr>
<td>B cells</td>
<td>14.61 (+/- 1.365)</td>
<td>12.77 (+/- 1.135)</td>
<td>0.3031</td>
</tr>
<tr>
<td>Total T cell</td>
<td>48.12 (+/- 1.941)</td>
<td>49.3 (+/- 1.433)</td>
<td>0.6250</td>
</tr>
<tr>
<td>CD4 T cell</td>
<td>31.73 (+/- 1.662)</td>
<td>32.56 (+/- 1.642)</td>
<td>0.7235</td>
</tr>
<tr>
<td>CD8 T cell</td>
<td>14.32 (+/- 0.9749)</td>
<td>14.27 (+/- 1.157)</td>
<td>0.9721</td>
</tr>
<tr>
<td>NK cells</td>
<td>11.29 (+/- 1.116)</td>
<td>11.71 (+/- 0.6905)</td>
<td>0.7488</td>
</tr>
<tr>
<td>NKT cells</td>
<td>5.838 (+/- 1.334)</td>
<td>4.342 (+/- 0.6668)</td>
<td>0.3149</td>
</tr>
<tr>
<td>Ratio non monocytes: monocytes</td>
<td>7.015 (+/- 0.5687)</td>
<td>6.863 (+/- 0.8768)</td>
<td>0.8868</td>
</tr>
</tbody>
</table>

#### 4.4.4.1. Monocytes

Total monocytes (CD14⁺ SSC<sup>high</sup>) from CRPS patients constituted a mean of 13.52% (+/- 0.882) of total PBMCs, compared to healthy controls, where 14.75% (+/- 1.105) of PBMCs were identified as total monocytes (Figure 4.17). Based on an unpaired Student’s T-test there was no significant difference between the percentage of monocytes in CRPS patients when compared to healthy controls.
Figure 4.17: Percentage of monocytes from total PBMCs in CRPS patients and healthy controls. A two-tailed T test showed no statistical significance of the monocyte percentage from CRPS patients (13.52%) when compared with healthy controls (14.75%).

As described above (Figure 4.16) monocytes were divided into classical CD14$^+$ CD16$^-$ monocytes and CD14$^+$ CD16$^+$ monocytes. These CD16$^+$ monocytes were further divided into intermediate CD14$^{\text{high}}$ CD16$^+$ monocytes and non-classical CD14$^{\text{low}}$ CD16$^+$ monocytes (see Figure 4.16D). Classical CD14$^+$ CD16$^-$ monocytes represented the majority of monocytes constituting 88.83% (+/- 1.016) of CRPS monocytes and 90.19% (+/- 1.389) of healthy controls monocytes. Intermediate CD14$^{\text{high}}$ CD16$^+$ monocytes were the next most frequent monocyte representing 8.97% (+/-0.841) of monocytes in CRPS patients compared to 7.53% (+/-0.987) in healthy controls. Non classical CD14$^{\text{low}}$ CD16$^+$ monocytes were the least frequent monocyte, constituting only 1.94% (+/- 0.264) and 1.76% (+/- 0.498) in CRPS patients and healthy controls, respectively. As a previous study showed a significant decrease in the number of classical CD14$^+$
CD16⁻ to CD14⁺ CD16⁺ (CD14<sup>high</sup> CD16⁺ and CD14<sup>low</sup> CD16⁺) monocytes, statistical significance was analysed by a one-tailed unpaired T-test (Ritz et al., 2011). This revealed no statistical significance in the subpopulations of CD14⁺ CD16⁻, CD14<sup>high</sup> CD16⁺ and CD14<sup>low</sup> CD16⁺ between CRPS patients and healthy controls (Table 4.6). However, when the ratio of CD14⁺ CD16⁻ to CD14⁺ CD16⁺ was assessed in this study, there were 9.74 (+/- 1.035) times more CD16⁻ monocytes to CD16⁻ monocytes in CRPS patients, with a higher ratio (13.93 +/- 1.84) of CD16⁻ monocytes to CD16⁺ monocytes in healthy controls (Figure 4.18). A one-tailed unpaired T test confirmed this was statistically significant (p = 0.029). However, when statistically analysed using a two-tailed unpaired T test, it was determined to not be significantly different (p = 0.058) (Table 4.6).

<table>
<thead>
<tr>
<th>Table 4.6: Subpopulations and percentage of total monocytes.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14⁺ CD16⁻ classical</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>CD14⁺ CD16⁺ (intermediate + non classical)</td>
</tr>
<tr>
<td>CD14&lt;sup&gt;high&lt;/sup&gt; CD16⁺ intermediate</td>
</tr>
<tr>
<td>CD14&lt;sup&gt;low&lt;/sup&gt; CD16⁺ non classical</td>
</tr>
<tr>
<td>Ratio CD14⁺ CD16⁻: CD14⁺ CD16⁺</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Figure 4.18: The ratio of CD14+ CD16- classical monocytes to CD14+ CD16+ monocytes (intermediate and non classical type). A one-tailed T test confirmed there was a significant decrease ($p = 0.029$) in CRPS patients (ratio: 9.74) when compared with healthy controls (ratio: 13.93).
4.4.4.2. B cells

Of total viable CD45⁺ leukocytes, B cells were identified as CD19⁺ CD3⁻ as shown in Figure 4.16E. In CRPS participants, 14.61% (+/- 1.360) of PBMC were B cells compared to 12.77% (+/- 1.135) in healthy controls (Figure 4.19), which was not statistically significantly different as determined by unpaired Student’s T test.

**Total PBMC % - B cells**

![Graph showing percentage of B cells in PBMC from CRPS patients and healthy controls.](image)

*Figure 4.19: Percentage of B cells from total PBMCs from CRPS patients and healthy controls. No statistical difference shown. CRPS B cells constitute 14.61% of the PBMC population, whereas healthy control B cells constitute 12.77% of the PBMC population.*
4.4.4.3. Total T cells and T cell subsets

Total T cells were identified as CD3$^+$ CD56$^-$ after gating for viable, CD14$^-$ CD19$^-$ cells as shown in Figure 4.16F. The mean percentage of total T cells in PBMC of CRPS participants was 48.12% (+/- 1.941) compared to 49.30% (+/- 1.433) in healthy controls (Figure 4.20A), which was not statistically significantly different as determined by unpaired Student’s T test.

For T cell subsets, CD4$^+$ T cells constituted 32.56% (+/- 1.642) of total PBMC of healthy controls, compared to 31.73% (+/-1.662) for CRPS participants (Figure 4.20B). Additionally, CD8$^+$ T cells comprised 14.27% (+/- 1.157) of total healthy control PBMCs compared to 14.32% (+/- 0.975) in CRPS participants (Figure 4.20C). The differences between CD4$^+$ T cells and CD8$^+$ T cells in CRPS and control participants were not statistically significantly different as determined by unpaired Student’s T test.

The percentage compositions of CD4$^+$ T cells, CD8$^+$ T cells, CD4$^+$CD8$^+$ DP T cells, CD4$^+$ CD25$^+$ and CD8$^+$ CD25$^+$ within the total T cell populations are shown in Table 4.7. Again, as shown for percentages of total PBMC above, there was no significant differences for these T cell subsets between CRPS patients and healthy controls. In addition, there were 2.45 (+/- 0.230) times more CD4$^+$ T cells than CD8$^+$ T cells in CRPS participants, which was not statistically significantly different from healthy controls, who had a CD4:CD8 ratio of 2.61 (+/- 0.251) (Table 4.7).
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Results

Total, CD4 and CD8 T cells of PBMCs

Figure 4.20: Percentage of T cells in PBMC population. A) Total (CD3+ CD56-) T cell percentage in PBMCs from CRPS patients (48.12% +/- 1.941) and healthy controls (49.30% +/- 1.433) was not statistically significantly different. The T cell population was split into CD4 (B) and CD8 (C) T cells. These were also not statistically significantly different in CRPS patients (CD4: 31.73%; CD8: 14.32%) when compared to healthy controls (CD4: 32.56%; CD8: 14.27%).

Table 4.7: Summary of the types of T cells found in PBMCs and their composition within the T cell population (+/- SEM).

<table>
<thead>
<tr>
<th></th>
<th>CRPS (% of T cells)</th>
<th>Healthy (% of T cells)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ helper T cells</td>
<td>65.56 (+/- 2.120)</td>
<td>66.11 (+/- 2.487)</td>
<td>0.888</td>
</tr>
<tr>
<td>CD8+ cytotoxic T cells</td>
<td>30.04 (+/- 2.164)</td>
<td>28.84 (+/- 2.008)</td>
<td>0.687</td>
</tr>
<tr>
<td>CD4+ CD8+ double positive</td>
<td>0.76 (+/- 0.217)</td>
<td>0.72 (+/- 0.315)</td>
<td>0.923</td>
</tr>
<tr>
<td>CD4+ CD25+ T regulatory cells</td>
<td>2.65 (+/- 0.320)</td>
<td>2.13 (+/- 0.256)</td>
<td>0.215</td>
</tr>
<tr>
<td>CD8+ CD25+ T regulatory cells</td>
<td>3.93 (+/- 0.959)</td>
<td>2.58 (+/- 0.536)</td>
<td>0.228</td>
</tr>
<tr>
<td>CD4: CD8 ratio</td>
<td>2.45 (+/- 0.231)</td>
<td>2.61 (+/- 0.251)</td>
<td>0.656</td>
</tr>
</tbody>
</table>
4.4.4.4. NK cells

Total viable NK cells were identified as CD56+ CD3- after gating for CD19- and CD14- cells as shown in Figure 4.16F. Of CRPS patients, NK cells constituted 11.29% (+/- 1.116) of total PBMC compared to 11.71% (+/- 0.691) for healthy controls (Figure 4.21). As shown in Figure 4.16G, NK cells were further separated on the basis of CD56: CD56\textsuperscript{bright} NK cells, which had a mean fluorescence intensity (MFI) for CD56 of 67.55 (+/- 1.782) and; CD56\textsuperscript{dim} NK cells, which had a MFI for CD56 of 14.94 (+/- 0.485). The CD56\textsuperscript{dim} NK cells were more frequent than the CD56\textsuperscript{bright} NK cells in both CRPS patients and healthy controls, constituting 79.79% (+/- 2.188) and 79.88% (+/- 2.49) of total NK cells respectively (Figure 4.22A). The CD56\textsuperscript{bright} NK cells were less frequent, representing only 3.04% (+/- 0.509) and 2.60% (+/-0.436) of total NK cells in CRPS and control participants respectively (Figure 4.22B). The differences between NK cells and NK cell subsets in CRPS and control participants were not statistically significantly different as determined by unpaired Student’s T test.
Figure 4.21: Percentage of NK cells from total PBMCs. CRPS patients (11.29%) were not statistically significantly different from healthy controls (11.71%).

Figure 4.22: CD56 dim (A) and CD56 bright (B) subsets of NK cells. CD56 bright constitute the majority of NK cell population in both CRPS (79.79%) and healthy (79.88%) participants compared to CD56 bright NK cells (CRPS: 3.04%; healthy: 2.50%).
4.4.4.6. NKT cells

Total NKT cells were identified as CD56$^+$ and CD3$^+$ after gating for CD19$^-$ and CD14$^-$ cells as shown in Figure 4.16F. Of the total PBMC population of CRPS participants, 5.84% (+/- 1.334) were NKT cells compared to 4.34% (+/- 0.667) for control participants (Figure 4.23), which was not statistically different as determined by unpaired Student’s T test.

![Figure 4.23: Percentage of NKT cells from total PBMCs across all 36 samples acquired. The percentage of NKT cells within the PBMC population were not statistically significantly different in CRPS patients (5.84%) when compared with healthy controls (4.34%).](image-url)
5. Discussion

The aim of this project was to examine the expression of $\alpha$-1$\text{AR}$, $\alpha$-1$\text{B}$AR, and $\alpha$-1$\text{D}$AR mRNA in PBMCs isolated from whole blood of CRPS patients and healthy controls. The populations of PBMCs in the peripheral blood of CRPS patients and healthy controls were also assessed, and the relevant percentages of these populations were determined. One CRPS patient and one healthy control expressed low levels of $\alpha$-1$\text{AR}$ mRNA. No other study participant expressed $\alpha$-1$\text{B}$AR or $\alpha$-1$\text{D}$AR mRNA in their PBMCs. Analyses of PBMC populations showed there was an overall increase of the concentration of PBMCs isolated from whole blood of CRPS patients compared to healthy controls, and a shift towards an increase of the proportion of CD14$^+$ CD16$^+$ monocyte subpopulation was found. There was no significant difference of T cell subsets, B cells, NK cells or NKT cells between CRPS patients and healthy controls.

5.1. Demographics of CRPS participants

The CRPS patients in this study mirrored the characteristics of CRPS in previous studies. In this study, there were three times as many females studied than men (Sandroni et al., 2003; de Mos et al., 2007). The mean age of patients in this study was 51 years (± 10.82), where previous studies state CRPS patients commonly develop the condition between the ages of 37 and 52. However, the age of the patients in this study did not signify the age of the patient at diagnosis, rather that it was documented at the time of blood extraction. Therefore, if the
duration of disease in CRPS patients was accounted for, the average age of the
CRPS patients at the time of diagnosis was 42.7 years.

Fractures, sprains and surgery of a limb are the most likely events provoking
CRPS onset (Veldman et al., 1993; Harden et al., 2010). This was reflective
among the participants of this study, as 75% of CRPS patients reported one of
these as their predisposing event. Tendon damage, hyperextension injury,
dislocation and crush injury were among the other predisposing events in this
study. Nerve conduction tests are commonly used in CRPS diagnosis for
determination of nerve injury and distinguishing between CRPS types I and II
(Borchers and Gershwin, 2014). However, several patients from this study had
not had prior nerve conduction tests, thus subdividing patients into type I or type
II could not be done. It is suggested that CRPS patients who immobilise their
affected limb to assist in the healing of the injury are more likely to develop type
I than type II, thus this study may have been able to speculate patients who were
inconclusive of nerve injury but who had immobilised their limb to assist in
healing as CRPS type I (Borchers and Gershwin, 2014; Wei et al., 2016). In this
study, the upper extremity was affected in 60% of the cases compared to 40% of
lower extremity cases, which correspond with previous studies (de Mos et al.,
2009). No children or adolescent CRPS cases were examined in this project
where only rare cases (<10% of sufferers) have been reported previously (Wilder
et al., 1992). In summary, the CRPS patients studied in this project were
reflective of CRPS characteristics as described in previous studies.
5.1.1. The use of a clinical severity score in CRPS research

Classifying CRPS patients on the basis of the severity of their condition can be used as a diagnostic aid to support criteria developed by the International Association for the Study of Pain (IASP) (Harden and Bruehl, 2005). Although not extensively used, the CRPS severity score (CSS) has emerged as a useful tool in recent years and could aid researchers in developing a clearer pathophysiological understanding of CRPS (Harden et al., 2010). Patients in this study were evaluated and tested for 13 possible symptoms (Appendix 1). The most severe CRPS patient had 12 of these 13 symptoms, and the least severe had 5 of these 13 symptoms.

One limitation of this study is that these patients were assigned a CSS at a single time point at the time of blood extraction. Therefore, this only documented the severity at one time point of their condition and did not take into account how the patients’ condition, or the type of symptoms, may have changed over time. A longitudinal study that observed patients over six years report 30% of chronic CRPS patients experience resolution of disease, 16% report progressive deterioration and the remaining 54% report stable symptoms (de Mos et al., 2009). Thus it is expected that the nearly half of the CRPS participants studied in this project may have experienced a change in their condition since diagnosis. Additionally, another study reported that patients initially diagnosed with “warm type” CPRS are more likely to experience disease resolution than those diagnosed with “cold type” CRPS (Vaneker et al., 2005). “Warm type” CRPS is the most common presentation in the acute CRPS phase, and is consistent with an acute inflammatory response. Despite this, this study did not categorise
patients into warm type or cold type CRPS. If, in future, the CSS could be utiliséd to capture the time point at which CRPS patients may begin to experience disease resolution, or where the condition intensifies, we may be able to grasp a better understanding of how this inflammatory process may switch off and resolve, or progress to chronic CRPS.

5.1.2. Correlation between disease severity and duration

Although this study demonstrated no correlation between the duration of disease and disease severity as measured by CSS, this study was limited in its ability to accurately describe this relationship. In order to determine if there was a relationship between the severity of CRPS with its duration, the same patient would need to be monitored over a period of time. This study correlated the CSS of all CRPS patients with the varied disease durations. As half of CRPS patients are expected to experience a change in condition, and self-reported symptoms and pain ratings used in the CSS developed are subjective, it may be inaccurate to assume there was no correlation between severity and duration of disease (de Mos et al., 2009). To confirm this, a larger scale study would need to be conducted to assess the severity of several CRPS patients over an extended period of time.

5.1.3. Correlation between age and total RNA concentration

Correlation analysis from this study showed there was a negative relationship between a participant’s age and the amount of RNA extracted from $1 \times 10^6$ PBMCs. Previous studies suggest that this decline in total RNA levels corresponds to a decline in growth, protein synthesis and metabolic rate with age.
(Chomczynski et al., 2016; Tahoe et al., 2003). This decline in RNA concentration did not affect the outcome of this study as the volume of total RNA used to qPCR analysis was modified across all samples to allow the same concentration of RNA to be used. However, since low levels of $\alpha$-1AR were detected in only two participants of this study and no $\alpha$-1AR expression elsewhere, levels of $\alpha$-1AR may have been more detectable with a larger total RNA concentration. Therefore, using a greater concentration of total RNA for $\alpha$-1AR analysis of PBMCs may need to be used in future.

5.2. Analysis of $\alpha$-1AR mRNA expression by PBMCs in CRPS

Previous studies have shown an increase in density, or responsiveness of $\alpha$-1AR in keratinocytes and nociceptors of CRPS patients’ affected limb that are subject to sensitisation by circulating catecholamines and cytokines (Dawson et al., 2011; Drummond et al., 2014a; Drummond et al., 2014b). This likely contributes to the malfunctioning of the SNS and the release of neuropeptides that evoke vasodilation and protein extravasation, facilitating local inflammation (Borchers and Gerschwin, 2014; Birklein and Schlereth, 2015). Additionally, in other chronic inflammatory diseases, the expression of $\alpha$-1AR in leukocytes is upregulated (Ricci et al., 1999; Szentivanyi et al., 1979; Wahle et al., 1996; Heijnen et al., 1996). As CRPS patients commonly present with persisting inflammation, and as it is known that $\alpha$-1AR expression is upregulated in other cell types in CRPS, the expression of $\alpha$-1AR on PBMCs in CRPS was assessed. From this study, no difference in expression of $\alpha$-1AR mRNA of PBMCs from CRPS patients when compared to healthy controls was observed. Low levels of
**α-1\textsubscript{A}AR** mRNA expression of PBMCs in one CRPS patient and one healthy control, and no mRNA expression of α-1\textsubscript{B}AR or α1\textsubscript{D}-AR in any of the study participants were shown. The detection of α-1\textsubscript{A}AR from CHO-1A cells, and α-1\textsubscript{B}AR and α1\textsubscript{D}-AR from HaCat cells, during optimisation and throughout the qPCR acquisitions supported the validity of these results. Additionally, the A260/A280 ratios of the RNA samples were consistently above 2.0, indicating no protein or DNA contamination (Desjardins and Conklin, 2010). However, the A260/A230 ratio was inconsistent with the recommended 1.8 value. A ratio below 1.8 in this respect indicates salt contamination from the washing step of the RNA extraction and may affect RNA integrity to convert to cDNA (Desjardins and Conklin, 2010). As the house-keeping gene UBE2D2 showed consistent Ct value across the qPCR analysis in any sample, I was confident that any salt contamination of extracted RNA did not affect the quality of cDNA used in this study.

A confounding issue was the non-transfected CHO cell line used as a negative control for α-1\textsubscript{A}AR optimisation showed low levels of endogenous α-1\textsubscript{A}AR expression. The CHO cell line was derived from Chinese hamster ovary epithelium. Since the CHO-1A cell line is transfected with the human α-1\textsubscript{A}AR gene, this primer may have detected a conserved genetic sequence of the hamster and human α-1\textsubscript{A}AR gene in the non-transfected CHO cell negative control. The level of α-1\textsubscript{A}AR expression in CHO cells compared to CHO-1A cells was substantially lower, thus it was used as a negative control. It would have been more appropriate to use a non-template control (NTC). However, this cDNA was a gift from another laboratory and we were not provided with a NTC alternative.
Despite these concerns, I was confident that the primers used in this study specifically detected $\alpha$-1AR of PBMCs from CRPS patients and healthy controls.

As there was no significant difference in $\alpha$-1AR mRNA expression between CRPS patients and healthy controls in this study, I concluded that $\alpha$-1AR mRNA expression of PBMCs was independent from CRPS. However, two female participants (one CRPS and one control) did show $\alpha$-1AR expression. The CRPS participant was 24 years of age and the healthy control was 45 years of age. As there were several other participants within these age ranges, and the majority of participants were female, I also concluded that the expression of $\alpha$-1AR in PBMCs was independent of age and sex in this study. In order to confirm this however, a larger number of samples would need to be analysed, which was beyond the scope of this project.

### 5.2.1. Expression of $\alpha$-1AR subtypes in PBMCs

Previous studies assessing the expression of $\alpha$-1AR in leukocytes showed mixed results (Casale and Kaliner, 1984; Kavelaars, 2002; Rouppe van der Voort et al., 2000). The majority of studies suggested that there was no baseline expression of $\alpha$-1AR in leukocytes; rather that it is only expressed following an inflammatory stimulus or within chronic inflammatory diseases (Ricci et al., 1999; Szentivanyi et al., 1979; Wahle et al., 1996; Heijnen et al., 1996). Although this project predicted that CRPS might have been an example of a chronic inflammatory...
disease provoking the expression of $\alpha$-1AR in PBMCs, this hypothesis was not supported.

In order to fully assess $\alpha$-1AR expression by PBMCs of CRPS patients, it may be necessary to identify the inflammatory stimulus provoking CRPS onset. This study assessed $\alpha$-1AR expression some time (up to 20+ years) after the initial injury, which may not have been an appropriate time to assess $\alpha$-1AR expression of PBMCs in CRPS patients. A previous study indicated that the expression of $\alpha$-1AR in leukocytes might be subject to change dependent on noradrenaline levels in the patients’ sera (Kalkoff et al., 2008). By examining cardiac surgery patients pre- and post surgery, they found that patients who had high and mid levels of $\alpha$-1AR and high levels of noradrenaline in leukocytes before surgery, showed a decrease in $\alpha$-1AR expression post surgery (Kalkoff et al., 2008). This suggests that CRPS patients who may had previously expressed $\alpha$-1AR in leukocytes at the time of injury, may not express it now, some time after the original inflammatory stimulus. This concept may also be applied to those participants who showed $\alpha$-1AR in PBMCs. Although undocumented, these participants may have experienced an inflammatory stimulus leading up to blood extraction resulting in $\alpha$-1AR expression on PBMCs. In order to validate this claim, $\alpha$-1AR expression in PBMCs from these patients would need to be reassessed some time later.

An alternative approach would include stimulating isolated PBMCs from CRPS patients and healthy controls \textit{in vitro} with an inflammatory stimulus and then examining $\alpha$-1AR expression. Previous \textit{in vitro} studies that assessed $\alpha$-1AR
expression on PBMCs used a T cell mitogen phytohemagglutinin (PHA) or lipopolysaccharide (LPS) as an inflammatory stimulus (Casale and Kaliner, 1984; Kavelaars, 2002; Rouppe van der Voort et al., 2000). Cytokines and other inflammatory mediators are known to regulate the expression of α-1AR from PBMCs. For example, one study found that when the THP-1 monocyte line was cultured in the presence of IL-1β or TNF-α, the expression of α-1\textsubscript{A}AR was upregulated and the expression of α-1\textsubscript{D}AR was reduced. (Heijnen \textit{et al.}, 2002). Since CRPS patients have increased levels of pro-inflammatory cytokines in their sera, it would be interesting to see if differing levels of these cytokines \textit{in vitro} affect the levels of α-1AR expression in PBMCs following an inflammatory stimulus (Schinkel \textit{et al.}, 2006; Uceyler \textit{et al.}, 2007).

Furthermore, incubating LPS-stimulated PBMCs that express α-1AR with noradrenaline activates ERK-2 (Rouppe van der Voort \textit{et al.}, 2000). This kinase targets transcription factors that regulate the production of cytokines. Coincubation of noradrenaline with an α-1AR antagonist completely blocks the noradrenaline effect. Additionally, cells that do not express α-1AR do not respond to noradrenaline with increased ERK-2 activation (Kavelaars, 2002). This indicates that noradrenaline induced ERK-2, through α-1AR, enhances immune activity by increased cytokine production. As increased cytokine production is evident in CRPS patients, future studies could also assess the effect of noradrenaline on inflammatory stimulated-PBMCs that have already been induced to express α-1AR by analysing cytokine production.
5.2.2. α-1AR expression may be masked in minority PBMC populations

Another possible reason for the failure to detect α-1AR expression by PBMC in this study is that the mRNA of α-1AR was only expressed by a subset of PBMC populations. Studies have shown that α-1AR is expressed in monocytes, which, from the results of this study, were found to be in the minority of the PBMC populations (13.5% of CRPS and 14.75% of healthy control PBMC) (Heijnen, 2002; Takahashi, 2005). If the monocytes from the participants in this study expressed α-1AR, this could have been masked by the greater proportion of non-monocytic PBMCs not expressing α-1AR. In this case, it would be advantageous to be able to isolate individual PBMC subpopulations to assess their α-1AR mRNA expression. In future studies, the antibody panel used in this study could be redesigned to include fluorescently labeled (BODIPY) prazosin, a specific α-1AR antagonist (O’Connell et al., 2014; Hirasawa et al., 1996; Calzada and de Artinano et al., 2001; Waugh et al., 1999; Marrow and Crease, 1986). Using the flow cytometry approaches described in this study, the separate PBMC populations could then be analysed to detect α-1AR at the protein level. There are several limitations to this approach, however. Firstly, the 10-colour flow cytometry panel used in this study would need to be redesigned to allow the inclusion of BODIPY-prazosin. Secondly, prazosin has equal affinity for α-1AAR, α-1BAR and α-1DAR (Calzada and de Artinano et al., 2001; Waugh et al., 1999; Marrow and Crease, 1986). This means we would not be able to differentiate between the α-1AR subtypes. Thirdly, although α-1AAR and α-1BAR are extracellular proteins, α-1DAR is entirely an intracellular protein. BODIPY-prazosin is used to identify extracellular proteins, thus the analysis of
extracellular proteins (and not intracellular) would inaccurately reflect the level of α-1AR expressed (McGrath et al., 1999; Mackenzie et al., 2000).

A second alternative for differentiating α-1AR expression among PBMC populations involves using technology that is more recent. Prime Flow (Invitrogen, ThermoFisher) is a technology that combines cell surface and intracellular protein analysis using fluorescently labeled monoclonal antibodies, with mRNA analysis as measured by flow cytometry. Cell surface (and intracellular) markers are labeled with antibody as used in the flow cytometry strategy for this project. These cells are then fixed and permeabilised to allow for hybridisation of gene-specific probes with the RNA transcript of interest. Rather than amplifying the target transcript as with RT-PCR and qPCR techniques, a pre-amplifier is used to amplify the signal of the probe-RNA hybrid. The fluorescence of this hybridisation is then built upon by continual hybridisation with the pre-amplifier with the addition of a fluorescently labeled probe. This approach could potentially assess α-1AAR, α-1BAR and α-1DAR mRNA expression, along with cell surface marker expression, simultaneously in PBMC populations to allow analysis of α-1AR subset expression in subsets of PBMC (Soh et al. 2016).

5.2.3. Relative shift of β-2AR to α-1AR expression in PBMCs

In addition, several studies suggest that α-1AR expression in PBMCs is regulated by β2 adrenergic receptors (β-2AR), particularly after prolonged exposure to AR agonists (Wahle et al., 1996; Goebel et al., 2010). This could
suggest that rather than an overall change in expression of $\alpha$-1AR in PBMCs, there could be a change in the ratio of $\beta$-2AR to $\alpha$-1AR. Noradrenaline inhibits the release of pro-inflammatory mediators through $\beta$-2AR (Grisanti et al., 2011). Thus, given that pro-inflammatory mediators are increased in the plasma of CRPS patients, we would expect to see a decrease in the level of $\beta$-2AR responding to noradrenaline (Schinkel et al., 2006; Uceyler et al., 2007). In a previous study, a human monocytic cell line (THP-1) known to express $\alpha$-1AR was cultured with the $\beta$-2AR agonist terbutaline and shown to increase $\alpha$-1$_B$AR and $\alpha$-1$_D$AR mRNA, without changes in the $\alpha$-1$_A$AR (Rouppe van der Voort, 1999). These findings suggest that noradrenaline may regulate the expression of $\alpha$-1AR in the immune system via binding to $\beta$-2AR that are constitutively expressed on most immune cells (Kavelaars, 2002). Thus, any future studies involving AR expression of PBMCs in CRPS could incorporate $\beta$-2AR mRNA analyses or stimulation in their study design.

5.3. PBMC subset analysis by flow cytometry

Using 10-colour flow cytometry analysis of PBMC subsets from CRPS and control participants, there were no significant differences in the relative percentages of any of the PBMC subpopulations identified between CRPS patients and healthy controls. However, there was a statistically significant increase in the total number of PBMCs per mL of whole blood in the CRPS patients compared to the healthy controls, and a shift of CD14$^+$ CD16$^-$ to CD14$^+$ CD16$^+$ monocytes in CRPS patients, which will be discussed in the following sections.
5.3.1. Viability and integrity of PBMCs for flow cytometry analysis

Due to the constraints of this project, PBMCs isolated from fractionated blood of CRPS patients and healthy controls were cryopreserved until flow cytometry analysis. Results showed a significant decline in cell viability following cryopreservation both by Trypan Blue and 7AAD. Interestingly however, there was also a significant difference in viability measured by these two techniques. Trypan Blue analysis showed a significant difference between the viability of CRPS patients (61.05%) when compared to healthy controls (70.45%), which was different again to 7AAD analysis. This showed viabilities of 84.02% and 86.10% for CRPS patients and healthy controls, respectively. As 7AAD was incorporated into the antibody panel of flow cytometry analysis, it measured the whole PBMC population of each sample in comparison to Trypan Blue, which only measured one hundredth of the amount. Therefore, viability analysis by 7AAD is expected to be more accurate than that by Trypan Blue. Using viabilities produced by 7AAD, there was one obvious outlier (P18: viability 24.58%). As the viability of this sample could affect the integrity of the PBMCs, it was excluded from PBMC percentage analysis.

Furthermore, the side scatter (SSC) and forward scatter (FSC) of cryopreserved PBMCs was plotted and compared to that of freshly-obtained PBMCs. The results show that there was a slight increase in the percentage of monocytes in the cryopreserved sample in comparison to the fresh sample, but no change in the percentage of lymphocytes. This could suggest that the loss in viable cells may have obtained from the lymphocyte population. However, as the plots show no
significant loss in cell populations, and all the PBMCs analysed by flow cytometry all were cryopreserved, I concluded this did not affect the outcome of results from this study.

5.3.2. Increased total PBMC count of CRPS patients

The results from this study showed CRPS patients have a higher total PBMC count when compared to healthy controls, but no differences in the counts of PBMC subpopulations. Correlation analysis of this study also showed there was no relationship between the concentrations of PBMCs isolated from CRPS patients and the severity of the condition, the duration of disease and the age of the participant. Often an increased PBMC count is used in the identification of inflammatory diseases as well as a prognostic parameter for monocyte leukaemia and multiple myeloma (Shin et al., 2013; Wu et al., 2011). Several studies have identified an association between increased PBMCs in the peripheral blood and poor outcomes with patients with heart failure, intracerebral haemorrhage, atherosclerosis and thrombosis, where subsets of PBMCs are involved in tissue damage as well as monocyte mediated pathways of inflammation and apoptosis (Greene et al., 2012; Walsh et al., 2015; Chapman et al., 2004; Rezende et al., 2014; Yin et al., 2003.).

One study found that a high PBMC count was associated with a loss in bone marrow density of elderly men, which was positively related to destructive bone diseases such as psoriatic arthritis (Lin et al., 2016). In addition, the sympathetic nervous system, and in particular α-1AR signalling, may play a role in the regulation of bone marrow density and proliferation of PBMC progenitors.
(Maestroni et al., 1992; Maestroni and Conti, 1994). Chemical sympathectomy by 6-hydroxydopamine (6-OHDA) significantly increased the number of peripheral blood leukocytes after syngeneic bone marrow transplant (Maestroni et al., 1992). The same study showed that prazosin, a potent α-1AR antagonist, can mimic and extend the effect of 6-OHDA with an additional rapid and significant increase of platelets, bone marrow granulocyte-macrophage colony forming units (GM-CFU) (the precursors for monoblasts and myeloblasts) and nucleated spleen cells. Furthermore, the α-1AR agonists noradrenaline and methoxamine can directly inhibit the in vitro growth of GM-CFU, while prazosin, and other α-1AR antagonists such as phentolamine can counteract this noradrenaline effect (Maestroni and Conti, 1994). Mechanical denervation results in significant mobilisation of cells into the peripheral blood within 24 hours, with a particular increase in progenitor cells (Afan et al., 1997). This demonstrates that α-1AR are present on bone marrow cells and participate in the regulation of hematopoiesis and proliferation. It also shows that the nervous system plays a selective role in the mobilisation of cells into the peripheral blood, which can ultimately result in an overall increase in cellular PBMC concentrations. For technical reasons, it would be difficult to assess α-1AR expression in cells of the bone marrow in CRPS patients. However, future studies may be able to target immature peripheral cells or progenitor cells in the periphery in conjunction with the previously mentioned addition of BODIPY-labeled prazosin into the antibody panel, to grasp a better understanding of this concept. Markers such as CD133, CD34 and CD38 are expressed on myeloid and lymphoid precursor cells that subside during maturation (Attar, 2014).
Additionally, the flow cytometry strategy used in this study only assessed the relative number of cells within a sample as a percentage of total PBMC. An alternative approach would be to assess the absolute number of subsets of cells within a sample using Tru Count tubes (BD Biosciences, San Jose, CA). Using a Tru Count tube decreases the variability of the percentages of cell populations assessed as it determines the overall number of PBMCs, rather than a specified number (Schnizlein-Bick et al., 2000). By adding a specified number of microbeads to a sample, the absolute cell number can be determined by comparing the number of stained cellular events to the absolute number of bead events as counted by the flow cytometer. This would provide a more accurate measure of total numbers of each PBMC subset.

5.3.3. Altered CD14\(^+\) CD16\(^-\) to CD14\(^+\) CD16\(^+\) monocyte ratios

The 10-colour flow cytometry panel used in this project included a series of conjugated monoclonal antibodies that identified CD4\(^+\) T cells, CD8\(^+\) T cells, CD4\(^+\) CD8\(^-\) double positive (DP) T cells, CD4\(^+\) CD25\(^+\) activated/regulatory T cells, CD8\(^-\) CD25\(^+\) activated/regulatory T cells, B cells, NK cells, NKT cells and monocytes, however there were no statistically significant differences in the percentage of these cell types in the PBMC of CRPS patients when compared with healthy controls. These results are in agreement with the study of Ribbers and colleagues who reported no association with lymphocyte subpopulations in CRPS type I, and the study of Kaufman and colleagues who reported no changes in the percentages of CD8\(^+\) T cells, NK cells and B cells in CRPS patients (Ribbers et al., 1998; Kaufmann et al., 2007).
However, the ratio of CD14⁺ CD16⁻ monocytes to CD14⁺ CD16⁺ monocytes in this study was lower in CRPS participants (9.742 +/- 1.035) when compared to healthy controls (13.93 +/- 1.84). As Ritz and colleagues have performed this analysis before and found a significant decrease in the ratio of CD14⁺ CD16⁻ to CD14⁺ CD16⁺ monocytes, a one-tailed unpaired Student’s T test was used in this study and also confirmed a significant decrease in this ratio ($p < 0.05$) (Ritz et al., 2011). An unpaired T test, rather than paired T test, was used because although the patients were aged and sex matched with healthy controls, these sample groups were independent. It became evident throughout this project that other factors, such as individual variability, other inflammatory stimuli and unknown pre-existing conditions, may have contributed to the results produced in this project. Furthermore, using a two-tailed unpaired T test to confirm this significance showed that this relationship was not quite significant ($p = 0.058$). Possibly an $n$ value of 19 for CRPS patients and 20 for healthy controls in this study could be a reason that this did not reach statistical significance, and in future an increased sample size could more accurately assess differences in this ratio.

The CD16⁺ monocytes represent a potent antigen-presenting and pro-inflammatory subpopulation of monocytes that have been shown to increase in inflammatory conditions (Radwan et al., 2016). They are a sole producer of the pro-inflammatory cytokine TNF-α, which CD16⁻ monocytes fail to produce, and are also involved in T cell activation (Wong et al., 2012) More recently, this group of monocytes have been further classified into CD14$^{high}$ CD16⁺ (intermediate monocytes) and CD14$^{low}$ CD16⁺ (non classical monocytes). This
study found no difference in the percentages between the two CD16 expressing monocytes of CRPS patients and healthy controls, although many studies report difficulty in identification of these two subsets by flow cytometry (Wong et al., 2012). Novel analysis show that intermediate type monocytes express the same level of CD14 as classical CD16− monocytes, whereas non-classical monocytes express CD14 at a much lower level (Wong et al., 2012). This gating strategy used in this study accounted for this, however as the percentage of these populations are so small, it may have not been entirely accurate. Furthermore, in CRPS, it has been shown that an increase in CD16+ monocytes is not correlated with the overall pain level of patients, but rather is correlated with patients who suffer from cold allodynia (Ritz et al., 2011). Interestingly, blocking α-1AR with a potent antagonist in CRPS patients inhibits dynamic allodynia, this can further suggest α-1AR expression on these cell subsets (Drummond et al., 2016).

5.3.4. Limitations of the flow cytometry panel

Another limitation of the flow cytometry strategy use in this study was that the panel was limited to only assess the most common subsets of PBMCs. Dendritic cells (DCs) are another type of PBMC involved in regulating the inflammatory response. They are antigen-presenting cells (APCs) that capture, process and present antigens through to naïve T cells, are thought to be involved in the functioning and maintaining of tolerance and maintain immune memory in tandem with B cells (Weider, 2003). Dendritic cells can derive from two lineages: myeloid DCs evolve from monocytes, whereas plasmacytoid DCs develop directly from hematopoietic stem cells. Myeloid DCs differentiate from monocytes in the presence of pro-inflammatory TNF-α and IL-4 and typically
express antigens CD11c, CD13, CD33 and CD11 but lack monocyte antigens CD14 and CD16. Plasmacytoid DCs lack myeloid antigens and are distinguished by CD13, CD303 and CD304. These antigens can all be targeted for flow cytometryic analysis (Collin et al., 2013). Co-stimulatory molecules CD80 and CD86 are also expressed, which are upregulated during activation. Since DCs are APCs, the major-histocompatibility complex class II (MHC II), which is involved in antigen presentation, is also a common target for flow cytometric analysis. Epidermal DCs (Langerhans cells) have been shown to be increase in the skin of CRPS patients, and to have upregulated α1-AR (Calder et al., 1998; Seiffert et al., 2002; Kavelaars, 2002). It was beyond the scope of this project to assess DCs in CRPS peripheral blood as this would have required expansion of the commercial flow cytometry panel beyond the 10-colour limit of the flow cytometer, however a future study could alter the flow cytometry strategy in this project to incorporate DC markers in the antibody panel, or use additional panels.

5.3.5. Limitations of the PBMC isolation method

The density gradient (Lymphoprep) isolation solution used in this project only isolated mononuclear cells from peripheral blood; polynuclear granulocytes have a higher density and therefore submerged to the bottom. However, there is a small chance of immature granulocyte contamination (1-5% of total cell number). The appearance of immature granulocytes in the peripheral blood indicates an early-stage response to infection, inflammation or isolation of other stimuli of the bone marrow (Senthilnayagam et al., 2011). The antibody panel used in this study did not stain for immature granulocytes, thus the proportion of immature granulocytes in the periphery was undetermined.
Mature granulocytes (neutrophils, eosinophils and basophils) were not isolated using this isolation procedure from whole blood. Neutrophils are decreased in CRPS patients which was shown to correlate with increased levels of stress hormone (Kaufmann et al., 2007). This suggests that CRPS patients have an impaired innate immunity, which may result in an adaptive immune response and chronic inflammation. In order to assess this however, in future it may be advantageous to analyse unfractionated blood and incorporate granulocyte markers into the antibody panel, or construct a panel assessing mononuclear cells, and a panel assessing polynuclear cells such as granulocytes.

5.4. Future directions

The results from this and other studies suggest that a number of factors regulate α-1AR expression, and that several reasons could explain why only two participants (one CRPS and one control) showed α-1AR expression in this study. With previous studies suggesting α-1AR may only be expressed on PBMCs after an inflammatory stimulus, in future a more advanced study incorporating in vitro analyses of α-1AR of CRPS patient PBMCs following experimental inflammatory stimuli (e.g. lipopolysaccharide or T cell mitogens) and/or co-incubation with noradrenaline or pro-inflammatory cytokines could be informative. Utilising the CSS, and by receiving samples from patients over a period of time, documenting the change in their condition may also provide a clearer understanding of the changes with time of the underlying inflammatory response. Additionally, since only few populations of PBMCs are known to express α-1AR following inflammatory stimulus, it would be advantageous to
distinguish between PBMC populations during α-1AR analysis using a modified
flow cytometry strategy incorporating fluorescently labeled BODIPY-prazosin
into the antibody panel, sorting individual PBMC subsets for PCR analysis, or
using the more technologically advanced Prime Flow strategy to detect mRNA in
PBMC subsets by flow cytometry. Monocytes have been one type of PBMC
shown to express α-1AR mRNA, and as shown in this and previous studies; the
ratio of CD14$^+$ CD16$^-$ to CD14$^+$ CD16$^+$ is increased in CRPS patients, which
indicates a pro-inflammatory response. The results from this study also showed
an overall cellular increase in PBMC of CRPS patients that could further indicate
the problem arises from progenitor cells within the bone marrow. For technical
reasons, it would be difficult to assess cells within the bone marrow of CRPS
patients, however a future study could incorporate the use of activation markers
or progenitor cell markers into this modified antibody panel.
6. Conclusion

This study established no difference in the expression of α-1AR mRNA of the total PBMC population isolated from whole blood of CRPS patients when compared to healthy controls. It did, however, show low level of α-1AR mRNA expression in one CRPS patient and one healthy control. In addition, this study showed no statistically significant difference in the percentages of CD4+ T cells, CD8+ T cells, CD4+ CD8+ T cells, CD4+ CD25+ activated/regulatory T cells, CD8+ CD25+ activated/regulatory T cells, B cells, NK cells and NKT cells of CRPS patients when compared to healthy controls, although an increase in the concentration of total PBMCs isolated from whole blood of CRPS patients and a shift to CD16+ from CD16- monocytes was found. In conclusion, based on the data generated, α-1AR expression of overall PBMC population is independent from that of CRPS, but was limited due to the inability to differentiate PBMC populations during this analysis. The increased PBMC concentration of CRPS patients and the shift to pro-inflammatory CD14+ CD16+ monocytes provide preliminary data that the aberrant inflammatory response of CRPS may stem from increased cell proliferation in the bone marrow that is regulated by the sympathetic nervous system via α-1AR.
References


Casale, T. B. and M. Kaliner. 1984. “Demonstration that circulating human blood cells have no detectable α1-adrenergic receptors by radioligand binding analysis.” *Journal of Allergy and Clinical Immunology* 74: 812-18.


Appendix 1: Clinical evaluation sheet and questionnaire.

**Interview and Pain Distribution Test**

Name: .......................................................... Date: .......

Age: ....  Sex: ....  Phone: ........................................ Dominate h

Examiner: ........................................

**History of injury**

Date of initial event:

Site of injury:

What happened and type of injury (crush, laceration, fracture, sprain, burn, etc.)

Evidence of nerve injury:

Sympathetic blocks:

Surgery (when, type of surgery):

Current drug treatment (type, dose, when last taken)

---

**Spontaneous pain**

Distribution of pain (local, fore-quarter, hemilateral, mirror image)

---

**Quality** (aching, stabbing, throbbing, burning, numb, pins-and-needles)

**Distribution of allodynia**

**Distribution of sensory loss**
### Distribution, description and persistence of pain in response to stimulation

<table>
<thead>
<tr>
<th>Dynamic stimuli (brushing, air movement)</th>
<th>Now</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Firm pressure (bumps, taps)</th>
<th>Now</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Light touch (clothing, sheets etc)</th>
<th>Now</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cold (ambient temperature, touching something cold)</th>
<th>Now</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Heat (ambient temperature, touching something hot)</th>
<th>Now</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Use (e.g., walking, carrying)</th>
<th>Now</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Other aggravating factors

<table>
<thead>
<tr>
<th>Stress (anxiety, anger, distress)</th>
<th>Now</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Startle or fright</th>
<th>Now</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other</th>
<th>Now</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Mechanical sensation (on the most painful medial/lateral side of the affected limb and at a similar contralateral site)

<table>
<thead>
<tr>
<th></th>
<th>Pressure</th>
<th>Pin</th>
<th>Hair: 1</th>
<th>Hair: 5</th>
<th>Light Touch Response (Brush): medial versus lateral, affected versus unaffected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affected Limb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unaffected Limb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Pressure:** pressure-pain threshold measured with an algometer

**Pin:** sharpness rating (0-10) to a 40 gm pin (NeuroPen)

**Hair:** 1 sharpness rating (0-10) to the single application of a 10 gm von Frey hair (NeuroPen)

**Hair:** 5 sharpness rating (0-10) after 5 1-s on 1-s off applications of a 10 gm von Frey hair (NeuroPen)

**Light Touch:** Sensations that might be experienced in response to light touch with a soft brush: a dull, a sharp, a painful, an abnormal, an uncomfortable or a normal sensation.

### Temperature

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Mid-dorsum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affected Side</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unaffected Side</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Appendix 2: Bioline Isolate II RNA Mini Kit

<table>
<thead>
<tr>
<th>Kit Contents</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISOLATE II Filter (violet)</td>
<td>Used to filter lysate once it was homogenized and reduces lysate viscosity.</td>
</tr>
<tr>
<td>ISOLATE II RNA Mini Columns (blue) &amp; Collection Tubes</td>
<td>Used for binding RNA to silicon membrane and steps throughout washing</td>
</tr>
<tr>
<td>Collection Tubes</td>
<td>Eluted RNA collection tube</td>
</tr>
<tr>
<td>Lysis Buffer RLY</td>
<td>Contains guanidinium thiocyanate used for lysing cells for RNA extraction</td>
</tr>
<tr>
<td>Wash Buffer RW1</td>
<td>Contains guanidinium thiocyanate and ethanol. Used for washing salts once RNA bound to silicon membrane.</td>
</tr>
<tr>
<td>Wash Buffer RW2</td>
<td>Concentrate. Contains added ethanol. Used for washing residual salts/</td>
</tr>
<tr>
<td>Membrane Desalting Buffer MEM</td>
<td>Contains guanidinium thiocyanate and ethanol. Desalts the silicon membrane before addition of DNase I. Salt reduces DNase I reactivity.</td>
</tr>
<tr>
<td>Reaction Buffer for DNase I RDN</td>
<td>Activates DNase I.</td>
</tr>
<tr>
<td>DNase I, RNase-free (lyophilised)</td>
<td>Digests genomic DNA to allow elution of pure RNA.</td>
</tr>
<tr>
<td>RNase-free Water</td>
<td>Ensures integrity of RNA.</td>
</tr>
</tbody>
</table>
Appendix 3: Bioline SensiFAST cDNA synthesis kit

**SensiFAST™ cDNA Synthesis Kit**

<table>
<thead>
<tr>
<th>Product Name</th>
<th>50 reactions</th>
<th>250 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Transamp Buffer</td>
<td>200ul</td>
<td>1ml</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>50ul</td>
<td>250ul</td>
</tr>
</tbody>
</table>

**Storage and stability:** SensiFAST cDNA Synthesis Kit is shipped on dry ice and should be stored at -20°C upon receipt. When stored under optimum conditions, the reagents are stable for a minimum of one year from date of shipment.

**Unit definitions:**
- **Reverse Transcriptase:** One unit catalyzes the incorporation of 1 fmol [32P]dATP in 30 minutes at 37°C in 50mM Tris-HCl, pH 8.3, 30mM KCl, 3mM MgCl₂, 0.5mM DTT, and 0.5mM each of 25mM dATP, dCTP, dGTP, and dTTP using 100μM oligo(dT)₅-primed poly(A)₅-terminated template.
- **RNAse Inhibitor:** One unit inhibits 200 U of RNase A by 50%.

**Safety precautions:** Harmful [if swallowed]: Irritating to eyes, respiratory system and skin. Please refer to the material safety data sheet for information regarding hazards and safe handling practices.

**Description**

SensiFAST cDNA Synthesis Kit provides a rapid and very sensitive method for first strand cDNA synthesis for use in real-time PCR studies. The 5x Transamp Buffer provides highly optimized components for efficient reverse transcription, and includes a unique blend of anchored oligo dT and random hexamer primers to ensure unbiased 3' and 5' coverage for enhanced data accuracy. An extremely efficient reverse transcriptase delivers highly robust first strand synthesis and higher cDNA yields from a wide range of input RNA concentrations. SensiFAST cDNA Synthesis Kit offers enhanced sensitivity, efficiency and reproducibility for exceptional performance in subsequent real-time PCR experiments.

**Components**

<table>
<thead>
<tr>
<th>Component</th>
<th>50 reactions</th>
<th>250 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Transamp Buffer</td>
<td>200ul</td>
<td>1ml</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>50ul</td>
<td>250ul</td>
</tr>
</tbody>
</table>

**DNAse I digestion of total RNA**

To eliminate any residual contaminating genomic DNA that can affect highly sensitive real-time PCR applications (e.g. probe-based quantification of a low abundant target), we recommend using a high quality RNase-free DNAse I during or after RNA extraction protocols. DNAse I removal by ethanol precipitation, or with a RNA clean-up kit e.g. ISOLATE II RNA Micro Clean-Up Kit is required prior to proceeding with first-strand cDNA synthesis.

**SensiFAST cDNA Synthesis Kit Protocol**

1. **Prepare the master mix on ice.**
2. **Vortex solutions and centrifuge briefly before use.**

<table>
<thead>
<tr>
<th>Total RNA or mRNA (up to 1μg)</th>
<th>nil</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Transamp Buffer</td>
<td>4μl</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>1μl</td>
</tr>
<tr>
<td>DNAse I/RNase-free water*</td>
<td>Up to 20μl</td>
</tr>
</tbody>
</table>

*Available separately (see Associated Products)

3. **Mix gently by pipetting.**
4. **Set up the following program in a thermal cycler:**
   - 25°C for 10 min (primer annealing)
   - 42°C for 15 min (reverse transcription)
   - Optional: 49°C for 15 min after high-strung RNA
   - 85°C for 5 min (inactivation)
   - 4°C hold (or chill on ice)
5. **Use up to 4μl (15μl volume) of cDNA synthesis reaction product in a 20μl volume real-time PCR.**
6. **Alternatively, store reaction product at 4°C for 1 week or -20°C for long term storage.** If desired, reaction product can be diluted in 10mM Tris-HCl (pH 8.0), 0.1mM EDTA and stored at -20°C.

**This protocol is intended for use as a guide only; conditions will vary from reaction to reaction and may need optimization.**
Appendix 4: RNA extraction concentrations from all samples and corresponding volumes used for cDNA synthesis

Table A4: Breakdown of total RNA concentration (ng/µL) from the 40 participants and the corresponding volumes of RNA that equates to 204.75ng of RNA.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Concentration of RNA eluded (ng/µL)</th>
<th>Volume of RNA used for cDNA</th>
<th>Control</th>
<th>Concentration of RNA eluded (ng/µL)</th>
<th>Volume of RNA used for cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>42.05</td>
<td>4.87</td>
<td>C1</td>
<td>53.9</td>
<td>3.8</td>
</tr>
<tr>
<td>P2</td>
<td>22.75</td>
<td>9</td>
<td>C2</td>
<td>30.95</td>
<td>6.62</td>
</tr>
<tr>
<td>P3</td>
<td>62.9</td>
<td>3.26</td>
<td>C3</td>
<td>30.3</td>
<td>6.76</td>
</tr>
<tr>
<td>P4</td>
<td>31.5</td>
<td>6.5</td>
<td>C4</td>
<td>28.1</td>
<td>7.29</td>
</tr>
<tr>
<td>P5</td>
<td>25.75</td>
<td>7.95</td>
<td>C5</td>
<td>22</td>
<td>9.31</td>
</tr>
<tr>
<td>P6</td>
<td>18.2</td>
<td>11.25</td>
<td>C6</td>
<td>20.65</td>
<td>9.91</td>
</tr>
<tr>
<td>P7</td>
<td>26.8</td>
<td>7.64</td>
<td>C7</td>
<td>30.4</td>
<td>6.73</td>
</tr>
<tr>
<td>P8</td>
<td>37.6</td>
<td>5.44</td>
<td>C8</td>
<td>28</td>
<td>7.31</td>
</tr>
<tr>
<td>P9</td>
<td>24.1</td>
<td>8.49</td>
<td>C9</td>
<td>34.95</td>
<td>5.86</td>
</tr>
<tr>
<td>P10</td>
<td>45.05</td>
<td>4.5</td>
<td>C10</td>
<td>49.8</td>
<td>4.11</td>
</tr>
<tr>
<td>P11</td>
<td>51.5</td>
<td>3.98</td>
<td>C11</td>
<td>46.15</td>
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<tr>
<td>P12</td>
<td>43.2</td>
<td>4.74</td>
<td>C12</td>
<td>34.9</td>
<td>5.87</td>
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<td>P13</td>
<td>25.55</td>
<td>8.01</td>
<td>C13</td>
<td>13.65</td>
<td>15</td>
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<tr>
<td>P14</td>
<td>76.9</td>
<td>2.66</td>
<td>C14</td>
<td>31.5</td>
<td>6.5</td>
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<td>P15</td>
<td>28.6</td>
<td>7.16</td>
<td>C15</td>
<td>60.55</td>
<td>7.16</td>
</tr>
<tr>
<td>P16</td>
<td>50.2</td>
<td>4.08</td>
<td>C16</td>
<td>23.3</td>
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<td>5.37</td>
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<td>6.96</td>
<td>C20</td>
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<td>5.58</td>
</tr>
<tr>
<td>Average</td>
<td>39.77</td>
<td>Average</td>
<td></td>
<td>34.66</td>
<td></td>
</tr>
</tbody>
</table>
# Appendix 5: Compensation matrix

<table>
<thead>
<tr>
<th></th>
<th>CD16</th>
<th>CD56</th>
<th>CD19</th>
<th>7AAD</th>
<th>CD14</th>
<th>CD4</th>
<th>CD8</th>
<th>CD3</th>
<th>CD25</th>
<th>CD45</th>
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<tbody>
<tr>
<td>FITC</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td>0.5</td>
</tr>
<tr>
<td>PE</td>
<td>20</td>
<td>8</td>
<td></td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7AAD</td>
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</tr>
<tr>
<td>PE-Cy7</td>
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<td>4</td>
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<td></td>
<td>0.5</td>
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<td></td>
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</tr>
<tr>
<td>APC</td>
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<td>A700</td>
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<td></td>
<td>0.7</td>
<td>28.5</td>
<td>12.48</td>
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<td></td>
</tr>
<tr>
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