Evaluating digital vascular perfusion and platelet dysfunction in Raynaud’s phenomenon and systemic sclerosis

submitted by
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A thesis submitted for the degree of Doctor of Philosophy
University of Bath
Department of Pharmacy and Pharmacology
April 2013

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Abstract

Raynaud’s phenomenon (RP) describes excessive vasoconstriction of the digital microvasculature in response to cold exposure and emotional stress. RP is typically the earliest clinical manifestation of systemic sclerosis (SSc); a complex multisystem disease of unknown aetiology characterized by vasculopathy, inflammation and fibrosis. Vasculopathy is considered an essential pre-requisite to tissue remodeling, characterized by excessive collagen synthesis and tissue fibrosis, which occurs in the skin and other organs of patients with SSc.

There is mounting evidence associating platelets with important biological functions beyond primary haemostasis. Upon activation, platelets release a large array of mediators implicated in vasoconstriction, inflammation and fibrosis, which has led me to consider the contribution of platelets to the pathogenesis of both RP and SSc.

The principal aim of this thesis is to explore the impact of targeted anti-thrombotic therapy on digital microvascular function, platelet activation, oxidative stress and eicosanoid biosynthesis in RP and SSc. It is not possible to examine the impact of anti-platelet therapy on microvascular function, without first identifying sensitive methods for assessing digital microvascular function in humans. I shall report the findings of work examining established (infrared thermography) and novel (laser speckle contrast imaging) methods for the objective assessment of digital microvascular function in RP and SSc. I shall critically appraise the application of these methods alongside subjective patient self-report assessment of digital vascular function. I shall examine associations between digital vascular function, platelet activation, oxidative stress and eicosanoid biosynthesis between primary RP and SSc. I shall also report the findings of an investigator-led early phase clinical trial of targeted anti-thrombotic medication in RP and SSc.

The major findings of this study were highlighting the strengths and limitations of established and novel methods for objective microvascular assessment in RP and SSc, and the poor agreement that exists between objective and subjective methods for assessing RP severity. I have identified similarities and differences in platelet activation and eicosanoid biosynthesis in primary RP and SSc. I shall present evidence of apparent efficacy of asasantin retard therapy on subjective, but not objective, assessments of digital microvascular function; the relevance of which shall be discussed in detail.
Declaration of work undertaken by myself and in conjunction with others

All of the work reported in this thesis was designed and undertaken by myself unless otherwise stated.

Urinary samples for eicosanoid analysis were sent to the Eicosanoid Core Laboratory at Vanderbilt University Medical Center in Nashville, TN, USA. This laboratory has pioneered the identification and characterization of novel eicosanoids including the isoprostanes. The Gas-Chromatography Mass-Spectrometry studies used to quantify urinary eicosanoids in this thesis were undertaken in collaboration with Professor Ginger L. Milne who is the Research Associate Professor of Medicine and Pharmacology within the Division of Clinical Pharmacology at Vanderbilt University.

The ELISA kit for human soluble CD40 ligand required the use of an orbital microplate shaker. These assays were undertaken with the help of Jean-Phillippe Walhin within the Centre of Excellence for DisAbility Sport & Health at the University of Bath.

I received support with the statistical analysis undertaken in Chapter 4 from Steve Raper within the Centre for Mathematical Biology at the University of Bath. I received support with the statistical analysis (reproducibility work) reported in Chapter 5 from Dr Anita McGrogan within the Department of Pharmacy and Pharmacology at the University of Bath.
Acknowledgements

First and foremost, I would like to thank Professor Neil McHugh for his encouragement, reassurance and support in undertaking this work. Without Neil’s support this work would not have been completed. I would like to thank Professor Steve Ward and Dr Malcolm Watson within the Department of Pharmacy and Pharmacology for their additional support and supervision.

I would like to extend that gratitude to all my colleagues at the Royal National Hospital for Rheumatic Diseases for their help in identifying potential patients and general support. I should like to extend particular thanks to Sue Brown for her support and Dr William Tillett who provided an ongoing source of advice, understanding, support and friendship.

I wish to thank Dr Jacqueline Shipley, Dr Nigel Harris and Darren Hart within the clinical measurement department of the RNHRD for their help, support and kindness, particularly during times of need.

I am hugely grateful to all my colleagues within the Bath Institute for Rheumatic Diseases for helping me undertake laboratory work. I would like to extend particular thanks to Juliet Dunphy for her support undertaking the ELISA studies.

Thank you to Professors Valerie O’Donnell and Ginger Milne for their collaborative advice and support in undertaking the eicosanoid work.

I am indebted to Mrs Anne Mawdsley and all the patrons of the Raynaud’s and Scleroderma Association, without whose support, this work could not have been undertaken. I owe a specific debt to the family of Mrs E Dando who bequeathed the funds necessary to undertake this work.

I would like to thank all of the healthy volunteers and patients who generously gave up their time to make such a vital contribution to this research. Finally, I wish to thank all of my family, and in particular my wife Shaney, for their endless support and encouragement. I dedicate this thesis to them.
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<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACA</td>
<td>Anti-centromere autoantibody</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>ARA</td>
<td>American Rheumatism Association</td>
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<tr>
<td>AVA</td>
<td>Arteriovenous anastamoses</td>
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<tr>
<td>BHT</td>
<td>2,6-Di-tert-butyl-4-methylphenol</td>
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<tr>
<td>β-TG</td>
<td>β-thromboglobulin</td>
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<tr>
<td>CTD</td>
<td>connective tissue disease</td>
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<tr>
<td>CTGF</td>
<td>connective tissue growth factor</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>dcSSc</td>
<td>diffuse cutaneous systemic sclerosis</td>
</tr>
<tr>
<td>DDD</td>
<td>distal dorsal difference</td>
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<tr>
<td>DP</td>
<td>digital pitting</td>
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<tr>
<td>DU</td>
<td>digital ulceration</td>
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<tr>
<td>EC</td>
<td>Endothelial cell</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
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<td>EMS</td>
<td>Electromagnetic spectrum</td>
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<tr>
<td>EMR</td>
<td>Electromagnetic radiation</td>
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<tr>
<td>EMW</td>
<td>Electromagnetic waves</td>
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<td>ERA</td>
<td>Endothelin receptor antagonists</td>
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<td>FLPI</td>
<td>full field laser perfusion imaging</td>
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<tr>
<td>GI</td>
<td>gastrointestinal</td>
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<td>Hep-2</td>
<td>Human Epithelial-2 cells</td>
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<tr>
<td>12-HETE</td>
<td>12-Hydroxyeicosatetraenoic acid (12-HETE)</td>
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<td>ILD</td>
<td>Interstitial lung disease</td>
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<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>IRT</td>
<td>infrared thermography</td>
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<tr>
<td>Laser</td>
<td>Light Amplification by Stimulated Emission of Radiation</td>
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<tr>
<td>lcSSc</td>
<td>limited cutaneous systemic sclerosis</td>
</tr>
<tr>
<td>LDF</td>
<td>laser Doppler flowmetry</td>
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<tr>
<td>LDPI</td>
<td>laser Doppler perfusion imaging</td>
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<tr>
<td>LOX</td>
<td>lipoxygenase</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>LSCI</td>
<td>laser speckle contrast imaging</td>
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<tr>
<td>MCP</td>
<td>metacarpophalangeal</td>
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<tr>
<td>MCTD</td>
<td>mixed connective tissue disease</td>
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<tr>
<td>MTP</td>
<td>metatarsophalangeal</td>
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<tr>
<td>NC</td>
<td>nailfold capillaroscopy</td>
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<td>OMERAECT</td>
<td>Outcome MEasures in Rheumatoid Arthritis Clinical Trials</td>
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<tr>
<td>PAH</td>
<td>pulmonary arterial hypertension</td>
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<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
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<tr>
<td>PF-4</td>
<td>platelet factor-4</td>
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<td>PGE$_1$</td>
<td>prostaglandin E$_1$</td>
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<tr>
<td>PGI$_2$</td>
<td>prostaglandin I$_2$</td>
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<tr>
<td>PFP</td>
<td>platelet free plasma</td>
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<tr>
<td>PPP</td>
<td>platelet poor plasma</td>
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<tr>
<td>PRP</td>
<td>platelet rich plasma</td>
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<td>RCS</td>
<td>Raynaud’s condition score</td>
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<td>RCT</td>
<td>Randomised controlled trial</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RP</td>
<td>Raynaud’s phenomenon</td>
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<tr>
<td>r$_s$</td>
<td>Spearman’s Rho or Spearman’s rank correlation coefficient</td>
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<tr>
<td>sCD40L</td>
<td>soluble CD40 ligand</td>
</tr>
<tr>
<td>SCTC</td>
<td>Scleroderma Clinical Trials Consortium</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosis</td>
</tr>
<tr>
<td>sP-selectin</td>
<td>soluble P-selectin</td>
</tr>
<tr>
<td>SRC</td>
<td>scleroderma renal crisis</td>
</tr>
<tr>
<td>SSc</td>
<td>systemic sclerosis</td>
</tr>
<tr>
<td>ssSSc</td>
<td>systemic sclerosis sine scleroderma</td>
</tr>
<tr>
<td>SSRI</td>
<td>selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>TxA$_2$</td>
<td>Thromboxane A$_2$</td>
</tr>
<tr>
<td>TxB$_2$</td>
<td>Thromboxane B$_2$</td>
</tr>
<tr>
<td>TGF-$\beta$</td>
<td>transforming growth factor $\beta$</td>
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<tr>
<td>vWF</td>
<td>von Willebrand’s factor</td>
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Conference contributions arising from the work of this thesis


Pauling JD, McHugh NJ. Platelet Aggregability, Eicosanoid Biosynthesis and Oxidative Stress in Primary Raynaud's Phenomenon and Systemic Sclerosis. Arthritis Rheum 2012;64(supplement 10):S641


Jenkins J, Pauling JD, McHugh NJ. Pulmonary function test abnormalities in patients with systemic sclerosis and anticentromere autoantibodies without established cardiopulmonary disease. Rheumatology 2012;51(supplement 3):iii84

Strickland G, Pauling JD, McHugh NJ. Autoantibody profile and clinical associations in a British cohort with scleroderma. Rheumatology 2012;51(supplement 3):iii83


Pauling JD, Shipley JA, Raper S, Watson ML, Ward SG, Harris ND, McHugh NJ

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1 Includes publications within the broader field of RP and SSc but not always directly related to the work reported in this thesis


Pauling JD, Flower V, Shipley JA, Harris N D, McHugh NJ. Influence of the cold challenge on the discriminatory capacity of the digital distal-dorsal difference in the thermographic assessment of Raynaud’s phenomenon. Rheumatology 2011;50(supplement 3):iii131

Publications arising from the work of this thesis²

Journal articles

Pauling JD, O’Donnell VB, McHugh NJ. The contribution of platelets to the pathogenesis of Raynaud’s phenomenon and systemic sclerosis. Platelets. 2012 Sep 11 [Epub ahead of print]


² Includes publications within the broader field of RP and SSc but not always directly related to the work reported in this thesis
Pauling JD, McHugh NJ. Does incorporation of a cold challenge provide diagnostic information in the thermographic assessment of Raynaud's phenomenon. Thermology International. 2010; 20(3):87-92


Book chapters

Prizes arising from the work undertaken as part of this thesis

May 2012  1st Place, BIRD Davies-Maitland Scholarship prize

April 2012  Royal National Hospital for Rheumatic Diseases Researcher of the Year Award

Sept 2010  Department of Pharmacy and Pharmacology PhD 6-month presentation prize, University of Bath
Chapter 1.0 Introduction

'The head was completely withered, of a uniform shade of bronze, exactly resembling the colour of an ancient icon painting; the nose was as thin as a knife-blade; the lips had almost disappeared—only the teeth and eyes gave any gleam of light... At the chin, where the quilt was folded back, two tiny hands of the same bronze colour slowly moved their fingers up and down like little sticks. And the face seemed all the more awesome to me because I could see that a smile was striving to appear on it, to cross its metallic cheeks—was striving and yet could not spread.'

With a shock, after the woman had said, "'Master, don't you recognize me?'... he realizes that she is Lukeria, who ten years before had been 'the greatest beauty among all the servants in our house, tall, buxom, white-skinned and rosy-cheeked, who used to laugh and sing and dance!'. But she says, "'Don't you pity me too much...I sing songs even now.'"

Turgenev
Living Relic 1874
Sketches from a Hunter's Album, translated by Richard Freeborn.

1.1 Introduction

This early literary description of systemic sclerosis (SSc) is striking, not least for the accuracy of the clinical features described, but also for its portrayal of the remarkable courage and tenacity that exemplifies patients afflicted by this devastating illness. The pathological hallmark of SSc is dysregulated tissue remodelling and excessive fibrosis. As indicated in the description of Lukeria above, fibrosis of the skin (leading to use of the synonym scleroderma) is the most clinically visible manifestation of established disease. Vascular dysfunction is typically the earliest manifestation of the disease and thought to represent an essential
prerequisite to subsequent disease characteristics. Raynaud’s Phenomenon (RP) is typically the presenting vascular feature of SSc and can manifest several years prior to the emergence of additional organ-specific features of SSc. RP describes episodic excessive vasoconstriction of the digital vessels in response to cold exposure and emotional stress. RP is common and typically occurs as an isolated phenomenon in otherwise healthy individuals (primary RP), with only a small minority of patients progressing to SSc.

The origin of this thesis was to evaluate platelet function in primary RP and SSc, and to explore the biological and peripheral microvascular effects of targeted anti-platelet therapy in primary RP and SSc.

In this chapter I shall describe the clinical features and pathogenesis of RP and SSc. I shall review the potential contribution of platelets to vascular function, inflammation and tissue remodeling in RP and SSc. I shall report the evidence to support platelet dysfunction in primary RP and SSc, before critically appraising the findings of previous studies of anti-platelet agents in these conditions. I shall outline some of the limitations of available subjective assessment of peripheral vascular function in RP and SSc, and discuss the use of objective non-invasive microvascular imaging techniques in the assessment of digital vascular function. The chapter will close with a summary of the key research hypotheses and main objectives I wish to address in this thesis.

1.2 Raynaud’s Phenomenon

1.2.1 Clinical Features of Raynaud’s Phenomenon

Maurice Raynaud (1834-1881, Figure 1.1 and 1.2) is generally credited with providing the earliest detailed description of episodic excessive vasoconstriction of digital vessels in response to cold exposure and emotional stress, to which he has become eponymously associated (Herrick, 2005, Wigley, 2002, Raynaud, 1862). Vasoconstriction in response to cold exposure is an important component of thermoregulation, leading an early 20th Century physician to dryly observe ‘we are all subjects of Raynaud’s phenomena to a greater or lesser degree’ (Hutchinson, 1901). Hutchinson advocated replacement of the original term disease with the alternative suffix “phenomenon”, and reserving use of the term for people in whom the degree of vasoconstriction is disproportionate to the precipitating stimulus.
Attacks of RP are typically associated with distinct cutaneous colour changes, reflecting the perfusion and oxygenation of sequestered blood within affected tissues during attacks. Blanching (white phase) occurs as a result of profound vasoconstriction of the digital arteries, pre-capillary arterioles and cutaneous arteriovenous anastamoses (AVAs) (Figure 1.3). This can be accompanied by digital cyanosis (blue phase) secondary to de-oxygenation of sequestered blood within affected tissue (Figure 1.3). Digital ischaemia is sometimes followed by a post-occlusive reactive hyperaemia during re-perfusion leading to erythema of the digits (red phase) (Wigley, 2002). Typically, a history of increased insensitivity to cold and the presence of 2 colour changes is sufficient, but not essential, to consider a diagnosis of RP (Brennan et al., 1993). The digits of the hands and feet are typically affected areas but other commonly affected sites include the nose, ears, cheeks and nipples. RP attacks are typically accompanied by ischaemic pain and paraesthesia, which can impair function. Other early observations of RP included bilaterality of the phenomenon, absence of gangrene of the digits and presence of adjacent palpable pulses (Allen, 1932).
Figure 1.3 The hands of a patient with RP secondary to SSc.

There is blanching and cyanosis of the digits representing the typical appearances of RP. The waxy appearance of the skin of the fingers is a consequence of cutaneous fibrosis (sclerodactyly). In addition, there is shortening of the index fingers bilaterally secondary to previous episodes of tissue ischaemia and atrophy.

1.2.2 Epidemiology of Raynaud’s phenomenon

RP usually occurs as an isolated phenomenon, referred to as primary RP. There is a strong predilection for females. The prevalence of primary RP varies according to geographical variation in climate (Maricq et al., 1997). For example, population based studies in the UK have estimated the prevalence of RP within the general population of 11% and 19% for men and women respectively, in comparison with a prevalence of only 3.7% in a Spanish population (Silman et al., 1990, Riera et al., 1993). In approximately 10% of cases of RP, there is an associated vascular, haematological, neurological or autoimmune disease, for which the term secondary RP is applied (Riera et al., 1993). Secondary RP can be associated with more profound peripheral vascular dysfunction, sometimes resulting in critical digital ischaemia and tissue necrosis. Approximately half of patients referred to secondary care for evaluation of RP symptoms have secondary RP (Edwards, 1990). The majority of patients with secondary RP have an underlying autoimmune condition although clinicians must remain vigilant to alternative causes and we have reported non-rheumatic examples of secondary RP that have presented to rheumatology services for further
investigation (Pauling et al., 2010, Flower et al., 2010). Older age at presentation and involvement of the thumbs have been identified as specific risk factors for secondary RP (Chikura et al., 2010). The presence of antibodies to ubiquitously expressed auto-antigens and nailfold capillaroscopic changes are useful markers for future progression to connective tissue disease (CTD), particularly SSc (Koenig et al., 2008). Approximately 13% of patients with RP referred to secondary care without features of CTD at presentation subsequently develop SSc (Koenig et al., 2008).

1.2.3 Pathogenesis of Raynaud’s Phenomenon

‘To sum up in a more definite form, I would say that in the present state of our knowledge, local asphyxia of the extremities ought to be considered as a neurosis characterised by enormous exaggeration of the excito-motor energy of the grey parts of the spinal cord which control the vaso-motor innervation.’

Maurice Raynaud
New Researches of the nature and treatment of local asphyxia of the extremeties. 1874
Selected Monographs, The New Sydenham Society, 1888
Translated by Thomas Barlow, MD

Raynaud correctly observed the absence of an obliterative vasculopathy, enabling episodic attacks to occur and completely resolve. He sensibly concluded that there must be an important contribution of neural innervation of the digital vessels in the development of RP. Great strides have been made over the last 30 years to elucidate the pathogenesis of RP, which is not yet fully understood. Abnormalities within the vessel wall (primarily the endothelium), the autonomic control of vascular smooth muscle tone and intravascular factors reciprocally modulate each other, resulting in the clinical features of RP, and making it difficult to treat the phenomenon by targeting one individual pathway in isolation (Herrick, 2005). Important pathophysiological differences are thought to exist between primary RP and RP secondary to SSc, despite the similarity in clinical presentation early in the course of SSc.

Vessel wall abnormalities
Primary RP is generally considered a functional disorder although minor structural abnormalities such as increased capillary dimensions have been identified on direct
visualization of the nailfold capillaries (Bukhari et al., 2000). In SSc, structural abnormalities are more pronounced with histopathological evidence of endothelial damage, intimal thickening and smooth muscle hypertrophy, resulting in progressive luminal obliteration (Herrick, 2005). The endothelium has an important role in mediating vascular tone through the production of vasoactive mediators. There is evidence to support an imbalance in endothelial production of vasodilators (such as NO and prostacyclin) and vasoconstrictors (primarily endothelin) in RP favouring vasoconstriction (Herrick, 2005). Impaired endothelial dependent vasodilation occurs in both primary RP and SSc, although to a greater extent in SSc (Herrick, 2005).

**Neural control of vascular tone**

The autonomic nervous system is of particular importance in thermoregulation and RP pathogenesis. Both autonomic and peripheral nerve dysfunction has been shown to occur in SSc in comparison with primary RP and healthy controls (Klimiuk et al., 1988, Schady et al., 1991). Vasoconstriction to noradrenaline may be enhanced due to increased expression of $\alpha_2$-adrenoceptors in digital arteries of patients with RP and SSc following cold exposure (Herrick, 2005). Biopsy specimens have also identified impaired vasodilatation due to a significant reduction in the number of vasodilatory calcitonin gene-related peptide (CGRP) immunoreactive neurons in skin from patients with primary RP and SSc compared with healthy controls (Bunker et al., 1990).

**Intravascular factors**

Vascular tone is also influenced by an array of intravascular factors including shear stress, haemorrhheological abnormalities, circulating sex hormones, oxidative stress and the action of circulating cells such as platelets. Indeed, intravascular abnormalities can be the direct cause of secondary RP in conditions such as cold agglutinin disease (Flower et al., 2010). All are thought to make important contributions to the pathogenesis of both primary RP and SSc. A comprehensive description of the potential contribution of platelets to the pathogenesis of RP and SSc is provided in section 1.4.3.

**1.2.4 Management of Raynaud’s phenomenon**

Identifying and managing the cause of RP can occasionally completely alleviate symptoms of RP e.g. cold agglutinin disease. In both primary RP and RP secondary to CTD, the
central components of management include advice on avoidance of precipitating or aggravating factors, alleviating acute episodes and the use of drugs to prevent vasoconstriction or promote vasodilatation. All patients are educated on cold avoidance measures and the importance of maintaining core temperature. The use of gloves and local heat packs can reduce the frequency and duration of RP attacks. Smoking cessation advice is given to all smokers with RP. There is RCT evidence to support the use of drugs designed to prevent vasoconstriction including selective serotonin reuptake inhibitors (SSRIs) such fluoxetine (Coleiro et al., 2001) and angiotensin II antagonists such as losartan (Dziadzio et al., 1999). Despite anecdotal reports, the endothelin receptor antagonists (ERA) have not been shown to be effective for RP symptoms in well-designed RCTs (Nguyen et al., 2010). Studies have also identified a role for drugs that promote vasodilation including calcium channel antagonists (Thompson et al., 2001), prostacyclin (McHugh et al., 1988), phosphodiesterase inhibition (Shenoy et al., 2010) and nitrates (Teh et al., 1995, Anderson et al., 2002).

1.3 Systemic sclerosis

SSc is a rare complex disease of unknown aetiology characterized by vasculopathy, fibrosis and inflammation. The hallmark of the disease is the overproduction and accumulation of extracellular matrix proteins such as collagen leading to progressive fibrosis of affected organs. This is most clinically evident in the skin, which becomes thickened and loses its elasticity leading to early and continued use of the synonym “scleroderma” (from the greek roots skleros meaning hard and derma relating to skin). As previously discussed, RP is typically the presenting feature of SSc and can predate the onset of other clinical manifestations by several years. Approximately 12.6% of patients referred to secondary care for assessment of RP symptoms subsequently develop SSc (Koenig et al., 2008). Activation of the immune system is a notable early feature of SSc and the identification of autoantibodies to ubiquitously expressed antigens and the use of nailfold capillaroscopy (to provide in vivo evidence of microvascular dysfunction) facilitates the early identification of patients with SSc, prior to the emergence of fibrotic complications of the disease (LeRoy and Medsger, 2001).
1.3.1 Early recognition and descriptions of systemic sclerosis

There were several reports of induration and thickening of the skin in the 18\textsuperscript{th} and 19\textsuperscript{th} century which utilised the term ‘scleroderma’. Retrospective analysis of these reports suggest many were likely to represent alternative diagnoses in which skin thickening occurs; most notably a likely case of ‘scleroedema’ reported by Curzio in 1753 (Benedek and Rodnan, 1982). The range of distinct disease entities characterised by skin thickening continues to cause diagnostic error and uncertainty to this day. In the appendix to his English translation of Raynaud’s original thesis and subsequent essays, Thomas Barlow describes a series of cases which were almost certainly SSc, accurately associating the vascular and fibrotic components of the disease. In one case he describes;

‘a women who for 5 years had suffered during the winter with hard yellowish patches on the extremeties, which subsided on the return of spring. Ultimately the fingers were permanently altered in that the extremeties became cold, hard, somewhat unsensitive and decidedly atrophied. The last phalanges were contracted in a state of semi-flexion....The patient was liable to crises from time to time during which the finger reddened and became painful, then ulceration occurred and tardy cicatrisation with loss of substance. After each crisis the affected digit became a little more atrophied and deformed than before. There was no trace of scleroderma in other parts of the body’

Thomas Barlow, 1888
Author’s appendix
Selected Monographs, The New Sydenham Society, 1888

In the mid 20\textsuperscript{th} century, in recognition of the major visceral involvement that occurs in SSc, Goetz first proposed use of the term “progressive systemic sclerosis” noting:

\textit{Obviously the term ‘scleroderma’ should be abandoned. ‘Scleroderma’ is only the obvious and striking symptom of a generalized disease and the most serious symptoms actually arise in the viscera.}

Goetz, 1945, as cited in (Benedek and Rodnan, 1982)
Much of the late 20th Century was devoted to sub-classifying this heterogeneous disease according to the extent of skin involvement, and recognition of the importance of such classification in terms of major organ manifestations, serological biomarkers and prognosis (LeRoy and Medsger, 2001, LeRoy et al., 1988).

1.3.2 Clinical features of systemic sclerosis
The varied clinical features of SSc led to delayed appreciation that they represented heterogeneous clinical manifestations of the same disease entity. It is easiest to consider the clinical features of SSc in terms of the sequelae of the main pathophysiological events driving the disease; vasculopathy, fibrosis and inflammation.

Vasculopathy
Microvascular dysfunction is considered an essential pre-requisite for subsequent clinical manifestations of the disease and RP occurs in virtually all patients with systemic sclerosis (LeRoy, 1996). Peripheral microvascular dysfunction in SSc is typically more pronounced than in primary RP. In addition to excessive vaso-reactivity to cold exposure, there is structural remodelling of the vessels with endothelial damage, intimal and adventitial thickening, smooth muscle hypertrophy and eventual obliteration of the vessel lumen. This leads to more persistent and profound tissue ischaemia, presenting as tissue necrosis in the form of digital ulceration (DU) or, in severe cases, critical digital necrosis and gangrene (Pauling et al., 2011a). Vascular dysfunction can occur in other vascular beds, most notably the lungs (pulmonary arterial hypertension [PAH]) and kidneys (scleroderma renal crisis [SRC]), which are leading causes of SSc-associated mortality. Whilst often clinically silent, vasculopathy can also be demonstrated in the tissue of organs complicated by fibrosis such as the gastrointestinal tract.

Fibrosis
Skin fibrosis is the most striking visible manifestation of the disease. SSc is typically sub-classified into 2 distinct variants according to the extent of cutaneous involvement. In limited cutaneous SSc (lcSSc), skin thickening is restricted to the face and extremities (distal to the elbows and knees) and is slowly progressive (LeRoy et al., 1988). In diffuse cutaneous SSc (dcSSc), skin thickening is more widespread and pronounced, with the potential for greater subsequent morbidity including joint flexion contractures in the hands and a restrictive lung
defect secondary to thickening of the skin of the chest wall. Fibrosis occurs elsewhere including, in order of decreasing frequency, the lungs, gastrointestinal tract and heart. Progressive fibrosis can result in organ failure, which remains a major cause of morbidity and mortality. The extent of cutaneous disease can help predict other SSc organ-specific manifestations. For example, lung fibrosis more oftenly occurs in dcSSc, whereas PAH is more commonly found in lcSSc. Such clinical associations, whilst helpful, are not absolute and any particular organ manifestation can occur in either lcSSc or dcSSc. Indeed, internal organ manifestations can occur in the absence of skin involvement (systemic sclerosis sine sclerodera [ssSSc]) highlighting the systemic nature and marked heterogeneity of the disease. For example, we have reported life-threatening PAH as the presenting feature of ssSSc (Pauling et al., 2008).

**Inflammation**

Activation of the immune system in SSc is evident by the early presence of auto-antibodies to ubiquitously expressed nuclear antigens. A detailed description of the various auto-antibodies that are found in SSc and their clinical relevance is beyond the scope of this thesis but has been reviewed elsewhere (Pauling, 2012). SSc-specific auto-antibodies provide vital prognostic information for clinicians that can facilitate earlier detection of disease manifestations e.g. SRC in patients carrying anti-RNA polymerase antibodies and PAH in patients carrying anti-nucleolar autoantibodies (Penn and Denton, 2008, Steen, 2005). Associations between auto-antibody carriage and disease manifestations are not absolute and unexpected disease associations do occasionally occur e.g. SRC in association with anti-centromere autoantibodies (ACA). Clinically evident immune dysfunction can be demonstrated by the presence of active inflammation in tissues including the joints (typically a non-erosive symmetrical polyarthropathy), tendons and muscle (typically a low-grade and often sub-clinical myositis) (Eisenberg, 2008).

**1.3.3 Epidemiology of systemic sclerosis**

The disease prevalence varies with geographical location and period of data acquisition. For example, the reported prevalence of SSc using data acquired from the hospital records from a single centre (Memphis and Shelby counties, USA) increased from 40 per million between 1947-52 and 280 per million between 1962-68. The highest estimated prevalence of SSc has been identified in a small population of Choctaw Native Americans in Oklahoma (660
per million), in whom a high prevalence of a polymorphism of the fibrillin gene has been identified. In a recent study, the prevalence of SSc within a large North American population was estimated at 276 cases per million adults with an annual incidence of 19.3 new cases per million adults per year (Mayes, A&R, 2003). Both prevalence (315.1 vs 224.7 per million) and incidence (23.7 vs 18.3 cases per million per year) of SSc is greater in black compared with white patients. Furthermore, black patients are 1.86 times more likely than non-black patients to have dcSSc. Even allowing for variation in study design, it would appear the prevalence of SSc in the US is higher than that of the UK. Comparable studies in the UK have estimated the adjusted prevalence at between 30.8 and 82 per million (Silman, Br J Rheumatol, 1988; Allcock, Rheumatology, 2004). The condition has a higher propensity for women, possibly reflecting the contribution of sex hormones, pregnancy-related events or gender-specific environmental exposures (Barnes, 2012). In the UK, the ratio between female to male is 5.2:1 and the ratio between limited to diffuse disease 4.7:1. Prevalence estimates in the UK have also risen in recent studies, which probably reflects increased recognition of patients with limited disease in recent studies. An unexpectedly low ratio of limited to diffuse disease (1.5:1) in early epidemiological studies would support this notion (Silman, Br J Rheumatol, 1988).

**1.3.4 Pathogenesis of Systemic Sclerosis**

The pathogenesis of SSc is complex and a detailed description is beyond the scope of this thesis. The pathogenesis is best considered as a triad of vascular injury, cellular and humoral autoimmunity, and connective tissue remodelling (leading to dysregulated fibrosis). These distinct pathophysiological processes are heavily inter-related and reciprocally modulate each other as the disease evolves.

Similar to many autoimmune diseases, the development of SSc is thought to follow a gene-environment interaction. Putative external triggers include toxins (e.g. silica, solvents and vinyl chloride) and infections (e.g. cytomegalovirus, parvovirus B19 and Epstein-Barr virus) although the cause in most cases is unknown. As previously discussed, polymorphisms of the fibrillin gene have been identified within populations with a high prevalence of dcSSc associated with anti-topoisomerase positive autoantibodies. Fibrillins play important roles in the storage and activation of growth factors including TGF-β. Mutation of the fibrillin gene is also responsible for the tight-skin mouse-1 (the most widely studied experimental animal
model of SSc). Other genetic associations in SSc have been identified which relate to genes responsible for the production of pro-inflammatory mediators such as cytokines (e.g. IL-1β and IL-2) (Hunzelmann and Brinckmann, 2010).

LeRoy, in his seminal paper of 1975, presented his ‘vascular hypothesis’ speculating that vascular dysfunction was an essential pre-requisite to late fibrotic manifestations of SSc (Campbell and LeRoy, 1975). This hypothesis is supported clinically by the early presence of RP, increased vascular permeability (sausage fingers) and telangiectasia in SSc. The vascular endothelium (a vital single cell thick layer of endothelial cells that regulate coagulation and fibrinolysis, vascular permeability and vascular tone) is thought to be a primary target in SSc (Kahaleh, 2012a). Endothelial cell (EC) injury (secondary to toxins, autoimmunity, microbial agents and/or ischaemia/reperfusion injury) help propagate a vicious cycle of vascular dysfunction favouring vasoconstriction and tissue hypoxia. A dysfunctional endothelium increases vascular permeability facilitating the migration of leukocytes leading to perivascular extracellular matrix (ECM) remodelling (Kahaleh, 2012a). The relationship between vasculopathy and fibrosis is complex. Chronic ischaemia and tissue hypoxia promote increased formation of growth factors (e.g. transforming growth factor β [TGF-β] and connective tissue growth factor [CTGF]) by fibroblasts attracted by EC cytokines (Hunzelmann and Brinckmann, 2010). TGF-β is thought to represent a key cytokine driving fibrosis in SSc, leading to the trans-differentiation of fibroblasts to myofibroblasts and increased collagen synthesis (Hunzelmann and Brinckmann, 2010). Platelet-derived growth factor (PDGF) is another important driver of fibrosis in SSc and shall be described later (see sections 1.4.3 and 1.4.4). These factors also play an important role in angiogenesis, which is also impaired in SSc. Histological analysis of early skin lesions demonstrates fibrosis occurring first around vessels, characterised by a dense peri-vascular infiltration of T cells, monocytes and activated fibroblasts (Hunzelmann and Brinckmann, 2010). In the late stages of the disease, fibrotic regions are characterised by a hypocellular connective tissue and an altered composition of the ECM characterised by excessive collagen deposition (Hunzelmann and Brinckmann, 2010).

No direct pathogenic role has yet been identified for the antibodies targeting ubiquitously expressed autoantigens that characterise SSc. There has been considerable interest in autoantibodies directed to candidate cells (e.g. endothelial cells), cell surface receptors (e.g. PDGF receptors) or extracellular matrix (ECM) proteins (e.g. fibrillin-1) which may
eventually provide a direct link between autoantibodies and endothelial damage and downstream fibrotic cell-signalling in SSc. The potential contribution of platelets to the pathogenesis of SSc will be described in section 1.4.3.

1.3.5 Management of systemic sclerosis

The outcome of therapeutic studies of SSc over the last 50 years has been largely disappointing and it remains one of few rheumatic diseases in which a range of effective disease modifying therapies has yet to emerge. Effective treatments targeting clinical manifestations of SSc are available and widely used e.g. the use of proton pump inhibitors to manage gastro-oesophageal reflux secondary to upper gastrointestinal dysmotility. A limited number of treatments directly targeting pathogenic mechanisms have been studied and in recognition of the central role of the immune system, much work has focused on immunomodulatory treatments. For many years, D-Penicillamine was the “anchor drug” in SSc before the disappointing findings of a large double-blind study led many to question its safety and efficacy (Wollheim, 2012). Glucocorticoids are largely avoided (in high dosage at least) in light of their potential to precipitate a SRC. Studies of methotrexate and cyclophosphamide have reported modest treatment efficacy in trials of fibrotic complications of SSc (Pope et al., 2001, Hoyles et al., 2006, Tashkin et al., 2006). The eagerly anticipated findings of the European Scleroderma Observational Study evaluating outcomes in SSc patients treated with methotrexate, mycophenolate mofetil or cyclophosphamide will hopefully provide further insight into the role of immunosuppressive regimes in the treatment of SSc. Large multicentre randomized trials of human autologous stem cell transplantation have recently reported potentially promising findings on mortality in early diffuse SSc (van Laar, 2012). The results of studies of recombinant human monoclonal antibodies to TGF-β and tyrosine kinase inhibition (imatinib mesylate) to augment downstream mediators of TGF-β and PDGF have failed to live up to expectations. The findings of early investigative studies of other ‘biological agents’ such as rituximab (anti-CD20 B cell depletion) and tocilizumab (anti-IL6) are potentially more promising.

Arguably the most effective disease modifying treatment for SSc has been angiotensin converting enzyme inhibitors (ACEi) which have significantly reduced the morbidity and mortality associated with SRC in SSc (Steen and Medsger, 2007). Targeting the renin-angiotensin pathway has been less effective for managing peripheral vascular complications in SSc (Dziadzio et al., 1999, Gliddon et al., 2007). Meta-analyses have indicated the
effectiveness of dihydropiridine-type calcium-channel blockers in the management of primary RP and RP in SSc (Thompson and Pope, 2005, Thompson et al., 2001). Intravenous prostanoids are effective for RP symptoms and improve DU healing in SSc (Wigley et al., 1994, McHugh et al., 1988). The use of selective serotonin re-uptake inhibitors (SSRIs) has also been shown to reduce the impact of RP symptoms in SSc (Coleiro et al., 2001). The advent of endothelin-receptor antagonists (ERA) and phosphodiesterase inhibitors may provide the first opportunity to modify the disease course of vascular complications of SSc. For example, bosentan has been shown to reduce the frequency of DU and improve exercise capacity, functional class and some haemodynamic measures in SSc-PAH (Kowal-Bielecka et al., 2009).

1.4 Platelets

1.4.1 Platelet structure and contents

Platelets were recognised by various physicians across Europe in the 18th century, but it is Bizzozzero who is credited with the first detailed description of their structure and significance as the central component of the cellular mechanisms responsible for primary haemostasis in 1882 (Ribatti and Crivellato, 2007). He termed these cells piastrine (Italian for small plates). It was not until 1906 that it was established that platelets were derived from megakaryocytes within the bone marrow, and much of the early part of the 20th century was devoted to establishing their role in the coagulation cascade and haemostasis. Indeed, for much of this period, haemostasis was thought to represent their sole function despite observations as early as 1896 for their phagocytic capacity (Klinger, 1997).

Platelets (or thrombocytes) are anucleate cytoplasts formed following cytoplasmic fragmentation from megakaryocytes under the control of humoral agents such as thrombopoietin. Megakaryocytes are primarily formed in the bone marrow although fragmentation can occur in other organs such as the lung. The normal platelet count is between 150 and 350x10^9/L (Kamath et al., 2001). Platelets have a lifespan of 8-10 days. Resting platelets are smooth biconcave discoid structures with a diameter of 1-3 μm and volume of 5-6 fl (Kamath et al., 2001). Despite the lack of a nucleus, platelets are capable of a small amount of protein synthesis due to the presence of megakaryocyte-derived
messenger RNA. Platelets contain around 35 round or oval α-granules which contain factors synthesized during megakaryocyte development and those endocytosed from plasma via the open canalicular system (OCS) following formation. In addition platelets contain around 2-7 dense bodies, which contain their own factors important in haemostasis and inflammation. Table 1.1 lists some of the major contents of platelet granules. Platelets also contain a dense tubular system, important in the regulation of calcium stores and arachidonic acid metabolism (namely the production of thromboxane A₂ [TxA₂]). The release of platelet-derived factors is facilitated by the OCS, sometimes referred to as the 2-way street owing to its equally important participation in exo- and endocytosis of particulate matter (Klinger, 1997).
Table 1.1 Major contents of platelet granules

<table>
<thead>
<tr>
<th><strong>Alpha granules</strong></th>
<th><strong>Electron dense granules</strong></th>
</tr>
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<tbody>
<tr>
<td>Fibrinogen</td>
<td>Serotonin</td>
</tr>
<tr>
<td>β-Thromboglobulin</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>Albumin</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>2-Antiplasmin</td>
<td>Calcium</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>Pyrophosphate</td>
</tr>
<tr>
<td>Osteonectin</td>
<td></td>
</tr>
<tr>
<td>Protein S</td>
<td></td>
</tr>
<tr>
<td>Factor V</td>
<td></td>
</tr>
<tr>
<td>Platelet factor 4</td>
<td></td>
</tr>
<tr>
<td>Plasminogen</td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td></td>
</tr>
<tr>
<td>Vitronectin</td>
<td></td>
</tr>
<tr>
<td>Plasminogen activator inhibitor 1 (PAI 1)</td>
<td>Lysosomes</td>
</tr>
<tr>
<td>Platelet derived growth factor (PDGF)</td>
<td>Glycohydrolases</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td></td>
</tr>
<tr>
<td>α2-Antitrypsin</td>
<td></td>
</tr>
<tr>
<td>α2-Macroglobulin</td>
<td></td>
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</tbody>
</table>

Adapted from references (Gurney et al., 2002, Kamath et al., 2001)

1.4.2 Platelet activation, adhesion and aggregation in primary haemostasis

In resting conditions the endothelium attempts to avoid platelet activation through the release of mediators such as NO, endothelial nucleotidases (ADPases\(^3\)) and prostacyclin that inhibit platelet activation. The von Willebrand factor (vWF) is a cell-adhesion molecule that facilitates binding between endothelial cells and collagen in the sub-endothelial basement membrane. Damage to the endothelial surface, such as following trauma, leads to exposure of collagen, tissue factor and vWF to platelets. The chief binding sites for collagen on platelets are the GpVI receptors (which have an important role in TxA\(_2\))

\(^3\) ADPases reduce local concentration of ADP through the conversion of ADP to adenosine, which itself further inhibits platelet function via adenosine receptors on the platelet surface
synthesis and release) and GpIa/IIa receptors (important in platelet adhesion), directly inducing granule release. TxA₂ release upregulates GpIIb/IIIa expression (the chief binding site of vWF and fibrinogen), that also promotes platelet activation.

There are several important circulating platelet activators such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), thrombin, adrenaline and thromboxane. A schematic outlining the key intracellular signalling pathways (and therapeutic targets) involved in platelet activation is presented in Figure 1.4.

ADP binds to the P2Y₁ and P2Y₁₂ G-protein coupled purinergic receptors. The P2Y₁₂ is considered the more important ADP receptor and induces platelet activation by antagonizing adenylyl cyclase activity and suppressing cAMP formation. The P2Y₁₂ receptor is coupled to G₁₂ which inhibits adenylyl cyclase and cytosolic cyclic adenosine monophosphate (cAMP) production (Kahner et al., 2006). cAMP is an important modulator of release of calcium from intracellular stores (and modulation of actin cytoskeleton dynamics involved in platelet shape change). In addition, P2Y₁₂ receptor activation modulates platelet activation and granule release through phosphoinositide-3-kinases (PI3K) (Kahner et al., 2006).

The P2Y₁ receptor is a G protein coupled receptor (GPCR) coupled to G₉ that activates phospholipase β2 (PLCβ2). PLCβ2 regulates production of inositol triphosphate (IP₃) and diacyl glycerol (DAG) which leads to calcium release from intracellular stores and protein kinase C (PKC) activation (Kahner et al., 2006). P2Y₁ activation is an essential component of shape change induction and platelet aggregation (Kahner et al., 2006).

Endothelial nucleotidases (e.g. CD39 and CD73) lead to extracellular breakdown of ATP and ADP to adenosine. Adenosine is a potent vasodilator and inhibitor of platelet activation due to its GPCR activation of adenylyl cyclase and increased intracellular production of cAMP. Adenosine is taken up by cells such as erythrocytes via equilibrative transporters (Johnston-Cox and Ravid, 2011). Purinergic signaling pathways in platelets can be targeted to reduce platelet activation e.g. P2Y₁₂ inhibition (clopidogrel) and adenosine reuptake inhibition (dipyridamole) (Figure 1.4).
Figure 1.4 Schematic outlining principal purinergic and intracellular signalling pathways involved in platelet activation.

The red text boxes and arrows indicate pharmacological methods for inhibiting platelet activation. ADP, adenosine diphosphate; ATP, adenosine triphosphate; TxA2, thromboxane A2; PLCβ2, phospholipase β2; PIP2, phosphatidylinositol 4,5-biphosphate; IP3, inositol triphosphate; DAG, diacyl glycerol; PKC protein kinase C; ER endoplasmic reticulum; cAMP, Cyclic adenosine monophosphate; PLA2, phospholipase A2; Ca2+, calcium; ENTs, equilibrative transporters; AA, arachidonic acid; PGI2, prostaglandin I2; AMP, adenosine monophosphate; PDE, phosphodiesterase; PI3K, phosphoinositide-3-kinases (PI3K)

G protein coupled receptor (associated text boxes indicate name of receptor and coupled G proteins)
ATP acts at non-selective ligand-gated cation channels (P2X\(_1\) receptors). Activation of P2X\(_1\) receptors results in early shape change (discoid to spherical) following transient influx of calcium. The more significant function of P2X\(_1\) receptor activation is to potentiate calcium signaling downstream of other GPCR activation e.g. P2Y\(_1\) (Figure 1.4).

Adrenaline is another platelet activator that inhibits adenyl cyclase, via \(\alpha_2\) adrenergic receptors on the platelet surface. Thrombin is a potent activator of platelets via protease-activated receptors 1 and 4 (PAR-1 and PAR-4) that are components of a 7-transmembrane group of GPCR that influence intracellular signaling within platelets (figure 1.4). Upon activation, platelets undergo a shape change, first becoming spherical before developing finger-like pseudopodes to assume a stellate shape. These events help promote the recruitment of platelets to the exposed subendothelial surface where the platelets adhere and aggregate leading to the formation of a platelet plug. Platelet aggregation is achieved using vWF and fibrinogen as bridging agents between platelets. The release of factors such as TxA\(_2\) and ADP from activated platelets further potentiates local platelet activation, endothelial adhesion and aggregation, leading to the formation of the platelet plug and secondary coagulation. The vasoconstrictive properties of platelet-derived mediators such as TxA\(_2\) and serotonin further limit blood loss following injury.

### 1.4.3 The potential contribution of platelets to the pathogenesis of Raynaud's phenomenon and systemic sclerosis

The biological actions of platelets are known to extend beyond primary haemostasis and there is now a greater appreciation of the shared characteristics of platelets and immune cells. Platelets release a surprisingly large array of mediators, many of which have important roles in inflammation, tissue repair and vascular function (Table 1.2).

These factors are either synthesized within the cells upon activation, or stored and released from cytoplasmic granules as previously discussed in section 1.4.1. There is strong phylogenetic evidence to suggest the precursors of mammalian platelets provided the front line in microbial defence and wound healing (Weyrich et al., 2003). The Atlantic Horseshoe Crab (Limulus polyphemus) is a marine arthropod and “living fossil” that has evolved little over 445 million years. It’s single nucleated circulating cell (ameobocyte) that contains bacterial infection and achieves primitive wound sealing through the release of clotting
factors from cytoplasmic granules and local aggregation (Weyrich et al., 2003). Despite the emergence of more specialised innate and acquired immunity, mammalian platelets have retained the ability to bind, encapsulate and destroy an array of pathogens in humans suggesting preserved inflammatory functions (Klinger, 1997). There is a growing appreciation of the potential contribution of platelets to diseases ranging from asthma (Kornerup and Page, 2007), inflammatory bowel disease (Danese et al., 2004), cystic fibrosis (O'Sullivan and Michelson, 2006) and cancer (Nash et al., 2002).

Unsurprisingly, a considerable body of work has evaluated associations between platelet function, and RP and SSc. In this section I shall provide a conceptual framework upon which to consider the potential contribution of platelets in RP and SSc, by discussing the role of key platelet-derived mediators in chemotaxis, cellular adhesion, altered vascular resistance, altered vascular permeability, angiogenesis, matrix degradation, and fibrosis. A schematic diagram of the putative influence of platelet-derived mediators in the pathogenesis of SSc is presented in Figure 1.5.
Table 1.2 Platelet derived mediators associated with inflammation, vascular function and tissue re-modelling.

<table>
<thead>
<tr>
<th>Inflammatory mediators</th>
<th>Growth factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitric oxide *</td>
<td>Platelet derived growth factors (PDGF)-A, B, C, D *</td>
</tr>
<tr>
<td>Thromboxane A2 *</td>
<td>Transforming growth factor-B1 and B2</td>
</tr>
<tr>
<td>Serotonin *</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Prostanoids (PGD₂, PGE₂, PGF₂α) *</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>Platelet activating factor</td>
<td>Vascular endothelial growth factor A and C</td>
</tr>
<tr>
<td>Platelet factor 4</td>
<td>Insulin like growth factor-1</td>
</tr>
<tr>
<td>β-Thromboglobulin</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>P-selectin</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>Soluble CD40 ligand</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>Adenosine *</td>
<td></td>
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<tr>
<td>Adenine nucleotides *</td>
<td></td>
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<tr>
<td>Histamine *</td>
<td></td>
</tr>
<tr>
<td>Neutrophil activating peptide-2</td>
<td></td>
</tr>
<tr>
<td>Matrix Metalloproteinases 1, 2 and 9</td>
<td></td>
</tr>
<tr>
<td>IL-1 and IL-8</td>
<td></td>
</tr>
<tr>
<td>Monocyte chemoattractant protein-3</td>
<td></td>
</tr>
<tr>
<td>Macrophage inflammatory protein-1</td>
<td></td>
</tr>
<tr>
<td>12-Hydroxyeicosatetraenoic acid</td>
<td></td>
</tr>
<tr>
<td>RANTES (regulated upon activation, normal T-cell expressed and secreted)</td>
<td></td>
</tr>
<tr>
<td>2-arachidonyl glyceride *</td>
<td></td>
</tr>
<tr>
<td>Dinucleoside polyphosphates</td>
<td></td>
</tr>
</tbody>
</table>

* also poiently vasoactive. Adapted from references (O'Sullivan and Michelson, 2006, Klinger, 1997, Postlethwaite and Chiang, 2007)
Figure 1.5 Putative role of platelet-derived mediators in the pathogenesis of systemic sclerosis.

TxA\textsubscript{2}, thromboxane A\textsubscript{2}; ECM, extracellular matrix; βTG, β-thromboglobulin; PF-4, Platelet factor 4; IL-1β, Interleukin 1β; IgG, immunoglobulin; 5-HT, 5-hydroxy tryptamine (serotonin); TGF-β, transforming growth factor β; PDGF, platelet derived growth factor; CTGF, connective tissue growth factor; MMP-1 & 2, matrix metalloproteinases 1 & 2; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; MIP-1α, Macrophage Inflammatory Protein-1α; ENA-78, epithelial neutrophil activating protein-78; sCD40L, soluble CD40 ligand; ROS, reactive oxygen species
**Chemotaxis**
Platelets are a rich source of RANTES (Regulated upon Activation Normal T cell Expressed and Secreted; CCL5), platelet factor 4 (PF-4; CXCL4), β-thromboglobulin (β-TG) and ENA-78 (epithelial neutrophil activating protein-78; CXCL5) (Klinger, 1997, Weyrich et al., 2003). Each has important leukocyte-chemoattractant properties attracting neutrophils, eosinophils, monocytes and fibroblasts to sites of inflammation (Deuel et al., 1981, Gurney et al., 2002, Weyrich et al., 2003).

**Cellular adhesion**
The outermost layer of platelets (the glycocalyx) is made of glycoprotein’s and has a vital role in adhesion, aggregation and leukocyte recruitment. The glycocalyx changes following platelet activation. For example, P-selectin translocates from the intracellular granule membrane to receptors on the glycocalyx following activation. P-selectin (also known as CD62p, GMP140 and PADGEM) is a single-chain trans-membrane glycoprotein from a family of important cell adhesion molecules (CAMs) whose names derive from “selective” and “lectins” (carbohydrate recognising proteins). They are classified according to the primary cell-type responsible for their production e.g. L-selectin derived from leukocytes. P-selectin translocates to the platelet membrane (the glycocalyx) following activation, facilitating adhesion, aggregation and leukocyte recruitment. P-selectin is the primary receptor for interaction between platelets and monocytes, neutrophils, memory T-cells and natural killer cells, modulating chemokine and cytokine expression in these cells (Klinger, 1997). It also facilitates neutrophil rolling, arrest and transmigration across the endothelial surface as well as potentiating their action by inhibiting leukocyte apoptosis (O'Sullivan and Michelson, 2006, Weyrich et al., 2003, Gurney et al., 2002).

**Immune cell activation**
Platelet granules release several important pro-inflammatory mediators. For example, dense bodies secrete ATP which enhances the oxidative burst in neutrophils and serotonin which has an important influences on T-cell responses, vascular permeability, and fibroblast mitogenesis (Klinger, 1997). Alpha granules secrete many pro-inflammatory factors including immunoglobulins, PDGF and cationic proteins (Klinger, 1997). An important platelet-derived pro-inflammatory cytokine is the CD40 ligand (CD154, CD40L). CD40 is a phosphoylated glycoprotein of the tumour necrosis factor superfamily found on B cells, monocytes, macrophages and non-haemopoietic cells including endothelial cells and
smooth muscle cells (Vogel and Noelle, 1998). Activation of this receptor by its ligand (CD40L) has important roles in thrombosis, oxidative stress and both humoral and cell-mediated immune responses (Vogel and Noelle, 1998, Urbich et al., 2002, Henn et al., 2001). The cytoplasm of platelets are the predominant source of sCD40L, and may account for up to 90% of circulating levels (Chen et al., 2005). Expression and cleavage of sCD40L occurs within seconds of ADP-induced platelet activation, appears distinct to granule secretion and is not responsive to treatment with aspirin (in contrast to clopidogrel) (Hermann et al., 2001, Henn et al., 1998). Platelet derived sCD40L leads to increased B cell production of immunoglobulins (IgG) in vivo highlighting a potential importance of platelet activation in the adaptive immune response (Cognasse et al., 2007). In addition, sCD40L stimulates endothelial cell production of reactive oxygen species, chemokines and facilitates the recruitment of leukocytes following increased expression of cellular adhesion molecules ICAM-1 and VCAM-1 (Henn et al., 1998, Henn et al., 2001). Other important platelet-derived cytokines such as IL-1β further modulate immune pathways (Postlethwaite and Chiang, 2007).

The eicosanoids are lipid-signalling molecules derived from 20-carbon essential fatty acids such as arachidonic acid (AA). They play central roles in inflammation and immunity, in addition to being potently vasoactive. The dense tubular system of platelets is a major site of pro-inflammatory leukotriene and prostaglandin synthesis. Eicosanoid metabolism by other inflammatory cells can be influenced by platelet-leukocyte interactions. For example, the transcellular passage of AA from platelets can promote formation of PMN-derived pro-inflammatory leukotrienes e.g. LTB-4 and platelet mediated metabolism of PMN-derived eicosanoids (O’Sullivan and Michelson, 2006, Klinger, 1997).

**Angiogenesis**
Platelets are a rich source of pro-angiogenic growth factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoietin 1, insulin-like growth factor (IGF-1) and platelet derived growth factor (PDGF) (Weyrich et al., 2003). These compete with the anti-angiogenic properties of other platelet-derived mediators such as PF-4, and transforming Growth Factor β (TGF-β). Their opposing angiogenic actions may be of particular significance in SSc, which is characterised by both capillary loss and dysregulated new vessel formation.
Matrix Degradation

Leukocyte recruitment, angiogenesis and fibrosis can only occur at sites of inflammation following controlled degradation of the extracellular matrix (ECM). Platelets promote matrix degradation by secretion of matrix metalloproteinases (MMP-1 and MMP-2) in addition to augmenting the degradative activity of other cell types (e.g. production of MMP-9 following platelet-monocyte interactions and the effects of platelet-derived CD40 ligand on endothelial proteolytic activity) (Weyrich et al., 2003). Platelet-derived IL-1 is also an important mediator of the degradation of the sub-endothelial matrix facilitating further extravasation of leukocytes (Klinger, 1997).

Fibrosis

Platelets are a major source of circulating transforming growth factor β (TGF-β), which is secreted from alpha granules on activation, and has important roles in the growth and differentiation of fibroblasts, wound repair, angiogenesis, immune tolerance and inflammation (Lev et al., 2007). Platelet-derived growth factor (PDGF) is another important platelet-derived regulator of angiogenesis, myofibroblast differentiation, vasoconstriction and smooth muscle proliferation (Liakouli et al., 2011, Trojanowska, 2008). PDGF-receptors are present on the surface of fibroblasts, and stimulate type I collagen-gene expression and conversion to a myofibroblast phenotype (Trojanowska, 2008). Similarly, connective tissue growth factor (CTGF, CCN2) is also released from activated platelets and has similar roles to TGF-β in extracellular matrix re-modeling and fibrosis.

Vascular tone and permeability

Platelets produce a panel of vasoactive mediators that can promote vasoconstriction (thromboxane, serotonin, PDGF and dinucleoside polyphophates) and vasodilation (nitric oxide), although the balance of vascular tone favours vasoconstriction. Thromboxane A₂ (TxA₂) is a particularly powerful vasoconstrictor, in addition to promoting further platelet activation. It is produced in platelets from prostaglandin H₂ (via thromboxane synthetase), synthesized from AA by platelet cyclo-oxygenase (COX). It has also been proposed that platelet derived mediators may influence production of vasoactive mediators by other cell types such as endothelial production of the potent vasoconstrictor endothelin-1 (Matsumura et al., 1994).
1.4.4 Evidence of platelet dysfunction in SSc

With growing appreciation of the contribution of platelets to vasoconstriction, inflammation and tissue re-modeling, it is unsurprising that platelets have attracted attention for their potential pathogenic role in RP and SSc. In this section, I shall describe the existing evidence to support excessive platelet activation in Raynaud’s phenomenon and systemic sclerosis, ranging from early studies of platelet aggregability and circulating platelet-derived mediators, to the fascinating findings of recent studies exploring the potential direct pathogenic role of platelets in established murine models of SSc. I shall review established anti-platelet treatments available and critically appraise the findings of previous therapeutic studies evaluating the use of such agents in RP and SSc.

Platelet number and structure

The platelet count in primary RP and SSc is generally within normal range. Cases of SSc associated with thrombocythaemia or thrombocytopenia are typically the result of other disease manifestations or overlap features e.g. vasculitis, malignancy, microangiopathic haemolytic anaemia (Frayha et al., 1980). One study did identify a higher total platelet count in whole blood from patients with the CREST variant of SSc compared with healthy controls (Price et al., 1991). In this study, the MPV and PDW did not differ between SSc and healthy controls (Price et al., 1991). In contrast, a recent study has demonstrated an increased MPV in primary RP compared with healthy controls (Shemirani et al., 2012).

Platelet aggregation

The vast majority of studies have identified increased ex vivo platelet aggregability to a panel of agonists including adrenaline (Hutton et al., 1984), ADP (Wilkinson et al., 1989, Cuenca et al., 1990, Lau et al., 1993), collagen (Goodfield et al., 1988, Wilkinson et al., 1989, Lau et al., 1993, Goodfield et al., 1993), serotonin (5-HT) (Beretta et al., 2008, Biondi and Marasini, 1989) and AA (Reilly et al., 1986) in both primary RP and SSc, in comparison with healthy controls. Furthermore, patients with RP and SSc are less sensitive to the inhibitory effects of PGI₂ and PGE₁ on platelet aggregation compared to healthy donor platelets (Hutton et al., 1984, Belch et al., 1985). Under-representation of a genetic polymorphism in the 5-HT receptor gene that blunts intracellular responses to 5-HT has been noted in patients with SSc and leads to increased aggregation to 5-HT (Beretta et al., 2008). In contrast, one study failed to show differences in aggregation to a panel of agonists (ADP, collagen and PAF) when comparing HC and SSc (Price et al., 1991). It was
postulated that harvesting of ‘exhausted’ platelets from the circulation may explain the findings, although long disease duration, limiting recruitment to patients with the CREST (Calcinosis, Raynaud’s phenomenon, (o)Esophageal dysmotility, Sclerodactyly and Telangiectasia) variant of SSc and the small study size may also account for the disparity in their findings with other studies (Price et al., 1991). There have been conflicting reports from studies evaluating the impact of disease duration on platelet aggregability in SSc (Friedhoff et al., 1984, Biondi and Marasini, 1989). Increased platelet aggregability appears to be more pronounced in SSc compared with primary RP (Cuenca et al., 1990, Biondi and Marasini, 1989, Wilkinson et al., 1989). Indeed, one study that solely evaluated primary RP failed to identify differences in aggregation to the agonists AA, ADP, PAF compared with healthy controls (Dowie et al., 1990). The differences in platelet responses between SSc and primary RP may explain the more profound microvascular compromise that characterises RP associated with SSc.

Platelet granule release products
PF-4 and β-TG were amongst the first alpha granule products found to be elevated in SSc (Hutton et al., 1984, Seibold and Harris, 1985, Lima et al., 1991, Macko et al., 2002, Reilly et al., 1986, Kahaleh et al., 1982, Cuenca et al., 1990). There are conflicting reports as to whether circulating β-TG and PF4 levels are associated with specific clinical disease characteristics in SSc (Seibold and Harris, 1985, Malia et al., 1988, Herrick et al., 1996). Circulating levels of β-TG are lower in primary RP compared with SSc and there are conflicting reports as to whether levels differ between primary RP and HC (Seibold and Harris, 1985). β-TG and PF-4 have been identified in the bronchoalveolar lavage (BAL) fluid of subjects with scleroderma lung disease (particularly early in disease course) in contrast to HC and SSc patients without ILD, providing an attractive link between platelet activation and the site of tissue injury (Kowal-Bielecka et al., 2005). The pulmonary circulation is highly relevant to platelet biology because megakaryocytes release their platelets on arrival in the lungs, providing an attractive hypothesis linking platelet activation within the lungs and the high incidence of pulmonary complications in SSc and other lung diseases associated with increased platelet activation (O’Sullivan and Michelson, 2006).

The von Willebrand factor (vWF) is a large multimeric glycoprotein produced by many cells including endothelial cells and platelets, facilitating platelet adhesion and aggregation. Several studies have reported increased circulating levels of vWF in SSc, variously
reporting their pathological significance in terms of either platelet or endothelial cell activation (Herrick et al., 1996, Kahaleh et al., 1981, Blann et al., 1993, Blann et al., 1995, Ames et al., 1997, Iannone et al., 2008, Mannucci et al., 2003, Cerinic et al., 2003). It has been proposed that a deficiency of the metalloprotease responsible for the cleaving of vWF may contribute to higher circulating levels of vWF in SSc (Mannucci et al., 2003). Circulating supra-normal multimers of vWF have also been identified in SSc (but not healthy controls) and decrease in number following aspirin therapy, suggesting the vWF multimers are platelet derived (Mannucci et al., 1989). Circulating vWF levels in SSc have been associated with various organ-specific complications including pulmonary vascular disease (Matucci-Cerinic et al., 1990, Scheja et al., 1994), pulmonary fibrosis (Kumanovics et al., 2008) and diffuse cutaneous involvement (Blann et al., 1993, Ames et al., 1997). There are conflicting reports as to whether levels of circulating vWF are also raised in primary RP (Marasini et al., 1992, Marasini et al., 2000, Herrick et al., 1996).

Increased expression of PDGF within BAL fluid (and skin) of patients with SSc has been demonstrated (Ludwicka et al., 1995, Yamakage et al., 1992). Circulating PDGF levels have been found to be raised in SSc and may account for increased mitogenic activity (Hummers et al., 2009, Riccieri V, 2011). In contrast, other studies have failed to identify increased circulating PDGF in the plasma of SSc compared to healthy controls. (Solanilla et al., 2009, Silveri et al., 2001, Dunbar et al., 1993) Furthermore, PDGF is not secreted in higher amounts from activated platelets from patients with SSc compared with healthy controls following activation (Solanilla et al., 2009). Cross-talk between TGF-β and PDGF is thought to make an important contribution to fibrosis in SSc (Trojanowska, 2008). For example, TGF-β up-regulates expression of PDGF receptors on scleroderma fibroblasts (Yamakage et al., 1992). TGF-β is another constituent of platelet alpha granules and is considered a key cytokine in the pathogenesis of pulmonary and dermal fibrosis in SSc (Atamas and White, 2003). TGF-β was originally identified in the alpha granules of platelets (Lev et al., 2007). A recent study has identified associations between platelet count and aggregability with plasma levels of TGF-β (Peracoli et al., 2008). Like PDGF, there are increased levels of TGF-β in BAL fluid from patients with SSc compared with healthy controls (Ludwicka et al., 1995). There have been conflicting reports as to whether circulating levels of TGF-β are elevated in SSc compared with healthy controls (Dziadzio et al., 2005, Solanilla et al., 2009, Snowden et al., 1994).
As previously mentioned, the majority of sCD40L is platelet-derived and sCD40L has attracted interest as a potential biomarker in SSc due to its potential contribution to inflammation and fibrosis (Doran and Veale, 2008). Increased expression of CD40L on CD4 (+) activated T lymphocytes has been demonstrated in SSc (Valentini et al., 2000). The CD40L-CD40 interaction has also been shown to be important in the formation of autoantibodies to topoisomerase-I in SSc (Kuwana et al., 1995). Increased circulating sCD40L levels have been identified in the plasma and serum of patients with SSc (Allanore et al., 2005a, Komura et al., 2004). Elevated levels of sCD40L may be associated with a more vascular phenotype (PAH and DU disease) (Allanore et al., 2005a). It has been proposed that platelet activation at the site of endothelial damage may be responsible for the local release of VEGF and secondary disordered angiogenesis that occurs in SSc (Solanilla et al., 2009). Circulating plasma levels of VEGF are higher in SSc compared with PRP and healthy controls although VEGF is produced by a variety of cell types including endothelial cells, fibroblasts and leukocytes in addition to platelets (Solanilla et al., 2009, Hummers et al., 2009, Kuryliszyn-Moskal et al., 2005, Distler et al., 2002). Elevated circulating VEGF levels have been associated with vascular features including pulmonary arterial hypertension, the Raynaud’s severity score and the presence of giant capillaries on nailfold capillaroscopy, but may confer some protection against ischaemic tissue injury such as digital ulceration (Hummers et al., 2009, Pendergrass et al., 2010, Distler et al., 2002).

Other granule contents are stored, but not synthesized in platelets and hence circulating levels may not reflect platelet activation (Kamath et al., 2001). For example, 5-HT is synthesized by enterochromaffin cells within the gastrointestinal tract and serotonergic neurons within the central nervous system, however the majority of circulating serotonin is stored and released from platelets upon activation. Serotonin is a potent vasoconstrictor and promotes fibrosis, attracting attention for it’s potential contribution to the pathogenesis of SSc (Seibold, 1985). Circulating levels of 5-HT in primary RP and SSc have revealed contrasting results (Coffman and Cohen, 1994, Biondi et al., 1988). Platelet 5-HT levels are lower in the CREST variant of SSc compared with healthy controls which may represent the harvesting of ‘exhausted’ platelets following in vivo platelet activation and degranulation in SSc (Klimiuk et al., 1989a). A genetic polymorphism of the serotonin (5-HT$_{2A}$) receptor gene that blunts intracellular responses to serotonin may protect against the development of SSc (Beretta et al., 2008). Plasma levels of other platelet granule constituents such as the matrix metalloproteinases have also been found to be elevated in SSc (Hummers et al., 2009).
Whilst the above studies provide evidence of an association between increased platelet activation and SSc (and possibly primary RP), few of the studies confirm a direct pathogenic role of platelets in these conditions.

**Platelet membrane glycoproteins**

Several studies have identified higher circulating levels of the soluble form of P-selectin (sP-selectin) in SSc compared with healthy controls (Olewicz-Gawlik et al., 2010, Blann et al., 2003, Sfikakis et al., 1999, Iannone et al., 2008, Gruschwitz et al., 1995). sP-selectin values correlate with circulating levels of vWF in SSc, particularly in diffuse cutaneous disease (Blann et al., 2003). Increased expression of P-selectin (CD62P) in the stratum granulosum of skin biopsies has been reported in patients with SSc compared with healthy controls, particularly early in the course of their disease (Koch et al., 1993, Gruschwitz et al., 1995). Platelet surface P-selectin expression is increased in SSc compared with HC and primary RP, and falls in response to prostacyclin administration (Pamuk et al., 2007, Solanilla et al., 2009). The endothelin receptor antagonist bosentan has also been shown to reduce circulating levels of sP-selectin and other adhesion molecules in patients with SSc to levels seen in healthy controls (Iannone et al., 2008). In contrast, P-selectin expression is not increased in primary RP, although platelet-monocyte and platelet-neutrophil interactions are enhanced providing evidence of enhanced platelet-leukocyte interactions in both primary RP and SSc compared with healthy controls (Pamuk et al., 2007). A recent study identified reduced expression of GpIIb/IIIa receptors on the platelets of patients with primary RP, the authors proposing this may be the consequence of enhanced thrombocytopoiesis in an environment of persistent platelet activation (Polidoro et al., 2010).

**Eicosanoid biosynthesis**

Circulating TxB$_2$ levels are higher in SSc compared with HC (Herrick et al., 1996, Wilkinson et al., 1989). Similarly, urinary levels of 2-3 dinor TxB$_2$ (a stable urinary metabolite of TxB$_2$) are elevated in SSc compared with HC, and rise in response to total body cooling. Local cooling (forearm and hand) does not have the same influence on TxA$_2$ biosynthesis (Hutton et al., 1984, Sakamoto et al., 1999). Studies evaluating plasma levels of TxB$_2$ in primary RP have yielded conflicting results (Dowie et al., 1990). Leukocytes can also synthesize TxA$_2$ and elevated expression of genes encoding thromboxane synthase has been identified in leukocytes from patients with SSc (Young et al., 2002).
**Platelet-leukocyte interactions**

Platelet-monocyte and platelet-neutrophil complexes are present in greater numbers of both PRP and SSc when compared with healthy controls (Pamuk et al., 2007). The formation and stability of adhesion bonds necessary for platelet-leukocyte interactions is regulated by phosphatidylinositol-3 kinase (PI 3-K). It has been postulated that T-cell auto-reactivity to collagen I may account for the enhanced activity of PI 3-K in SSc platelets (Postlethwaite and Chiang, 2007).

**Platelet-derived microparticles**

Microparticles are small membrane bound vesicles with important pro-inflammatory and pro-thrombotic functions released from activated or dying cells. Increased circulating levels of platelet-derived microparticles have been identified in SSc (and mixed connective tissue disease) and are independently associated with symptoms of RP in connective tissue disease (Oyabu et al., 2011).

**Experimental models of systemic sclerosis**

Genetic and chemically induced animal models of systemic sclerosis have been developed, particularly in the context of skin and lung fibrosis. Much of the experimental work undertaken has explored pro-fibrotic signaling pathways and until recently, few studies had considered the contribution of platelets to the clinical phenotype in such models. In recent years, studies are beginning to emerge that have begun to address this shortfall. For example, Dees et al. have identified the important role of platelet-derived serotonin (5-HT) in the induction of ECM synthesis and fibrosis secondary to TGF-β/Smad signaling mediated by 5-HT₂B receptors which are themselves over-expressed in the skin of patients with SSc (Dees et al., 2011). Targeting the 5-HT₂B receptor through pharmacological inhibition, or genetic nullification of the 5-HT₂B receptor, ameliorates inducible and genetic experimental models of fibrosis. Furthermore, inhibition of platelet activation with the P2Y₁₂ receptor antagonist clopidogrel decreased dermal thickening, collagen content and number of activated fibroblasts in the skin of both Tsk-1 and bleomycin-challenged mice (Dees et al., 2011). Platelet trapping appears to be an important initiating event in chemically-induced murine models of fibrosis (Piguet and Vesin, 1994, Piguet et al., 1997). Komura et al. neatly demonstrated reduced dermal fibrosis and decreased generation of anti-topoisomerase-I autoantibodies in the tight skin mouse (Tsk-1) following blockade of the CD40/CD40L but
did not consider the implications of their findings in terms of the potential pathogenic role of platelets to this interaction (Komura et al., 2008).

The recent development of animal models exhibiting characteristic microvascular and fibrotic features of SSc, such as the fos-related antigen 2 (Fra-2) transgenic mouse, provide exciting novel opportunities to evaluate the contribution of platelets to both vascular and tissue remodelling in animal models of SSc. To date, no studies specifically exploring the contribution of platelets in these novel animal models of SSc have been undertaken.

1.4.5 Use of anti-platelet agents in the management of RP and SSc

The evaluation of anti-platelet therapy has been a largely neglected area of therapeutics in RP and SSc (Herrick, 2005). A summary of the therapeutic studies evaluating the use of anti-platelet agents in RP is presented in Table 1.3.
Table 1.3. Summary table of previous clinical trials evaluating the use of anti-platelet therapy in the management of RP and SSc

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Study Medication</th>
<th>Patients</th>
<th>Study Design</th>
<th>Endpoints</th>
<th>Outcome and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kahelah et al.</td>
<td>1982</td>
<td>ASA(150mg every 3rd day) and DIP (400mg/day)</td>
<td>SSc (n=10)</td>
<td>Open-label</td>
<td>Platelet aggregates and B-TG levels</td>
<td>Reduction in platelet aggregates and B-TG levels. No assessment of clinical efficacy.</td>
</tr>
<tr>
<td>Jones et al.</td>
<td>1983</td>
<td>3 weeks Dazoxiben 100mg qds vs. placebo</td>
<td>Severe RP (n=8, 2 patients had positive ANA but no clinical CTD)</td>
<td>Double-blind cross over study</td>
<td>Severity of RP symptoms and pain (4 point scales). Skin temperature (thermistor) and digital blood flow (plethysmography)</td>
<td>No significant improvement following treatment.</td>
</tr>
<tr>
<td>Coffman et al.</td>
<td>1984</td>
<td>Dazoxiben 100mg qds vs. placebo</td>
<td>PRP (13) SRP (12)</td>
<td>2 week Double-blind crossover study</td>
<td>Patient diary of frequency, pain intensity and duration of RP attacks. Fingertip blood flow, capillary flow, systolic blood pressure and radioisotope disappearance rate.</td>
<td>Decrease in number of RP attacks and percentage attack-free days in PRP group. Trend for increased fingertip blood flow following dazoxiben.</td>
</tr>
<tr>
<td>Luderer et al.</td>
<td>1984</td>
<td>Dazoxiben 100mg qds vs. placebo for 2 weeks</td>
<td>PRP (n=5) SSc (n=13) Other (3)</td>
<td>Double-blind, placebo-controlled cross over study</td>
<td>Frequency, duration and intensity of RP attacks. Both in vivo and ex vivo TxB₂ and 6 keto PGF₁α formation. Digital and forearm blood flow (plethysmography). Capillary blood flow (radioisotope disappearance rate).</td>
<td>Decreased in vivo and ex vivo TxB₂ production. Increased ex vivo 6 keto PGF₁α. No change in digital vascular or clinical outcomes.</td>
</tr>
<tr>
<td>Rustin et al.</td>
<td>1984</td>
<td>UK-38,485 (thromboxane synthetase inhibitor) vs. placebo</td>
<td>SSc (n=7), PRP (n=3)</td>
<td>Randomised double-blind cross-over</td>
<td>Number, severity and duration of RP attacks. 10cm pain VAS. Forearm blood flow (plethysmography), hand temperature (thermography), digital blood flow (photoplethysmography).</td>
<td>Significant reduction in pain VAS. No improvement in vascular assessments. Small study. The authors questioned dose of study medication.</td>
</tr>
<tr>
<td>Beckett et al.</td>
<td>1984</td>
<td>Aspirin (975mg/day) and dipyridamole (225mg/day) vs. placebo</td>
<td>SSc (n=28)</td>
<td>Double Blind RCT</td>
<td>Multisystem endpoints evaluated as better, same or worse.</td>
<td>No improvement in clinical or laboratory endpoints at 1 year. Exclusion of CREST patients. Highly damped subjective clinical endpoints.</td>
</tr>
</tbody>
</table>

PRP, primary Raynaud's phenomenon; SRP, secondary Raynaud's phenomenon; SSc, systemic sclerosis; PEP, photoelectric plethysmography; ASA, aspirin; DIP, dipyridamole; RCT, randomized controlled trial; CREST, Calcinosis, Raynaud's, (o)Esophageal dysmotility, Sclerodactyly, Telangiectasia; βTG, β-thromboglobulin; vWF, von Willebrand's factor; COX, cyclo-oxygenase; PF-4, Platelet factor 4; TI, thermographic index; LTG, longitudinal thermal gradient; TxB₂, thromboxane B₂; sig, significant; PGF₁α, Prostaglandin F₁α.
<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Study Medication</th>
<th>Patients</th>
<th>Study Design</th>
<th>Endpoints</th>
<th>Outcome and Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tindall et al.</td>
<td>1985</td>
<td>Acute effects of 4 doses of dazoxiben 100mg vs. placebo</td>
<td>PRP (n=3), MCTD (n=9) and healthy controls (n=9)</td>
<td>Randomized cross over study*</td>
<td>Finger blood flow (plethysmography), capillary blood cell velocity (videomicroscopy) and finger skin temperature (radiometry) during cold challenge</td>
<td>Significantly increased finger blood flow 15 minutes post-cold challenge after dazoxiben in patients. Increased 6-oxo-PGF&lt;sub&gt;1α&lt;/sub&gt; production following dazoxiben. Cold challenge increased local TxB&lt;sub&gt;2&lt;/sub&gt; production (reduced 65% following dazoxiben) *No improvement in finger skin temperature or capillary blood cell velocity in subset of patients who also entered 12 week placebo-controlled double blind RCT extension</td>
</tr>
<tr>
<td>Destors et al.</td>
<td>1986</td>
<td>6 weeks Ticlodipine (250mg B.D) vs. placebo</td>
<td>PRP (n=39), SSc (n=12) Other (n=12)</td>
<td>Double-blind RCT</td>
<td>Weekly frequency of RP attacks. Subjective 3 point ordinal scale; RP improved, same or worse</td>
<td>No improvement versus placebo. Heterogeneous population. Major placebo effect (65% reported improvement in RP)</td>
</tr>
<tr>
<td>Van der Meer et al.</td>
<td>1987</td>
<td>Aspirin 80mg od + placebo/ Aspirin 80mg od + Dipyridamole 75mg qds/placebo</td>
<td>Primary RP (n=18) and secondary RP (n=7, 1 with SSc)</td>
<td>Prospective placebo-controlled double-blind study</td>
<td>Severity of RP (subjective) PEP during cooling and re-warming Platelet count and morphology LTA to ADP Plasma BTG and PF-4 levels</td>
<td>Significant reduction in plasma BTG and PF-4 after treatment with ASA/Placebo and ASA/Dipyridamole. Majority of patients had primary RP</td>
</tr>
<tr>
<td>Lau et al.</td>
<td>1991</td>
<td>ICI 192,605 (a TxB receptor antagonist) vs. placebo 1 and placebo 2</td>
<td>SSc (n=8) and vibration white finger (n=8)</td>
<td>Double-blind randomised cross over study</td>
<td>Platelet aggregation to Thromboxane mimetic (U46619) Laser Doppler flowmetry (LDF) response to warming/cooling/re-warming</td>
<td>Significant inhibition of platelet aggregation No significant differences in LDF perfusion at baseline or following cooling. Significantly higher perfusion during re-warming following treatment vs. one of placebo arms only (possible confounding hang-over effect from active arm)</td>
</tr>
<tr>
<td>Pancera et al.</td>
<td>1997</td>
<td>Picotamide (300mg bd) vs. losartan (12.5mg od)</td>
<td>PRP (n=15)</td>
<td>Single-blind cross-over study</td>
<td>Number and severity of RP attacks, Digital blood flow (plethysmography)</td>
<td>No response to picotamide. Significant improvement in clinical and plethysmographic endpoints following losartan.</td>
</tr>
<tr>
<td>Easter et al.</td>
<td>2005</td>
<td>Aspirin (600mg)</td>
<td>PRP (n=15)</td>
<td>Open-label</td>
<td>Endothelial dependent vasodilation assessed using laser Doppler</td>
<td>Aspirin potentiates endothelial dependent vasodilation in PRP Aspirin may affect production of endothelial-derived vasoconstrictor COX products</td>
</tr>
</tbody>
</table>
Aspirin
Aspirin is the most commonly used anti-platelet agent, irreversibly acetylating COX-1 suppressing platelet production of TxA$_2$ for the duration of the platelet’s lifespan (due to the lack of platelet RNA necessary to resynthesize COX-1). Low-dose aspirin (40mg/day) is sufficient to reduce serum TxB$_2$ levels by over 90% (after one week’s treatment) with associated decreased platelet aggregability (Tohgi et al., 1988). Endothelial cells, meanwhile, rapidly resynthesize COX preserving production of endothelial COX-derived prostaglandins such as PG-I2 (prostacyclin: a potent vasodilator) despite treatment with low dose aspirin (Jaffe and Weksler, 1979, Tohgi et al., 1992, Moncada, 2006). No studies have specifically evaluated the long term safety or clinical efficacy of aspirin therapy in primary RP or SSc. A small number of studies have demonstrated potentially useful biological effects of aspirin within these patient groups. For example, low dose aspirin reduces circulating amounts of supranormal vWF multimers in SSc (Mannucci et al., 1989). Aspirin has also been shown to potentiate endothelium-dependent vasodilation measured using laser Doppler in patients with primary RP, possibly due to the reduced synthesis of endothelium-derived vasoconstrictor COX products (Easter and Marshall, 2005). In contrast, other studies have demonstrated persistent expression of P-selectin and platelet-leukocyte interactions in patients with SSc despite treatment with aspirin (Agache et al., 2007, Pamuk et al., 2007). This may be the result of persistent platelet activation secondary to non-COX derived platelet activators, such as collagen expression on damaged endothelial surfaces in SSc.

Thromboxane signalling inhibition
After promising pilot work, subsequent therapeutic trials of TxA$_2$ synthetase inhibitors (primarily dazoxiben) in the treatment of RP yielded generally disappointing results (Table 1.3) (Coffman and Rasmussen, 1984, Belch et al., 1983, Jones and Hawkey, 1983, Rustin et al., 1984, Luderer et al., 1984, Tindall et al., 1985, Pancera et al., 1997). These treatments effectively suppress TxA$_2$ formation, with the added benefit of re-directing AA metabolism into prostacyclin production (Belch et al., 1983, Luderer et al., 1984, Tindall et al., 1985). Several studies of TxA2 synthetase inhibition have incorporated objective microvascular imaging techniques (such as thermography); although only 2 have reported improvements following therapy (Belch et al., 1983, Tindall et al., 1985). Pancera et al. failed to identify improvement in clinical severity or objective vascular function (plethysmography) in primary RP following picotamide therapy (inhibits both the synthesis
and receptor of TxA$_2$ (Pancera et al., 1997). The effects of a specific thromboxane receptor antagonist in a small number of patients with SSc (n=8) revealed a significant reduction in aggregation to a thromboxane mimetic (U46619) but no significant alteration in digital perfusion assessed using LDF, although a hang-over effect following treatment with the active drug may have confounded the findings within the placebo arms (Lau et al., 1991). Whilst the findings of the aforementioned studies fail provide strong support for antagonising TxA$_2$ signalling, it is important to note the chief limitations of these early studies that included their small size, inclusion of a heterogeneous mix of primary and secondary RP, and the lack of validated endpoints by which to assess peripheral vascular function in RP.

**Adenosine diphosphate (ADP) signalling inhibition**

P2Y$_{12}$ receptor inhibitors (e.g. Clopidogrel, Ticlodipine) block ADP-dependent platelet activation. Only one study has considered this class of treatments for the management of Raynaud’s phenomenon and failed to identify any clinical improvement in a heterogeneous population of RP patients after 6 week’s therapy with ticlodipine (Destors et al., 1986). This study did not evaluate the effects of ticlodipine on laboratory markers of platelet function or incorporate any objective measures of vascular perfusion. The previously discussed study by Dees et al. meanwhile would support further assessment of P2Y$_{12}$ receptor inhibition on the dermal and visceral fibrosis in SSc (Dees et al., 2011).

**Adenosine reuptake inhibition**

Platelets and endothelial cells indirectly produce adenosine as a breakdown product of ATP. Adenosine is a potent vasodilator and powerful inhibitor of platelet aggregation. Dipyridamole antagonises the nucleoside transporter responsible for the uptake of adenosine, thereby inhibiting platelet function (Kim and Liao, 2008). In addition, dipyridamole actively promotes vasodilation through the direct local effects of adenosine and selective inhibition of phosphodiesterase (potentiating the action of local NO) (Kim and Liao, 2008). Additional pleiotropic effects of dipyridamole include increased endothelial prostacyclin production, antioxidant properties and anti-inflammatory actions such as inhibition of the monocyte-platelet interaction and reduced recruitment and activation of leukocytes at sites of injury (Kim and Liao, 2008). It is therefore an attractive potential therapeutic agent in SSc. Dipyridamole has been shown to selectively reduce mitogenic activity in the sera of patients with systemic sclerosis, possibly by reducing PDGF secretion (Takehara et al., 1987, Takehara et al., 1990). Studies evaluating the effects of
dipyridamole (with and without aspirin) on circulating platelet aggregates and levels of α-
granule release products (PF-4 and β-thromboglobulin) have yielded conflicting results
(Takehara et al., 1990, Kahaleh et al., 1982).

The results of two randomised controlled trials (RCT) evaluating treatment with combination
aspirin and dipyridamole therapy in patients with RP were disappointing (Beckett et al.,
1984, van der Meer et al., 1987). The first evaluated combination aspirin (975 mg/day) and
dipyridamole (225 mg/day) for 1 year in 28 patients with early SSc, and failed to identify
improvements in clinical endpoints. Limitations of the study included small study size (n=14
in each arm) and subjective insensitive endpoints. For example, peripheral vascular
assessment was based upon a physician clinical assessment and rated as same, better or
worse. The dosing was possibly sub-optimal with the high-dose aspirin capable of inhibiting
endothelium-derived prostacyclin synthesis along with a relatively low dose of dipyridamole
(Moncada, 2006). A subsequent double-blind RCT study evaluated the short-term effects of
aspirin (80mg daily), combination aspirin (80mg daily) and dipyridamole (300 mg/day) or
placebo in a heterogeneous population of RP (18 primary and 7 secondary RP; only one of
whom had SSc) (van der Meer et al., 1987). This study revealed a significant reduction in
aggregation to ADP, and reduced circulating BTG and PF-4 following treatment with aspirin
alone or in combination with dipyridamole (van der Meer et al., 1987). Subjective (patient
classified response to therapy as improved, unchanged or worsened) and objective
(photometric plethysmography) measures of peripheral vascular function did not improve
following therapy with either aspirin alone or combination aspirin/dipyridamole (van der
Meer et al., 1987). These findings are disappointing as this combination of anti-platelet
agents is the most attractive in terms of therapeutic potential in RP/SSc in view of their
direct vasodilatory, anti-oxidant and anti-fibrotic potential.

Pleiotropic anti-platelet effects of other drugs
It has been suggested that the clinical benefits of a raft of treatments commonly used for the
management of RP symptoms including prostacyclin,(McHugh et al., 1988, Solanilla et al.,
2009) calcium channel blockers (Wigley et al., 1987, Malamet et al., 1985), piracetam
(Moriau et al., 1993), fluoxetine (Coleiro et al., 2001), ketanserin (Seibold, 1985) and Ginkgo
Biloba (Stucker et al., 1997) are thought to be in part the result of the pleiotropic effects of
such treatments on platelet function.
1.4.6 Challenges in establishing a role for anti-platelet therapy in RP and SSc

The results of early clinical trials of anti-platelet agents in RP and SSc have been generally disappointing. It would be premature to conclude that such studies confirm the lack of efficacy of anti-platelet agents, particularly in SSc. Major limitations of these studies include small study sizes, short study duration and the incorporation of a heterogeneous mix of RP patients (the majority of whom had primary RP). Whilst platelet activation may contribute to both immune dysfunction and fibrosis in SSc, the slowly progressive nature of tissue fibrosis would require long-term RCTs involving large numbers of patients to demonstrate the efficacy of anti-platelet therapy for such patients. The use of experimental models of fibrosis such as the previously discussed work by Dees et al. provides an exciting opportunity to establish a proof-of-concept in pre-clinical studies (Dees et al., 2011). Evaluating the contribution of platelets to vascular function in RP and SSc is more feasible in clinical practice. The lack of sensitive and validated clinical and vascular endpoints by which to evaluate digital vascular function was a major limitation of previous therapeutic trials of anti-platelet agents. The emergence of validated clinical outcome measures and novel methods for objectively assessing vascular function in RP and SSc provides the opportunity to re-appraise the vascular effects of anti-platelet therapy in such patients. It is to quantifying peripheral vascular function in RP and SSc that I shall now divert our attention.

1.5 Quantifying digital vascular function in the assessment of RP and SSc

Due to the episodic nature of RP, objective assessment of digital vascular function can not easily be undertaken in the clinical setting. This has led to reliance upon subjective patient self-reports and it remains standard practice for clinicians to appraise the efficacy of new treatments for RP in clinical practice by simply enquiring as to whether or not the patient feels the treatments have been beneficial for their symptoms. With novel vasoactive treatments costing in excess of £21K per annum, there is an urgent need for validated assessment tools for the quantification of digital vascular function in RP. In this section, I shall describe the various subjective and objective methods that have emerged for the assessment of digital vascular function in RP and SSc. To better understand such methods,
I will first describe healthy cutaneous vascular anatomy and physiology in humans, with particular reference to the digits.

1.5.1. Normal digital cutaneous vascular anatomy and physiology

The cutaneous circulation has functions beyond nutritional support including wound repair, immunity and thermoregulation, which are of particularly importance when considering vascular physiology within the digits. The radial and ulnar arteries are the principle large vessels supplying oxygenated blood to the hand. Distal to the wrist, these arteries divide and anastamose in the deep and superficial palmer arches. From these arches arise the common palmer digital arteries that supply the proper digital arteries extending into the digits (Figure 1.6). The palmer tissues of the digits are supplied by small palmer branches arising from the main digital arteries (Voche and Merle, 1996). The dorsal aspect of the fingers, meanwhile, are supplied proximally by terminal branches of the dorsal metacarpal artery at the MCP and distally by dorsal cutaneous branches arising from the proper palmer digital arteries (Endo et al., 1992). The nailbed has proximal and distal arterial arches formed from anastamoses of branches of the digital arteries. From these arterioles arise a capillary loop system that can be directly visualised at the nailfold (which is the basis of nailfold capillaroscopy).

All areas of skin have an arcade of arterioles at the interface between the dermis and deeper structures. Arterioles arise from this arcade and ascend into the superficial dermis. Capillary loops arising from this second network of arterioles extend into the sub-papillary plexus at the dermal-epidermal interface. The capillaries drain into venules and the deeper veins that accompany the arteriolar supply within the dermis (Rhoades, 2009).

It has long been recognised that the cutaneous circulation is not uniform across the body. The advent of non-invasive measures of microvascular function allowed, for the first time, the assessment of the variability of cutaneous blood flow across the body. Tur et al. used photopulse plethysmography and laser Doppler velocimetry to measure basal skin blood flow at 52 anatomical sites, identifying regions in the hands and face for which cutaneous perfusion was much higher (Tur et al., 1983).
The regions identified by Tur et al. can be broadly separated into glabrous (from Latin for hairless) and non-glabrous areas. Glabrous skin differs anatomically due to the presence of multiple arterio-venous anastamoses (AVAs). These thick walled, low resistance vessels allow blood to pass directly from arterioles to venules. Alteration of vascular tone within these AVAs can have substantial effects on cutaneous blood flow. Non-glabrous skin has few, if any, AVAs (Charkoudian, 2003). The sympathetic nervous system is responsible for control of AVA function and the cutaneous circulation. Under ambient temperatures significant sympathetic mediated vasoconstriction is required to maintain basal tone of the vascular smooth muscle. Blockade of sympathetic function leads to maximal cutaneous vasodilation and cessation of sympathetic noradrenergic activity is an important mediator of vasodilatation in response to heat.

α₁ adrenoceptors are the predominant receptor type on the larger arteries and arterioles. Selective α₁ adrenoceptor antagonists such as doxazosin are potent vasodilators of these

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4 Permission not required as copyright has lapsed into the public domain
vessels and are effective in the management of macrovascular diseases such as hypertension. α2-adrenergic receptors are the predominant adrenergic receptor residing on terminal arterioles, venules, veins and AVAs and are of greater importance in thermoregulation and microvascular disorders such as Raynaud’s phenomenon (section 1.2.3). α2-adrenergic receptors promote vascular wall contraction through influx of extracellular calcium through voltage-dependent (dihydropyridine sensitive) channels which can be antagonised using dihydropyridine calcium channel antagonists such as nifedipine (Pollock et al., 1996).

In a warm environment dilated AVAs can provide the pathway for 80% of digital blood flow, making a crucial contribution to thermoregulation. The remaining 20% of blood passes through the capillary bed where metabolic exchange can take place. This is usually described as the nutritional blood supply. The skin’s relatively low metabolic activity requires relatively low perfusion to meet its nutritive needs (around 40mls/min) and hence the large shifts in cutaneous perfusion (up to 2.8L/min) which occur are primarily to facilitate thermal homeostasis at times of increased heat generation (e.g. exercise). In ambient conditions basal cutaneous blood flow is around 400mls/min. Following cold exposure, AVAs virtually close following α2-adrenergic receptor stimulation, diverting the majority of cutaneous blood flow away from the dermis. The nutritional capillary vascular bed remains patent in all but the most extreme cold exposure to prevent cutaneous ischaemic necrosis (Berlin and Pehr, 2004, Charkoudian, 2003, Krogstad et al., 1995, Rhoades, 2009). Glabrous regions within the fingertips and nailbeds are densely populated with AVAs providing an important role in thermoregulation (Baran, 2003). The dorsum of the hand is less densely populated with AVAs (non-glabrous skin) resulting in lower blood flow relative to glabrous regions under ambient conditions when AVAs are dilated. The varied cutaneous perfusion within the digits at rest and altered response to cold exposure forms the basis of non-invasive assessment of digital vascular function in RP and SSc (section 1.5.3).

1.5.2 Subjective patient self-reports for the assessment of digital vascular function in Raynaud’s phenomenon

The episodic nature of RP attacks prevents useful objective assessment of disease activity in the clinical setting, leading to a reliance on patient self-report. In early therapeutic studies of RP, treatment efficacy was often reported by asking patients to categorise responses following treatment using nominal scales such as better, same or worse (Yamaoka, 1987,
Martin et al., 1981, Dowd et al., 1982, Clifford et al., 1980). Analyses were largely descriptive and used comparisons of the proportion of patients represented in each group. These self-report measures were gradually superceded by linear psychometric scales such as rating scales and visual analogue scales (VAS) which provided the opportunity to quantify and explore specific components of RP such as pain intensity and the severity of attacks (McHugh et al., 1988, Kyle et al., 1985, Kyle et al., 1992, Klimiuk et al., 1989b). Latterly, the use of diary cards has facilitated quantification of the frequency and duration of RP attacks, often incorporating VAS scores of RP severity (Selenko-Gebauer et al., 2006, Herrick et al., 2000, Dziadzio et al., 1999, Coleiro et al., 2001). The Raynaud’s Condition Score (RCS) diary was originally developed for 2 separate studies of oral iloprost (Black et al., 1998, Wigley et al., 1998). This diary collected daily information over a 2 week period of the frequency and duration of RP attacks whilst also incorporating a 10 point rating scale (the Raynaud’s Condition Score, RCS) that enabled patients to report the severity of their RP that day (Black et al., 1998, Wigley et al., 1998). The RCS diary performed well in a post hoc analysis of the validity, feasibility and reliability of the tool (Merkel et al., 2002). The RCS diary has been recommended for use for the subjective assessment of digital vascular function in therapeutic trials of RP and SSc (Merkel et al., 2003, Khanna et al., 2008). Additional description of the development, validation and use of the RCS diary can be found in sections 2.3.3 and 5.1.

There are major limitations to reliance on self-report measures of RP severity. These tools are subjective, influenced by health beliefs and psychological factors and sensitive to placebo effect. They require a prolonged period of assessment and are laborious for patients (with the subsequent potential for ‘diary fatigue’). Furthermore, the frequency, duration and severity of RP attacks are likely to be influenced by both seasonal variation in environmental temperature and the effectiveness of coping strategies adopted by patients to avoid the conditions responsible for RP attacks and ameliorate attacks when they occur. There are additional challenges using the RCS diary in SSc. Habituation to digital ischaemia occurs along with difficulty distinguishing acute attacks of RP from background digital ischaemia (secondary to irreversible morphological vascular changes) which can lead to underestimation of the extent of digital vascular dysfunction. These limitations have led to considerable work to develop objective methods for assessing digital vascular function.
1.5.3 Established objective non-invasive assessment tools of digital microvascular function in RP and SSc

A variety of non-invasive microvascular imaging tools have been developed to objectively assess digital vascular function and they are already recommended for the classification of conditions such as primary Raynaud’s phenomenon (RP) and systemic sclerosis (SSc) (LeRoy and Medsger, 2001). As previously discussed, structural vascular morphology can be assessed in vivo using nailfold capillaroscopic assessment of capillary loops (Herrick and Cutolo, 2010). This approach is of particular value in identifying patients at risk of SSc early in the course of the disease, but does not allow assessment of digital vascular response following treatment. To this end, various methods for the dynamic assessment of digital vascular function have emerged (often incorporating a cold challenge in an attempt to recreate the conditions responsible for RP attacks). I shall not describe all the available methods in detail here but shall devote some attention to infrared thermography (IRT) which is the most widely evaluated technique (sometimes considered the “silver standard”) and laser-derived methods which have emerged over the last 30 years. Some knowledge of the basic physics of electromagnetic radiation, heat transfer, the Doppler effect and light interference is assumed. Similarly, a comprehensive discussion regarding the mathematical principles adopted in quantifying tissue perfusion using laser techniques has been described previously and is beyond the scope of this thesis (Briers, 2007, Briers, 2001, Briers, 2006, Briers, 1996a).

**Infrared thermography**

Radiation describes the process by which energy is transmitted through objects or space. Objects radiate thermal energy through electromagnetic waves (EMW). The electromagnetic spectrum (EMS) has been arbitrarily divided into distinct bands according to wavelength, extending between radiowaves (with the longest wavelengths) and gamma rays (the shortest). The “visible spectrum” of the EMS is a narrow band (390-750nm) of EM radiation that can be detected by the human eye. Infrared (IR, from the latin *infra*, below) radiation is electromagnetic radiation (EMR) with a wavelength between 700nm and 300 micrometres (spanning the limit of visual perception at short wavelengths to “microwave” radio wavelengths at the long-wavelength end of the IR spectrum). The IR spectrum includes most of the thermal radiation emitted by objects at near room temperature. Mammals at around 300 Kelvin emit radiation of a wavelength of around 10 micrometers in
the far infrared. Wein’s displacement law implies that as an object’s temperature increases, the shorter the wavelength at which it will emit most of its radiation. Around the “visible spectrum”, this phenomenon is occasionally observed e.g. when a metal object becomes ‘red hot’. Infrared thermography (IRT) cameras quantify IR emissivity to estimate the surface temperature of an object. The temperature of the human skin increases with increasing perfusion, allowing IRT to provide a safe, non-invasive, indirect measure of vascular function (Ammer, 2009).

A large number of studies have reviewed the use of IRT in the assessment and diagnosis of RP. A detailed description of this work is beyond the scope of this thesis but has been reviewed previously by others including myself (Pauling, 2010b). The longitudinal thermal gradient within the hands has consistently performed well as an endpoint for both diagnosing and differentiating between disease states in RP. This gradient provides information on AVA function within the fingertips. In healthy digital vascular function, perfusion is greater at the fingertips (due to dilation of the thermoregulatory AVAs described in section 1.5.1) leading to a positive thermal gradient (Figure 1.7). In RP and SSc, a negative longitudinal thermal gradient arises due to excessive vasoconstriction of digital AVAs (Figure 1.7 and 1.8). Various methods for calculating this gradient have been developed including the “longitudinal temperature difference” (Schuhfried et al., 2000), “distal-dorsal difference” (Anderson et al., 2007) and the “combined thermal gradient” (Ring, 1990, Ring, 1988). Many thermographic protocols incorporate a local cold challenge (e.g. placing the hands in a cooled water bath at 15-20°C for 60s) to allow dynamic assessment of digital vascular function during the conditions typically responsible for an attack of RP in vivo (Figure 1.8).
Figure 1.7 Examples of thermal images in a healthy control and a patient with systemic sclerosis.

On each image regions of interest have been placed over the dorsum of the hands (proximal [dorsal] region) and over the digits to allow calculation of the longitudinal thermal gradient (or distal dorsal difference).

Thermal images obtained from our laboratory with proximal and distal regions of interest are marked (adapted from (Ring et al., 1981)). A) Healthy control (right proximal mean surface temperature 33.23°C, right distal mean temperature 34.05°C, gradient +0.82°C). B) Systemic sclerosis (right proximal mean surface temperature 30.87°C, right distal mean temperature 28.07°C, gradient -2.8°C).
Figure 1.8 Examples of baseline assessment and re-warming curves following cold challenge in a healthy control (HC) and a patient with SSc.

Data collected from our laboratory. In the HC there is a positive distal-dorsal thermal gradient at baseline and rapid rewarming of the fingertips following cold challenge. In SSc the distal-dorsal thermal gradient is negative at baseline and there is delayed re-warming of fingertips following cold challenge. Baseline skin temperature and response to cold challenge over the dorsum of the hands is similar for both patients reflecting the absent contribution of thermoregulatory AVAs influencing cutaneous perfusion.
Due to initial difficulties in obtaining and analysing thermal images, many early studies incorporating a cold challenge were restricted to a single post-cold challenge thermographic assessment (Ring et al., 1981). The ability to capture multiple thermographic images with modern IRT cameras has allowed repeated assessment following cold challenge and mapping of re-warming curves (Figure 1.8). This has led to the emergence of additional parameters such as the maximum gradient of recovery, maximum re-warming percentage and the lag time to commencement of re-warming which have been used successfully in discriminating between disease states in RP (O’Reilly et al., 1992). It remains unclear whether baseline assessment of the longitudinal thermal gradient alone has comparative, if not superior, discriminatory capacity in differentiating between disease states in RP, to those parameters derived from the re-warming curve characteristics (Schuhfried et al., 2000, Anderson et al., 2007, Clark et al., 1999). The additional diagnostic value derived from undertaking a standardised cold challenge assessment is unclear, although it unquestionably provides information on dynamic vascular responses that baseline assessment alone can not provide (Pauling et al., 2011b, Pauling, 2010a). There are several limitations to IRT, particularly when considering the effects of the cold challenge. IRT assessment following cold challenge fails to consider the influence of conductive and convective heat exchange on surface skin temperature, unrelated to vascular perfusion. Translation of altered microvascular tone into changes of surface skin temperature is influenced by the thermal capacity of the surrounding tissue, limiting the temporal resolution of IRT. The spatial resolution, meanwhile, is influenced by thermal conduction of heat from adjacent and/or deeper tissues, which may not be a true reflection of superficial cutaneous perfusion.

Several studies have attempted to use IRT as an endpoint in the evaluation of treatments for RP (McHugh et al., 1988, Dziadzio et al., 1999, Coleiro et al., 2001), although no effort has been made to systematically review these studies to establish a preferred thermographic parameter for use in therapeutic trials of RP. As a result, IRT has remained a largely experimental endpoint in clinical trials of RP (Merkel et al., 2003).

**Laser Doppler imaging of vascular function**

When there is relative movement between the source of a wave and an observer, the frequency of the wave changes. Christian Doppler (1803-53) was the first to describe this effect in 1842. The audible drop in pitch as an object fitted with a siren travels towards us
which we are all well acquainted with is the commonly reported example of a Doppler shift in action. The magnitude of the frequency change elicited through the Doppler effect can be used to calculate the velocity of an object. Light scattered from moving particles within a stationary object causes a similar shift in frequency in relation to non-shifted light from stationary matter. Lasers (Light Amplification by Stimulated Emission of Radiation) are highly amplified coherent radiation of one or more discrete frequencies. When lasers are focussed on an object, the manner in which the reflected radiation is scattered will be influenced by both the surface of the object and the presence of moving particles within the object (such as blood cells in the skin). The emergence of lasers in the 1960’s, along with the mathematical application of Doppler principles, led to the development of laser Doppler imaging (Briers, 2007, Briers, 2001). The high frequencies of light waves render them difficult to measure directly. It is, however, possible to measure the beat frequency generated by interference between the original light source and the shifted reflected wave. This frequency shift (or beat frequency) is much lower than the frequency of the original waves and therefore provides a more accessible means of estimating the velocity of particles within objects (Briers, 2001). Laser Doppler relies on certain assumptions such as the object remaining stationary and the isotropic distribution of light within the illuminated tissue (Ruth, 1994). Perfusion should be strictly defined as volume per unit area per unit time, however, as no laser instrument is capable of directly measuring blood flow, measurements derived from laser imaging tools are typically described in arbitrary flux units.

The first laser Doppler devices (laser Doppler flowmetry [or velocimetry], LDF) measured blood flow at a single point using a contact probe. The major limitations of this approach were its limited spatial resolution and poor repeatability (due to the challenge of attaching the LDF probe in the same place at each assessment). The tissue contact associated with laser Doppler flowmetry (LDF) may also influence flux measurement, damage tissue and precludes assessment of perfusion at inaccessible sites such as the retina (Ruth, 1994).

The need for non-contact tools that could map perfusion of tissue over a larger area led to the development of laser Doppler perfusion imaging (LDPI). This technique uses a non-contact scanning laser that measures blood cell velocity at multiple single points, generating a 2-dimensional map of tissue perfusion (Briers, 2001). LDPI overcame some of the limitations of LDF, although the temporal resolution was limited by prolonged scanning times (initially lasting up to several minutes) precluding useful assessment of cutaneous
microvascular responses to physiological stimuli (Stewart et al., 2005, Roustit et al., 2010b, Briers, 2001).

1.5.4 Laser speckle contrast imaging (LSCI)

Laser speckle contrast imaging (LSCI) is a novel non-invasive microvascular imaging modality that provides near real-time dynamic vascular assessment over large areas of tissue (Briers, 1996a, Briers, 2006, Briers, 2001). The historical development, mathematical assumptions and processing techniques used as part of laser speckle contrast imaging (LSCI) have been described elsewhere (Draijer et al., 2009, Briers, 2007, Briers, 2001, Briers, 1996b, Briers, 2006, Briers, 1996a). I shall not emulate this work other than providing a brief introduction to the principle of LSCI but will instead devote my attention to previous work applying this technique within the field of cutaneous vascular physiology.

Background to laser speckle imaging

Laser speckle is a random interference effect that was originally regarded as an optical nuisance, limiting resolution in techniques such as holography (Briers, 2001, Briers, 2006). Directing a laser source at an object (such as a wall) generates a speckle pattern following scattered reflection from the uneven surface. If the object remains static then this speckled interference pattern will not change. Movement of the object, or of particles within an object, meanwhile will alter the scattering of light as it strikes the object leading to speckle variation and a subsequent reduction in speckle contrast. From the study of the temporal statistics of the speckle fluctuations, information can be gathered on the degree of motion within the object. This principle forms the basis of laser speckle contrast imaging (LSCI). On face value, the physics of LCSI appear distinct from laser Doppler however the dynamic (blurred) speckle pattern produced following the interaction of laser light with moving particles within an illuminated object is primarily the result of Doppler shifts and the mathematical formulae used to interpret images do not differ greatly from those used in laser Doppler imaging (Briers, 2001).

The study of time-varying speckle can be applied to the study of blood flow within living tissues. Within areas of high blood flow, the contrast of the speckle becomes blurred, whereas areas of relatively lower perfusion have the appearance of a more static contrast speckle pattern (Stern, 1975). Fercher and Briers adopted this principle to develop single
exposure speckle photography which could be used to map, but not quantify, tissue perfusion (Briers and Fercher, 1982). The advent of digital imaging and processing has allowed real time quantification of speckle contrast (and hence vascular flux) along with the generation of a false colour map of speckle contrast. Speckle contrast is quantified by calculating the ratio of the standard deviation to the mean of the intensities recorded for each pixel within squares of 5x5 or 7x7 pixels (Briers, 2006). This technique was initially termed LASCA (LAser Speckle Contrast Analysis) and its chief strengths were its simplicity and low cost (Briers, 2006). Early LASCA cameras were limited by a restricted field of view which suited early studies examining cerebral and retinal perfusion in animal models (Durduran et al., 2004). The extended field of view provided by modern cameras (e.g. the Moor FLPI LSCI) has greatly increased the potential clinical application of the method and over last 5 years there has been a considerable growth in the number of studies evaluating the use of LSCI to measure tissue perfusion (Figure 1.9).

**Figure 1.9 Number of publications of studies evaluating LSCI retrieved from the US National Library of Medicine National Institutes of Health 2001-2012**

There are important differences between laser Doppler and speckle contrast imaging that deserve mention. Firstly, Laser speckle contrast imaging lacks the spatial resolution of conventional laser Doppler, but this is offset by rapid image acquisition, providing the opportunity for near real-time dynamic assessment of vascular perfusion over a wide area.
LSCI and LDI respond differently to the optical characteristics of an illuminated object. LDI perfusion flux values increase with increasing tissue absorption whereas LSCI flux values demonstrate a slight decrease (Forrester et al., 2004). Furthermore, the depth of tissue penetration is dependent on the wavelength of the laser used. The deepest penetration is achieved using infrared (>700nm, several mm penetration), red (~630nm, about 1-2 mm), green (~530, around 0.15mm) and virtually none for blue (Briers, 2006). This may provide an attractive opportunity to study microvascular function at varying levels within the skin such as comparing superficial nutritive capillary flow compared with deeper vascular function within the dermis and thermoregulatory AVA function. Modern LSCI devices (such as the moorFLPI) uses a laser wavelength of 775nm achieving a depth of penetration in human skin of approximately 1mm. LDPI scanners have variable laser wavelengths of between 633nm and 830nm providing depth of penetration of between 0.1 and 2mm. These differences prevent direct comparison of flux values elicited using the 2 techniques on different tissues, although assessment of relative changes in tissue perfusion overcomes such concerns.

Use of laser speckle imaging in the assessment of cutaneous perfusion
The limited processing capability of early LASCA cameras restricted use to retinal or cerebral perfusion in animal models. More recent studies have extended the use of LSCI to the evaluation of cutaneous perfusion (Ruth, 1994, Forrester et al., 2002, Bezemer et al., 2010, Roustit et al., 2010b, Murray et al., 2009, Forrester et al., 2004). A recent study has successfully applied LSCI to demonstrate impaired endothelium-dependent vasodilatory responses in patients with cardio-metabolic disease (Cordovil et al., 2012). Several studies have compared LSCI with other non-invasive microvascular imaging tools including nail fold capillaroscopy (NC) (Bezemer et al., 2010, Murray et al., 2009), LDPI (Stewart et al., 2005, Forrester et al., 2004, Forrester et al., 2002, Millet et al., 2011) and LDF (Roustit et al., 2010b).

Good correlation between LSCI and LDF was identified in the dynamic assessment of forearm perfusion in healthy volunteers (Roustit et al., 2010b). As expected, subsequent studies identified superior reproducibility with LSCI compared to LDF due to the challenges correctly re-positioning the LDF probe (Tew et al., 2011, Roustit et al., 2010a). Comparison of LSCI and LDPI in the dynamic assessment of cutaneous perfusion has been undertaken in healthy controls (Millet et al., 2011, Forrester et al., 2002) and the evaluation of burn
injuries (Stewart et al., 2005). There is strong correlation between the 2 imaging modalities (Forrester et al., 2002, Stewart et al., 2005, Millet et al., 2011). The improved temporal resolution favours LSCI (Stewart et al., 2005, Forrester et al., 2002). LSCI has also been shown to have a lower inter-site variability compared with LDPI (Millet et al., 2011). LSCI has also demonstrated superior reproducibility to LDPI (Roustit et al., 2010a).

Comparison of dynamic assessment of perfusion at the nailfold using both LSCI and capillary red blood cell velocity measured using sidestream dark field (SDF) imaging (i.e. capillary microscopy) has also been undertaken in healthy controls with good correlation between the 2 techniques (Bezemer et al., 2010). No previous studies have compared IRT and LSCI in the assessment of cutaneous vascular function.

**Use of laser speckle imaging in RP and SSc**

Only one previous study has evaluated the use of LSCI in scleroderma spectrum disorders. LSCI assessment of perfusion at the nailfold of the non-dominant ring finger was undertaken in healthy controls and patients with RP and SSc (Murray et al., 2009). No significant differences were observed between groups and there was poor correlation between LSCI and NC at the nailfold, although the choice of site may have accounted for these findings. As previously discussed, morphological vascular changes occur at the nailfold in SSc which include neoangiogenesis (e.g. elongated dilated and tortuous vessels) which may have influenced perfusion values at the nail fold. Reproducibility of LSCI in this study was poor (ICC 0.15) although repeatability was assessed in a relatively small number of subjects (n=5) and reproducibility needs to be re-evaluated in larger studies (Murray et al., 2009). To date, no studies have evaluated the use of LSCI in the dynamic assessment of vascular function in RP or SSc.

1.6 Hypotheses

Understanding the influence of platelets on vascular function, inflammation and tissue remodelling provides an attractive conceptual framework to consider the potential contribution of platelets to the pathogenesis of RP, particularly in the context of SSc. There is a considerable body of evidence associating excessive platelet activation in both RP and SSc. Recent work has begun to consider the potential direct pathogenic effects of platelets in
animal models of SSc. The findings of such studies suggest the release of growth factors from platelets may make an important contribution to downstream pro-fibrotic signalling.

The results of previous therapeutic trials of anti-platelet therapy in RP and SSc have been disappointing. Major limitations of these studies include small study sizes, short study duration and the incorporation of a heterogeneous mix of RP patients (the majority of whom had primary RP). The lack of sensitive and validated clinical and vascular endpoints by which to evaluate digital vascular function and other clinical manifestations of SSc was an additional major limitation of these early trials. Anti-platelet agents have revolutionized the management of cardiovascular diseases, although it is unlikely that similarly designed studies to those undertaken in RP and SSc could have provided evidence of their efficacy. We feel it is premature to conclude that previous studies confirm the lack of efficacy of anti-platelet agents in these conditions.

Due to the high cost and lack of industry motivation to support such work, it is feared that major trials of anti-platelet therapy in RP and SSc may never be undertaken. This is disappointing considering the low cost, established safety and widespread availability of anti-platelet agents. Animal models, despite their many limitations, may provide a useful pre-clinical model to elucidate the key platelet-derived mediators in SSc and the anti-platelet treatments (or combination of agents) that offer the greatest therapeutic. We feel there is also a role for further proof-of-concept clinical trials in humans. The emergence of validated clinical vascular endpoints and non-invasive microvascular imaging techniques now provide the opportunity to re-appraise the effects of anti-platelet therapy on peripheral vascular function in these related conditions. In addition to re-appraising established clinical and objective vascular endpoints (namely IRT) in clinical trials, novel microvascular imaging modalities such as LSCI may have an important role in future exploratory studies and further validation work of this technique in RP and SSc is now needed.

The principal hypotheses of this thesis can be broadly summarized into the following categories:

1. Objective measurement of digital vascular function (with established and novel methods such as IRT and LSCI) has advantages over subjective self-report in the
assessment of RP activity and should be at the core of future therapeutic trials of RP.

2. LSCI is a novel method for the objective assessment of digital vascular function in the both diagnosis and assessing treatment efficacy in RP and SSc.

3. Platelets are central to the cellular mechanisms driving vascular dysfunction in RP, particularly in the context of SSc.

4. The competing action of platelet and endothelial derived vasoactive mediators are associated with clinical and objective vascular endpoints of digital vascular function in primary RP and SSc.

5. The combination of aspirin and dipyridamole in asasantin retard, through their effects on platelet function, eicosanoid biosynthesis and oxidative stress, can improve peripheral vascular function in primary RP and SSc.

1.7 Aims

In order to explore these hypotheses, the principal aims of each chapter of this thesis are outlined below:

2) In chapter 2 I shall outline the materials and methods used to undertake the work in this thesis.

3) In chapter 3 I shall systematically review the use of IRT as an endpoint in previous clinical trials of Raynaud’s phenomenon and systemic sclerosis.

4) In chapter 4 I shall report the findings of early validation work exploring the use of LSCI (and IRT) in the dynamic assessment of digital vascular function in healthy volunteers.

5) In chapter 5 I shall extend this validation work reporting the findings of work evaluating clinical vascular endpoints and the use of LSCI (and IRT) in the dynamic assessment of digital vascular function in patients with primary RP and SSc.

6) In chapter 6 I shall evaluate determinants of platelet function, eicosanoid biosynthesis and oxidative stress in primary RP and SSc, and explore associations with subjective and objective vascular endpoints.
7) In chapter 7 I shall report the findings of an exploratory proof-of-concept study evaluating the biological, clinical and microvascular effects of asasantin retard therapy in the management of primary RP and SSc.

8) In chapter 8 I shall bring together the findings of the previous chapters and discuss their relevance in the context of existing knowledge and future work.
Chapter 2.0 Materials and Methods

2.1 Materials

2.1.1 Chemical Reagents

<table>
<thead>
<tr>
<th>Name of reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine-5-Diphosphate (ADP)</td>
<td>Alpha Laboratories, UK</td>
</tr>
<tr>
<td>Arachidonic Acid (AA)</td>
<td>Alpha Laboratories, UK</td>
</tr>
<tr>
<td>2,6-Di-tert-butyl-4-methylphenol (BHT)</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Hydrochloric acid (HCL, A.C.S grade, 12N)</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Sodium Hydroxide (NaOH, A.C.S grade, 10N)</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>HEPES (^5) (Reagent Grade, M.W. 238.3)</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Ethanol (200 proof, &gt;99.5% A.C.S grade)</td>
<td>Sigma-Aldrich, UK</td>
</tr>
</tbody>
</table>

2.1.2 Buffers and Solutions

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 N HCL</td>
<td>8.2ml 12.2 N HCL in 91.8 ml ddH(_2)O</td>
</tr>
<tr>
<td>10 N NaOH</td>
<td>4.8g NaOH in 12 ml ddH(_2)O</td>
</tr>
<tr>
<td>1.2 N NaOH/0.5 M HEPES</td>
<td>75ml ddH(_2)O + 12 ml 10 N NaOH + 11.9g HEPES (made up to 100ml with ddH(_2)O)</td>
</tr>
</tbody>
</table>

2,6-Di-tert-butyl-4-methylphenol (BHT)                | 2.5 mg in 500 \(\mu\)L ethanol                  |

2.1.3 Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermovision camera</td>
<td>FLIR systems, Sweden</td>
</tr>
<tr>
<td>FLIR A40 camera(^6)</td>
<td>FLIR systems, Sweden</td>
</tr>
<tr>
<td>Full-Field Laser Perfusion Imager</td>
<td>Moor Instruments, UK</td>
</tr>
<tr>
<td>Bio Data PAP-4 Aggregation Profiler</td>
<td>Alpha Laboratories, UK</td>
</tr>
<tr>
<td>Jouan BR4i Centrifuge</td>
<td>Jouan, UK</td>
</tr>
<tr>
<td>Zenit SP+ Plate reader</td>
<td>A. Menarini Diagnostics, Italy</td>
</tr>
<tr>
<td>HTIII Spectrophotometer</td>
<td>Anthos Labtec Instruments Ltd.</td>
</tr>
<tr>
<td>Wellwash 4 MK 2</td>
<td>Thermo Fisher Scientific</td>
</tr>
</tbody>
</table>

\(^5\) 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

\(^6\) This camera was used for 2 assessments only following equipment malfunction September 2011 (see section 2.4.1)
2.1.4 Commercial kits

- Human soluble CD40 ligand ELISA: R&D systems, UK
- Human soluble p-selectin ELISA: R&D systems, UK
- Human TGF-β ELISA: R&D systems, UK

2.2 Patient recruitment

2.2.1 Regulatory approval

Ethical approval was obtained for the work involving healthy controls from Bath Research Ethics Committee (Bath REC 09/H010/79). The Clinical Trial of an Investigational Medicinal Product (CTIMP) received ethical approval from the South West 3 REC (reference number: 10/H0106/69). The CTIMP received approval from the Medicines and Healthcare products Regulatory Agency (MHRA). We sought guidance from the Human Tissue Authority prior to the storage of plasma and urine as part of this study. The study did not require an HTA license for storage of samples as the study had NHS REC approval. Each study received approval from the Royal National Hospital for Rheumatic Diseases (RNHRD) Research and Development (R&D) committee.

2.2.2 Healthy controls

Healthy Controls were recruited from the staff of the RNHRD. Healthy subjects aged 18-75 years were approached for inclusion in the study. Participants were issued with a participant information sheet (PIS, Appendix 1) and given the opportunity to discuss the study prior to enrollment. Subjects with hypertension were eligible if they had remained on a stable dose of vasodilator medication for the previous month. Subjects were excluded if they fulfilled proposed clinical criteria for RP (Brennan et al., 1993), were pregnant or had a history of peripheral vascular disease, diabetes or other conditions associated with secondary RP. All participants provided informed written consent (Appendix 2). Further details regarding study design and recruitment can be found in section 4.3.2.
2.2.3 Prospective patient recruitment

All patients with primary RP and SSc were recruited to a single prospective study with several sub-studies (which shall be reported sequentially in Chapters 5-7). An outline of the study design incorporating a flow chart of the contribution of patients at each of the study is presented in Figure 2.1.

Figure 2.1. Summary of study design.

The initial 2-week run-in phase forms the basis of the work described in chapters 5 and 6. Eligible patients were given the opportunity to enter the second interventional stage of the study, which involved open-label use of asasantin retard prior to a post-treatment assessment at the end of week 4 (reported in chapter 7).

All patients recruited to the study could take part in the cross-sectional microvascular imaging work that is described in Chapter 5. Participants had to fulfill strict eligibility criteria to take part in individual subsequent components of the study. For example, patients were unable to take part in the cross-sectional study exploring platelet function in primary RP and SSc if they were already receiving anti-platelet therapy (full eligibility criteria reported in section 6.3.1). Patients with a history of duodenal ulceration meanwhile were unable to enter the interventional phase of the study (when they receive asasantin retard). Full details of eligibility criteria for the interventional study are outlined in section 7.3.2. For this reason, participant numbers become smaller as the results chapters progress.
Potential participants were identified using the RNHRD CTD database and from the outpatient clinic department of the RNHRD. All patients were initially provided with a PIS outlining the trial (Appendix 3) along with study interest form (Appendix 4) which they could return in an enclosed stamp addressed envelope. If interested, arrangements were made for prospective participants to attend the unit to discuss the study in further detail. To reduce the burden of multiple visits on participants, the screening visit could coincide with the first assessment if they chose to. All participants were asked to provide informed written consent (Appendix 5) before taking part in the study. Further details regarding study design and recruitment can be found in sections 5.3.1, 6.3.1 and 7.3.1.

2.3 Patient characteristics and clinical endpoints

2.3.1 Patient demographics and clinical phenotype

Patient demographics
Information on patient demographics and clinical phenotype were recorded following direct questioning and a review of the medical case notes. Patient demographics included age, gender, smoking history (current, previous and never, including an estimate of pack-year [1 pack year representing the equivalent of 20cpd for 1 year] history) and disease duration (defined as both year of onset of RP symptoms and year of physician diagnosis) were recorded.

Clinical phenotype of patients with SSc
Details of the classification and eligibility criteria used to recruit patients with primary RP and SSc can be found in sections 5.3.2, 6.3.1 and 7.3.1.

Patients with SSc were classified according to extent of cutaneous involvement was classified according to the LeRoy and Medsger criteria (LeRoy et al., 1988). In brief, lcSSc comprises skin changes distal to the elbows, knees and clavicles i.e. forearms, hands, calves, feet and face. In dcSSc, skin thickening is present proximal to these sites e.g. thighs, upper arms and torso (LeRoy et al., 1988). In the absence of documented skin involvement, the term limited SSc (ISSc) was adopted as previously described (LeRoy and
Medsger, 2001). The extent of skin thickening was not assessed clinically but the highest previous modified Rodnan skin score (mRSS, section 2.3.4), where documented, was recorded (Clements et al., 1993).

Clinical evidence or a documented history of calcinosis and/or telangiectasia was recorded. A past history of digital ischaemic complications was based on a previously documented history of either digital ulceration (DU) or digital pitting (DP). A diagnosis of ILD required previously reported characteristic findings on HRCT or lung biopsy. The results of most recent pulmonary function tests (forced vital capacity [FVC] and diffusing capacity [DLco]) undertaken within the last five years were documented. A diagnosis of pulmonary arterial hypertension (PAH) required a mean pulmonary artery pressure of >25mmHg on right heart catheter (Badesch et al., 2009). Cardiac involvement was based on a documented history of any cardiac involvement, other than ischaemic heart disease, and included left ventricular dysfunction, arrhythmias, myocardial fibrosis, or pericardial effusion. Upper GI involvement was defined as a documented clinical history of symptomatic gastro-oesophageal reflux (GORD) and/or dysphagia, endoscopic evidence of GORD or stricture, or abnormal peristalsis/transit on oesophageal manometry and/or barium swallow. Patients were documented as having lower GI involvement if there was a recorded history of chronic frequent and/or loose stools, faecal incontinence or a documented history of intestinal pseudo-obstruction. A scleroderma renal crisis (SRC) was defined as the new onset of hypertension accompanied by acute renal failure. Muscle involvement required the presence of an unexplained elevation in serum creatine kinase and/or characteristic changes on muscle biopsy. Joint involvement was considered present if there was a documented history of inflammatory arthritis (erosive or non-erosive).

**Serological assessment of patients with SSc**

The results of serological analysis, based upon indirect immunofluorescence studies using a rapidly dividing human laryngeal epithelial cell carcinoma cell line (HEp-2 cells), followed, where appropriate, by immunoblotting and/or immunoprecipitation to aid further characterisation of SSc-specific autoantibodies, was recorded.

**2.3.2 The Scleroderma Health Assessment Questionnaire**

The Scleroderma Health Assessment Questionnaire (SHAQ) is a modified version of the Stanford Health Assessment Questionnaire Disability Index (HAQ-DI) that is recommended
for use in clinical trials of SSc (Merkel et al., 2003, Steen and Medsger, 1997). The 20-item HAQ-DI is a patient self-report of disability. It is divided into eight domains: dressing and grooming, arising, eating, hygiene, reach, grip and activity. Patients rate their ability to perform these activities of daily living on a scale from 0 (without difficulty) to 3 (unable to do). The use of aids or devices to assist with these activities is also recorded. The HAQ-DI score is calculated by dividing the summed component scores by the number of components answered and yields a score between 0 (no disability) to 3 (severe disability) (Fries et al., 1982, Bruce and Fries, 2003). The SHAQ has been demonstrated to have high face validity and good test-retest reliability in SSc (Steen and Medsger, 1997). The SHAQ comprises a standard HAQ-DI along with six visual analogue scales relating to the major organ manifestations of SSc (pain, gastrointestinal, respiratory, Raynaud’s, digital ulceration and a patient global assessment) each of which is converted into comparable 0-3 scores (Steen and Medsger, 1997). The VAS RP of the SHAQ is recommended for use in both the provisional core set of outcome measures for clinical trials of SSc and by OMERACT7 (Khanna et al., 2008). An example of the SHAQ used in this study can be found in Appendix 6.

2.3.3 The Raynaud’s Condition Score

The Raynaud’s Condition Score (RCS) forms part of an RP attack diary completed by patients over 14 days (Black et al., 1998). Each day, patients record the number and duration in minutes of each RP attack. Patients also record a separate RCS, which is a daily self-report assessment of the impact of RP symptoms using 0-10 ordinal scale. Patients are encouraged to consider the number, duration, severity and impact on functional capacity when completing the RCS. The RCS (and the frequency and duration of daily RP attacks derived from the diary) has been used successfully in multicentre clinical trials and is the recommended outcome measure for use in clinical trials of SSc (Merkel et al., 2002, Merkel et al., 2003). Patients taking part in the study were issued with a RCS diary and coached on its completion. Each page of the diary was numbered to help participants. Spare pages were provided in case of error, or should participants experience greater than 12 attacks in a single day. The mean daily frequency of RP attacks, duration of attacks and RCS score were calculated over the 2 week period. Participants were required to have correctly completed 70% of the diary (i.e. at least 10 days) for the results to be used in subsequent analysis. On occasions were the RCS score had been left blank (when no attacks of RP had

7 See section 4.1.4 for further details
occurred) it was confirmed with participants whether or not this indicated a score of 0. An example of a single day from the RCS diary used in this study can be found in Appendix 7.

2.3.4 The modified Rodnan skin score (mRSS)

The modified Rodnan skin thickness skin score (also referred to as the TSS) has been validated and is recommended for use in clinical trials of SSc (Merkel et al., 2003, Clements et al., 1993). Skin thickening is evaluated by palpation of the skin at seventeen sites of the body (fingers, hands, forearms, arms, feet, legs, thighs, face, chest and abdomen) and scored using a 0-3 ordinal scale (0=normal, 1=mild thickening, 2=moderate thickening, 3=severe thickening). The mRSS is a simple composite score ranging from 0-51 (Clements et al., 1993). The mRSS was not assessed during the study. Instead, the medical notes were scrutinized for the highest previous recorded mRSS. The reason for this was because the natural history of diffuse cutaneous SSc is for there to be a gradual improvement in skin thickening (and mRSS) over time. As such the mRSS at a single timepoint sometimes provides less information on the overall disease phenotype than the highest previously recorded mRSS. All previous mRSS assessments had been undertaken by a single trained assessor (Sister S. Brown).

2.4 Digital vascular assessments

2.4.1 Cold challenge assessment

All assessments were undertaken by the same assessor (JDP) between 09:00hrs and 12:30hrs in a temperature-controlled laboratory within the clinical measurement department of the Royal National Hospital for Rheumatic Disease. Subjects were asked to attend in light clothing (exposed the forearms) and asked to refrain from consumption of tobacco, alcohol caffeine and vigorous exercise for 4 hours prior to the assessment time. On arrival, subjects were acclimatised for a period of 20 minutes at 23°C +/- 0.5°C (with the exception of assessment 3 in healthy control study reported in Chapter 4) in a climate-controlled laboratory. Where possible, jewellery and watches were removed. Subjects were seated comfortably in the examination chair with their hands placed in front of them on a vacuum-stiffened beanbag on a table in front of the participants at waist height. A vacuum-stiffened beanbag ensured the hands remained stationary, and maintained participant comfort, for the duration of the assessment. Subjects sat with their right hand dorsum facing up and left hand volar surface facing up to allow simultaneous assessment of different regions of
interest (ROI) within the hands to be undertaken (Figure 2.2). The IRT camera was manually focussed to maximise image quality. Simultaneous assessment of digital vascular function using IRT and LSCI was undertaken at baseline over a 3-minute period. The subject then placed both hands into cellophane gloves (to avoid evaporative cooling during re-warming phase) and from, their seated position, submerged both hands up to the level of the radio-carpal joints into a water bath cooled to 15°C (+/- 0.1°C) for a period of 60 seconds. The gloves were removed and hands placed back into baseline position. In the event of direct contact between the skin and water, the hands were gently dabbed dry using a paper towel before re-positioning them on the vacuum-stiffened beanbag.

**Figure 2.2 Set-up of patient during cold challenge assessment**

The IRT camera is attached to a stand with an orthogonal distance of ~80cm between the scan head and the patient's hands. The LSCI is angled at ~30º at a distance of ~30cm from the hands. The hands are supported using a vacuum stiffened bean bag placed on a table in front of the seated participant. The left hand is positioned palm up where as the right hand is palm down. The red container is the water bath that is cooled to 15°C for the cold challenge. On the shelf next to the sink is the thermometer for continually assessing room temperature during assessments.

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8 written consent obtained for use of image
Continuous assessment of digital vascular function using both IRT and LSCI was undertaken for a period of 15 minutes following cold challenge. This signified the end of the assessment.

Significant thought was given to the temperature of the cold challenge. A waterbath of 15°C has been successfully used by other groups and may represent the optimum temperature to elicit a cold-induced vasoconstrictor response (Anderson et al., 2007). The temperature chosen for cold challenge tests is not considered to be critical provided the test is standardised across subjects. Higher temperatures of 20°C have been used (McHugh et al., 1988, Ring et al., 1981). Temperatures above 20°C (approaching room temperature) would generally be considered to provide too mild a stimulus to promote vasoconstriction. Temperatures below 15°C is arguably too great a cold challenge and should be avoided for the following reasons; Firstly, this level of stimulus can be unnecessarily uncomfortable for patients with RP (leading to a degree of pain-mediated sympathetic activation). Secondly, at temperatures of 10-13°C or below vascular smooth muscle cells lose some of their muscular contractility, in addition to a sudden reduction in the release of neurotransmitters from sympathetic nerves innervating AVAs, leading to vasodilation (Lewis, 1930). This cold induced vasodilatation (CIVD) was first described by Lewis (Hunting Reaction) and manifests itself as reddening of exposed skin. CVID is a commonly encountered symptom in healthy individuals during cold weather (Lewis, 1930). Cold-induced vasodilation usually occurs after 5-10 minutes cold exposure. It helps maintain forearm blood flow, nutritive perfusion, manual dexterity and tactile sensitivity during brief cold exposure at the potential cost of risking a fall in core temperature and hypothemic complications after prolonged exposure (Daanen, 2003). People regularly encountering cold exposure through work or sports can develop an enhanced CIVD response.

2.4.2 Infrared Thermography (IRT)

IRT assessment of surface skin temperature was undertaken using a camera attached to an upright stand with an orthogonal distance of ~80cm from the subject’s hands. The majority of images were captured using a Thermovision (FLIR systems, Danderyd, Sweden) camera. Following an equipment malfunction (15/09/2011), 2 assessments (~1% of total) were undertaken using a replacement camera (FLIR Thermovision A40 camera). Subsequent calibration studies led to the application of a correction factor (multiply by 1.064 and subtract 1.475) to ensure comparable findings in temperature values using the 2 cameras (e.g. 30°C
was adjusted to 30.4°C). Thermal images of the hands were taken at 13 second\(^a\) intervals for a period of 3 minutes at baseline and for 15 minutes following cold challenge. All images were processed using the commercially available CThERM software (Version 2.3, University of Glamorgan). An example of a thermal image from a patient with SSc is provided in Figure 2.3.

\(^a\) Allowing for subsequent delay for shutter closure and processing, setting the CThERM computer software at an image capture rate of 10 seconds led to a real time image capture rate of 13 seconds.
Figure 2.3. Example of IRT image from a patient with SSc following cold challenge.

Skin emissivity is converted into a colour scale with blue areas representing low surface temperatures and red areas representing warmer, better perfused regions. The colour scale on the right of the image provides a guide to surface skin temperature at individual areas. The left forearm is supinated with the volar aspect of the hand (the palm) facing the camera. The right forearm is pronated with the dorsum of the hand facing the camera. Regions of interest (ROI) can be placed over pre-defined areas of the thermal image and the mean estimated temperature for that ROI can be calculated.

2.4.3 Laser Speckle Contrast Imaging (LSCI)

All LSCI assessments of digital vascular perfusion was undertaken using a Full-Field Laser Perfusion Imager (Moor Instruments FLPI, Axminster, UK). Image analysis was undertaken using the accompanying moorFLPI Imager software version 2.0. The laser wavelength of moorFLPI is 775nm. Assessment of digital perfusion using the moorFLPI LCSI was undertaken simultaneously with IRT. The laser head was placed at a standardised distance of ~30cm (+/- 2cm) although distance between laser head and skin has not been shown to significantly influence flux values (Mahe et al., 2011). The laser was maintained at an angle of ~30° (+/- 2.5°) to the hands as recommended. The polarising filter orientation was adjusted to minimise specular reflections (mirror-like reflection from shiny surfaces such as the nails). The sample rate was fixed at 25 Hz (with single values of flux calculated every 40ms). The time constant (which influences absolute flux values) was set at 1.0 second to minimise noise relating to the pulsatility of the cardiac cycle and minor hand movements. As
with other laser-based imaging modalities e.g. Doppler and flowmetry, LSCI quantifies perfusion in arbitrary flux units (fu). Unlike other laser based imaging modalities, the moorFLPI is not hazardous to the eye and laser safety goggles are not required to be worn by the operator or patient. The moorFLPI incorporates a diverging infra-red laser beam having accessible power below the limits of Class 1 as defined in IEC60825-1:2001. Images were taken continuously during the assessment and then sub-divided into individual images at 13 second intervals (to correspond with IRT assessment). An example of typical appearance of images taken using LSCI in a patient with SSc before and following cold challenge are presented in Figure 2.4.
Figure 2.4 Typical appearance of images taken using LSCI before and following cold challenge in a patient with SSc

Continuous LSCI assessment is edited into multiple images at 13 second intervals. The images presented demonstrate perfusion in a patient with SSc during 3-minute baseline and following cold challenge. No images are available between images 14 and 22 as this was the period of the cold challenge. Speckle contrast is converted into a colour scale with red areas representing high flux (perfusion) and blue areas representing lower flux as indicated on accompanying flux scale (on the left hand side of the image). The values on the flux scale are arbitrary flux units with higher values indicating greater perfusion. The restricted field of view precluded assessment of the whole of the hands as was achieved using IRT. Note the stable and higher perfusion at baseline compared to following cold challenge. Regional differences in perfusion are also evident and shall be discussed in later chapters. Image 11 at baseline demonstrates the unfortunate consequence of movement artefact on the LSCI image of the left hand (wherever possible such data was excluded from analysis).

2.4.4 Image analysis

Imaging software for both IRT and LSCI enabled mean temperature (°C) and arbitrary flux units (fu) to be calculated over pre-defined identically sized regions of interest (ROI). In contrast to IRT which could visualise the entire surface of both hands, the LSCI camera's field of view was limited to approximately 20cm x 10cm allowing visualisation of the middle fingers of both hands when placed along side each other (Figure 2.4 for examples of images obtained). Three ROI were chosen for analysis: the dorsal aspect of right middle fingertip (ROI 1), dorsal aspect of the middle phalanx of the right middle finger (ROI 2), and
the palmer aspect of left middle fingertip (ROI 3) (the ROIs are demonstrated in Figure 4.1). These ROIs were chosen to allow comparison between dorsal vs. palmer assessment of digital perfusion, and to allow evaluation of tissue perfusion between different regions of the hands known to differ anatomically by the presence, or absence of arteriovenous anastamoses (section 1.5.1). Using data generated at 13 second intervals before and following cold challenge it was possible to assess perfusion at specific time points and compile re-warming/reperfusion curves for subsequent analyses. The same endpoints were chosen for both IRT and LSCI. These included absolute measurements such as baseline (abbreviated hereafter as B), immediately following cold challenge (t0), at 5 minute intervals post-cold challenge (t5, t10 and t15) and the maximum measurement during re-warming (Max). Dynamic assessments dependent on the characteristics of the reperfusion/reperfusion curve were also assessed. These included maximum % recovery (R%max, calculated as [Max - t0]/(B – t0) x100). The maximum gradient of re-warming (Gmax) for the reperfusion/reperfusion curves was also calculated. For illustrative purposes, an example of a re-warming curve generated using IRT, highlighting some of the endpoints evaluated is presented in Figure 2.5.

\[ \text{R}\%\max = \frac{\text{Max} - \text{t0}}{\text{B} - \text{t0}} \times 100 \]

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\( ^a \) The ring finger of the right hand was used to assess perfusion at ROIs 1 and 2 for one patient who had previously undergone amputation due to ischaemic complications of his SSc.
2.5 Blood and urine collection

2.5.1 Collection of whole blood

All participants provided informed written consent to blood collection. Blood collection was undertaken by Dr J Pauling who is medically trained and experienced at phlebotomy. Blood samples were drawn at the end of the cold challenge assessment to ensure all platelet function analyses were undertaken within 1 hour of sample collection and within the 2-4 hour recommended time-frame (Harrison, 2005). Local cooling (such as the cold challenge undertaken in our experiments) is not thought sufficient to precipitate significant in vivo platelet activation (Hutton et al., 1984). Platelets are susceptible to ex-vivo activation and desensitisation during collection and the preparation of PRP. Blood was collected from the antecubital fossa using a 21 gauge needle and Vacutainer ® system. Early tourniquet release was used to minimise artefactual platelet activation (Harrison, 2005). The choice of anticoagulant for platelet studies is important. For example, anticoagulation with calcium
chelation is not suitable for aggregometry studies as it would interfere with calcium dependent platelet activation. Blood for platelet aggregometry was gently anticoagulated in 3.2% sodium citrate in a ratio of 1:9 (1 part anticoagulant to 9 parts blood). All samples were stored at room temperature and processed within 1 hour of collection. Blood for circulating biomarkers was gently anticoagulated in K2EDTA and stored on ice prior to sample processing (in accordance with recommendations of the ELISA manufacturer).

The initial ~8mls of blood was not used for platelet studies (as recommended); the first ~5mls was drawn into a serum separation tube (SST™II) and the subsequent ~3mls was collected into K2EDTA. These samples were sent, anonymised with the unique identifier code (e.g. ART001JDP), to the Royal United Hospital laboratory for assessment of full blood count (FBC), Plasma Viscosity (PV) and urea and electrolytes (U&E). A further ~7mls was collected in K2EDTA bottles (x2) and ~7mls in 3.2% sodium citrate bottles (x3) for platelet function analyses. Immediate gentle mixing ensured effective anti-coagulation. Citrated blood was kept at room temperature whereas EDTA blood was placed in ice prior to further sample preparation. Blood and urine samples were transferred within a secure specimen transport carrier (in accordance with local policy) to the Bath Institute for Rheumatic Disease (BIRD) research laboratory by Dr J Pauling for processing.

2.5.2 Extraction of Platelet Rich Plasma (PRP)
Platelet rich plasma (PRP) was prepared by centrifuging citrated blood at 150 x gravity (g) (equates to 970rpm) for 10 minutes at room temperature. The plasma layer was examined for red cells which if present, prompted a further centrifugation at 150 x g for 5 minutes. Using a plastic transfer pipette the supernatant (PRP) was carefully transferred to a test tube labeled PRP, which was capped and left to stand at room temperature. The resulting 2.7-3mls of PRP was generally sufficient to undertake 6 platelet aggregometry analyses (see section 2.7.5).

2.5.3 Extraction of Platelet Poor Plasma (PPP)
Following removal of the PRP, the remaining citrated blood sample was centrifuged at 2500 x g (2900 rpm) for 20 minutes at room temperature. The platelet poor plasma (PPP) layer was inspected for haemolysis and transferred to a plastic tube labeled PPP.
2.5.4 Extraction of Platelet Free Plasma (PFP)
Platelet Free Plasma (PFP) was prepared by centrifuging EDTA blood collected (within 30 minutes and stored on ice) at 1000 x g (2400rpm) for 15 minutes at 8-10°C. The plasma layer was transferred into a test tube and immediately centrifuged at a greater speed of 3000 x g (4100rpm) for 10 minutes in an attempt to remove all remaining platelets (Christie, 2008). To avoid multiple freeze thaw cycles when assaying multiple biomarkers, PFP samples was stored in x3 1ml screw-top storage tubes.

2.5.5 Collection and preparation of urine
Participants were issued a plastic universal container to provide a fasting mid-stream urine sample on the morning of their 2nd (and 3rd when applicable) assessment. The urine samples were stored at the Bath institute for Rheumatic Diseases for later batch analysis of eicosanoid metabolites. Urine was stored both unaltered (x3, 1ml storage tubes) and containing 10μL of the antioxidant 0.005% BHT to prevent ex vivo lipid peroxidation which could influence F2-isoprostane synthesis (x3, 1ml storage tubes).

2.5.6 Labelling and storage of samples
All plasma and urine samples were anonymised with a unique patient identifier code (e.g. ART001JDP), sample details, and date of collection in accordance with the requirements of the Ethics committee. An electronic record of the sample details was recorded on a password protected Microsoft Access Research Sample Database in accordance with local policy. To avoid multiple freeze thaw cycles when assaying for multiple biomarkers, samples were stored in 1ml screw-top storage tubes. In total, 6 urine samples were stored (3 containing 10μL of 0.005% BHT), 1 citrated platelet poor plasma and 3 EDTA plasma samples. All samples were stored at -80°C for subsequent batch analysis. The study number was written on the screw-top to facilitate sample identification and as a fail-safe mechanism should sample labels be lost. An electronic record of the sample details is recorded on a password protected Microsoft Access Research Sample Database in accordance with local policy. This is accessed using my BIRD account log-in details → S-drive → Research Daybook → Research Samples. A log of all samples uploaded onto the research sample database is also made in the research daybook which remains behind locked doors in the BIRD research laboratory.
2.6 External laboratory investigations

2.6.1 Blood samples

The initial 8mls of blood was not used for platelet studies; the first ~5mls is drawn into a serum separation tube (SST™II) and the subsequent ~3mls is collected into K2EDTA vacutainer tubes. These samples were anonymised and sent for analysis of Full Blood Count (FBC), plasma viscosity (PV), and serum creatinine (Cr) at the accredited haematology and biochemistry diagnostic laboratories of the Royal United Hospital, Bath. Written reports of the results of these investigations were sent to the Chief Investigator (J Pauling) for review and documentation within the case report forms (CRF).

2.7 Platelet aggregometry

Since its development in the early 1960’s by Born, light transmission aggregometry (LTA) has long been considered the “gold standard” of platelet function analyses (Born and Cross, 1963, Born, 1962).

2.7.1 Technique and Choice of Agonists

LTA provides a quantitative analysis of the degree of aggregation stimulated by a panel of agonists such as adenosine diphosphate (ADP), arachidonic acid (AA), collagen and adrenaline. The use of varying concentrations of these agonists allows the assessment of dose response curves. The principle of LTA has been well described elsewhere (Harrison, 2005). In brief, a sample of PPP is placed in the aggregometer and set as 100% light transmission. A PRP sample is incubated between a light source and luminometer, which is set as the 0% light transmission. A known concentration of agonist is spiked into the PRP activating the platelets and stimulating aggregation. Upon addition of the agonist, the platelets first undergo a shape change. The increased surface area of the platelets initially decreases light transmission that can occasionally be identified on the aggregation trace. As aggregation ensues, the light transmission increases as the plasma changes from cloudy to clear. The rate and extent of aggregation can be read from the subsequent trace. Examples of the macroscopic appearance of PPP, PRP and PRP following the addition of agonist are presented in Figure 2.6. An example of typical aggregation traces to varying concentrations of the agonist ADP is presented in Figure 2.7. Platelet function analysis using LTA is limited by the supply of platelet rich plasma. As such, significant thought was given to both the
choice of agonists and concentrations used in this study. A panel of agonists has emerged for use in LTA and includes many of the agents responsible for platelet activation in vivo such as adenosine diphosphate (ADP), arachidonic acid (AA), collagen, and adrenaline (see section 1.4.2 for mechanisms of action). Each agonist provides specific information regarding platelet activation pathways.

Agonists are usually categorized as either strong or weak agonists. Strong agonists e.g. thrombin, thromboxane and collagen directly induce platelet aggregation and platelet granule secretion. Weak agonists, such as ADP and adrenaline meanwhile can induce aggregation without necessarily inducing granule secretion (a primary wave of aggregation). At low agonist concentrations, ADP-mediated aggregation is reversible and de-aggregation can be observed as a reduction in light transmission following primary wave aggregation. If the working concentration of ADP is sufficient to precipitate storage granule release during platelet aggregation, an irreversible secondary wave of aggregation can occur. Adrenaline is another weak agonist.

Figure 2.6. The macroscopic appearance of A) Platelet Poor Plasma (PPP), B) Platelet Rich Plasma (PRP) and C) PRP following the addition of agonist.

The initial cloudy of appearance of PRP (B) is lost following the addition of agonist which leads to the formation of visible clumps of aggregated platelets (C). The turbidity assumes the appearance of PPP (A) achieved through high-speed centrifugation of citrated blood.

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9 I am very grateful for the advice and support of Professor Paul Harrison from the University of Oxford for his advice and guidance on the use of LTA and choice of agonists.
Collagen, thrombin, TxA\textsubscript{2} and its precursor AA are strong agonists of platelet aggregation and are associated granule release with a single wave of aggregation. Ristocetin is an antibiotic and agonist, but stimulates platelet agglutination as opposed to aggregation.

We chose to evaluate platelet aggregation responses to ADP for the following reasons. Aspirin therapy abolishes the secondary wave, thereby influencing responses to high working concentrations of ADP (e.g. 10\textmu L and 5\textmu L) in LTA analyses. At lower working concentrations (e.g. 2.5\textmu L and 1.25\textmu L), ADP provides an \textit{ex vivo} assessment of P2Y\textsubscript{1} and P2Y\textsubscript{12} G-protein receptor signaling. Dipyridamole has several modes of action (see section 1.4.5) including inhibition of phosphodiesterase enzymes responsible for the breakdown of cyclic AMP (cAMP). Increasing intracellular cyclic AMP levels, and thereby reducing platelet responses to ADP. For this reason, previous studies have used ADP in \textit{ex vivo} aggregometry studies assessing platelet responses to dipyridamole (e.g. (Gregov et al., 1987) In addition, dipyridamole inhibits re-uptake of adenosine increasing adenosine A2-receptor-mediated activation of adenylate cyclase further increasing intra-platelet cAMP (Figure 1.4).

Arachidonic acid (AA) is the obvious choice for the quantification of platelet responses to COX inhibition (Gachet, 2008). Whilst COX inhibition with low-dose aspirin effectively suppresses platelet synthesis of thromboxane, exogenous AA can still stimulate platelet aggregation through the production of non-enzymatic oxidative products of AA metabolism such as the F\textsubscript{2} isoprostanes. The use of AA had an unintended advantage of identifying patients who had inadvertently received treatment with COX inhibitors (aspirin and NSAIDs) during the run-in phase of the study. Patients in whom aggregation did not occur in response to AA were contacted to clarify their drug history. The many OTC remedies available for corysal illnesses and musculoskeletal pain make it very easy for patients to inadvertently receive treatment with COX inhibitors.

\textsuperscript{10} This effect is best observed \textit{ex vivo} using whole blood impedance aggregometry as impaired re-uptake of adenosine by erythrocytes is the major source of increased local concentrations of adenosine following treatment with dipyridamole.
2.7.2 Use of adenosine diphosphate as agonist

Adenosine-5-Diphosphate (ADP) was reconstituted at room temperature using 1ml of purified water and stored for a maximum of 30 days at 2-8°C in its original tightly sealed container. This concentration was $2 \times 10^{-4}$ M. In view of the 1:10 dilution with PRP, this provided a working concentration for the aggregometry study of $2 \times 10^{-5}$ M (equivalent concentration 20μmol/L). A series of 1:1 dilution steps with 50μl of purified water allowed subsequent agonist working concentrations of 10μmol/L, 5μmol/L and 2.5μmol/L to be prepared at the time of analysis for use in aggregometry analyses.

2.7.3 Use of arachidonic acid as agonist

Arachidonic acid (AA) was reconstituted at room temperature using 0.5ml of purified water and stored for a maximum of 8 weeks at <20°C in its original tightly sealed container. This reconstituted AA provided a working concentration for the aggregometry study of 1.64mmol/L. A single of 1:1 dilution step with 50μl of purified water prepared at the time of analysis allowed a second agonist working concentration of 0.82mmol/L (equivalent concentration 250μl/mL) to be used for a second aggregometry analysis.

2.7.4 Platelet Aggregometry Procedure

Platelet aggregometry (PA, also known as Light Transmission Aggregometry, LTA) is generally regarded to be the “gold standard” of platelet function analyses. All platelet aggregometry studies were undertaken using the Bio Data PAP-4 Aggregation Profiler (Alpha Laboratories). Four channels allow simultaneous performance of aggregation profiles with automated 0% and 100% baselines. The printed results provide aggregation patterns and report slope value (maximum gradient of aggregation) and final percent aggregation. The 5-minute percent aggregation can be read manually using time bars present on the printed report.

All PA studies complied with guidelines developed through the Clinical and Laboratory Standards Institute consensus process.(CLSI, 2008) All LTA studies were undertaken within 1 hour of sample collection.(Harrison, 2005) An aggregometer blank (100%) was prepared using 0.5ml of PPP into a siliconized11, flat bottomed 8.75 x 50mm cuvette (Alpha Laboratories). 0.45mls of PRP was pipetted into additional cuvettes, which were incubated at 37°C for 3 minutes before adding a plastic coated macro stir bar (stir speed 920-940rpm).

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11 Platelets are activated on negatively charged surfaces such as glass
It remains unclear whether adjusting PRP for platelet count (through dilution with PPP) is required in LTA studies. Adjusting PRP for platelet count does not appear to affect platelet aggregation (Mani et al., 2005). We undertook all analyses using native PRP samples for the following specific reasons; it is inappropriate to adjust the platelet count in the PRP samples for analyses using AA and the use of PPP-adjusted PRP is known to alter the local environment of the platelets potentially falsifying the results. The platelet count of all subjects was assessed separately. The 100% and 0% baselines were set using PPP and PRP samples respectively. 0.05mls of agonist was pipetted directly into the PRP samples, carefully avoiding the reagent from running down the side of the cuvette. We allowed the aggregation pattern to generate for 5 minutes. The maximum % aggregation, %aggregation at 5 minutes and slope of aggregation were recorded from the aggregation trace and automated analysis respectively. An example of typical aggregation patterns to decreasing concentrations of ADP is illustrated in Figure 2.7.

2.7.5 Insufficient sample

Seven millilitres of citrated plasma typically yields approximately 3mls of PRP easily allowing 6 PA studies (2.7mls PRP required) to be performed. On occasions, we noted insufficient PRP sample to undertake the full complement of PA studies. In such instances, a decision was made to attempt to preserve the lower doses of agonists (in most cases) at the expense of higher doses (e.g. 20μmol/L ADP and 1.64mmol/L AA) on the basis that such concentrations may provide better discriminatory capacity between primary RP and SSc.

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12 This was not noted on initial training exercises prior to commencement of the study and only became apparent after commencement of the study. In the early stages of the study, there were occasions when the lower concentrations of agonists were excluded as opposed to the higher doses which accounts for some of the missing data in the LTA analyses reported in section 6.4.1.
Figure 2.7 Examples of aggregometry curves using varying concentrations of the agonist adenosine diphosphate (ADP).

20μmol/L, B) 10μmol/L, C) 5μmol/L and D) 2.5μmol/L ADP. The arrows indicate the moment the samples were spiked with agonist. A light transmission deflection of 2-4% is the result of the diluting effects of the agonist. The small rebound is the result of platelet shape change following early platelet activation. Note the rapid extensive primary waves of aggregation to higher concentrations of ADP. At the lower concentration of 5μmol/L, there are distinguishable primary and secondary waves of aggregation (representing initial aggregation to ADP and a secondary wave following platelet granule release). Following addition of the lowest concentration of ADP (2.5μmol/L), there is no secondary wave of aggregation and subsequent reversal of the primary wave due to failure to stimulate granule release and subsequent de-aggregation during the 5-minute period following addition of the agonist.

2.7.6 Quality Control Assessments of PAP-4 Aggregometer

Details of Quality Control assessments undertaken to ensure acceptable instrument functionality and operator expertise can be found in Appendix 8.
2.8 Plasma biomarkers of platelet activation

2.8.1 Choice of circulating biomarkers

Each plasma biomarker was assessed using commercially available kits, which employed a quantitative sandwich enzyme linked immunosorbent assay (ELISA) technique. All reagents and controls were prepared as directed. PFP samples were not diluted (unless stated) as the expected concentrations of each plasma biomarker were generally well within the range of the standard curve generated using the standards. In addition to running known control values on each plate, additional QA work was undertaken by calculating the coefficient of variation (CV) for each assay to establish inter-assay and intra-assay reliability; the results of which are outlined below.

2.8.2 Soluble P-selectin ELISA

Soluble P-selectin (sP-selectin) levels were assessed using a commercially available ELISA (R&D systems, UK). In brief, plasma samples were pipetted into the plate wells. The sP-selectin binds to a monoclonal antibody which has been pre-coated onto the microplate wells. An enzyme-linked (horse radish peroxidase) polyclonal antibody specific for sP-selectin was added to the wells (sandwiching the immobilized sP-selectin) followed by a 1 hour incubation period. After removal of unbound conjugated antibody (aspirate and wash x3), a substrate solution (stabilized tetramethylbenzidine) was added that develops colour proportional to the quantity of bound sP-selectin concentration. Colour development was stopped (using an acid solution provided with the ELISA kit) and intensity measured using the Zenit SP+ plate reader (A. Menarini Diagnostics, Italy). An automated standard curve was plotted using supplied standards. The quality of the assay was assessed using controls of known value obtained from R&D systems. All samples were run in duplicate and an average of the 2 values used for final analysis. The intra-assay and inter-assay reliability CVs for the sP-selectin assays were 8.57% and 8.73% respectively.

2.8.3 Soluble CD40 ligand ELISA

Soluble CD40 ligand (sCD40L) levels were assessed using a commercially available ELISA plate (R&D systems, UK). In brief, undiluted plasma samples were pipetted into the plate

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13 In addition to the assays described, attempts were made to measure Platelet Derived Growth Factor BB (PDGF-BB) levels using a commercially available ELISA plate (R&D systems, UK). The initial assay was successful with control values within target range. We made several attempts at assaying the 2nd batch of samples using the same kit but struggled to establish reliable standard curves. It was felt the data was not strong enough for inclusion in the final thesis.
wells. The sCD40L binds to a monoclonal antibody which has been pre-coated onto the microplate wells (during 2 hour incubation at RT on a horizontal orbital microplate shaker with a 0.12" orbit, set at 500 rpm. The samples were then aspirated and washed 4 times. An enzyme-linked (horseradish peroxidase) polyclonal antibody specific for sCD40L was added to the wells (sandwiching the immobilized sCD40L) followed by a further 2 hour incubation period at room temperature on the shaker. After removal of unbound conjugated antibody (aspirate and wash x4), a substrate solution was added that develops colour proportional to the quantity of bound sCD40L concentration. The substrate solution was a combination of stabilized hydrogen peroxide and stabilized tetramethylbenzidine that were mixed within 15 minutes of use. Colour development was stopped (using 2N sulphuric acid stop solution provided with the ELISA kit) and intensity measured using a HTIII Spectrophotometer plate reader. An automated standard curve was plotted using supplied standards. The quality of the assay was assessed using controls of known value obtained from R&D systems. All samples were run in duplicate and an average of the 2 values used for final analysis. The intra-assay and inter-assay reliability CVs for the sCD40L assays were 13.43% and 23.12% respectively.

2.8.4 Transforming Growth Factor β (TGF-β) ELISA

Transforming Growth Factor β (TGF-β) levels were assessed using a commercially available ELISA plate (R&D systems, UK). TGF-β is secreted as an inactive latent complex. Plasma TGF-β was activated using an acidification process (10 minutes incubation of 80μL PFP sample with 40μL of 1 N HCL) followed by a neutralization process (addition of 40μL of 1.2 N NaOH/0.5 M HEPES) in polypropylene test tubes prior to assay. 50μL of activated sample was added to each well. Activated TGF-β binds to a monoclonal antibody pre-coated onto the microplate wells during 2 hour incubation. After washing (x4), an enzyme-linked polyclonal antibody specific for TGF-β1 was added to the wells (sandwiching the immobilized TGF-β). Subsequent washes (x4) removed unbound antibody-enzyme reagents before the addition of a substrate solution that develops colour proportional to the quantity of bound TGF-β. The substrate solution was a combination of stabilized hydrogen peroxide and stabilized chromogen (tetramethylbenzidine) which were mixed together within 15 minutes of use. Colour development was stopped (using a stop solution of diluted hydrochloric acid provided with the kit) and intensity measured using the Zenit SP+ plate reader (A. Menarini Diagnostics, Italy). A standard curve was plotted using supplied standards. The quality of the assay was assessed using controls of known value obtained
from R&D systems. All samples were run in duplicate and an average of the 2 values used for final analysis. The intra-assay and inter-assay reliability CVs for the TGF-β assays were 9.65% and 16.84% respectively.

2.9 Urinary biomarkers of eicosanoid biosynthesis

All eicosanoid analysis was undertaken using established techniques at the worlds leading eicosanoid research laboratory in collaboration with Professor Ginger Milne at Vanderbilt University Medical Center in Nashville, Tennessee (Morrow and Roberts, 2002, Morrow and Roberts, 1999, Milne et al., 2007). Quality control samples were analysed within each batch and all fell within the laboratories acceptable range.

2.9.1 Measurement of urinary F2-Isoprostanes

To analyse F2-isoprostane levels, 1.0 ng of [2H4]-15-F2t-IsoP (Cayman Chemical, Ann Arbor, MI) internal standard is first added to 0.200 ml of each sample of urine in 5mL water. The solution is adjusted to pH 3 with 1N HCl. The sample is then applied to a C-18 Sep-Pak cartridge that has been prewashed with 5 ml methanol and 5 ml 0.01N HCl. The cartridge is then washed with 10 ml 0.01N HCl, followed by 10 ml heptane, and compounds are then eluted with 10 ml ethyl acetate:heptane (50:50, v/v). The eluate is applied to a silica Sep-Pak cartridge prewashed with ethyl acetate (5 ml). It is rinsed with 5 ml ethyl acetate and compounds eluted with 5 ml ethyl acetate:methanol (50:50, v/v). The eluate is dried under nitrogen. Compounds are converted to the pentafluorobenzyl (PFB) esters by the addition of 40 μl of a 10% solution of pentafluorobenzyl bromide in acetonitrile and 20 μl of a solution of 10% diisopropylethanolamine in acetonitrile and allowed to incubate for 30 min at 37°C. Reagents are dried under nitrogen and the residue reconstituted in 30 μl chloroform and 20 μl methanol and chromatographed on a silica TLC plate to 13 cm in a solvent system of chloroform:methanol (93:7, v/v). The methyl ester of PGF2α is chromatographed on a separate lane and visualized with 10% phosphomolybdic acid in ethanol by heating. The Rf of PGF2α methyl ester in this solvent system is 0.15. Compounds migrating in the region 1 cm below the PGF2α standard to 1.0 cm above the standard are scraped from the TLC plate, extracted with 1 ml ethyl acetate, and dried under nitrogen. Following TLC purification, compounds are converted to trimethylsilyl (TMS) ether derivatives by addition of 20μl N,O-bis(trimethylsilyl)trifluoroacetamide and 10μl dimethylformamide. The sample is incubated at 37°C for 10 minutes and then dried under nitrogen. The residue is re-dissolved for GC/MS analysis in 20μl undecane that has been
stored over a bed of calcium hydride. GC/NICI-MS is carried out on an Agilent 5973 Inert Mass Selective Detector that is coupled with an Agilent 6890n Network GC system (Agilent Labs, Torrance, CA) that is interfaced with an Agilent computer (Milne et al., 2007). GC is performed using a 15 m, 0.25 um film thickness, DB-1701 fused silica capillary column (J and W Scientific, Folsom CA). The column temperature is programmed from 190°C to 300°C at 20°C per minute.

The major ion generated in the NICI mass spectrum of the PFB ester, TMS ether derivative of F2-IsoPs is the m/z 569 carboxylate anion [M-181 (M-CH2C6F5)]. The corresponding ion generated by the [2H4]-15-F2t-IsoP internal standard is m/z 573. Levels of endogenous F2-IsoPs in a biological sample are calculated from the ratio of intensities of the ions m/z 569 to m/z 573. Employing this assay, the lower limit of detection of F2-IsoPs is in the range of 4pg using an internal standard with a blank of 3 parts per thousand. The precision of this assay in biological fluids is ±6% and the accuracy 94%.

2.9.2 Measurement of urinary 2,3-dinor-6-keto-PGF1α

Urinary 2,3-dinor-6-keto-PGF1α is the major urinary metabolite of prostacyclin (PGI2, PGI-M). To 3 ml of urine is added 1 ng [2H4]-2,3-dinor-6-keto-PGF1α. It is then acidified to pH 3 with 1N HCl and allowed to stand at room temperature for 5 min. Under these conditions, 2,3-dinor-6-keto-PGF1α exists essentially entirely as a lactone. The sample is then applied to a C18 Sep-Pak preconditioned with 5 ml methanol followed by 5 ml 0.01N HCl. The sample is rinsed with 10 ml 0.01N HCl followed by 10 ml heptane. The sample is then eluted with 10 ml ethyl acetate-heptane (50/50, v/v). The eluate is dried under 10 g of anhydrous sodium sulfate and the solvent evaporated under nitrogen. The residue is reconstituted in 1 ml of dichloromethane and washed twice with 2 ml of a 50mM sodium borate buffer (pH 8.1), discarding the aqueous layer after each wash. At this step, 2,3-dinor-6-keto-PGF1α still remains in the lactone form and does not extract into the aqueous phase. Non-lactonized fatty acids that are present as carboxylate anions, however, are readily extracted into the buffer layer. Subsequently, the organic phase is dried under nitrogen and reconstituted in 50 μl of acetonitrile. To this is added 200 μl of a 3% solution of methoxyamine hydrochloride in water and the mixture is incubated for 45 min at room temperature. This step results in opening of the lactone ring of the molecule and simultaneous derivatization of the keto group. The sample is then extracted with 1 ml of dichloromethane and dried under nitrogen. The residue is derivatized to the pentafluorobenzyl (PFB) ester by the addition of 40 μl PFB bromide (10% by volume in acetonitrile) and 20 μl N,N-diisopropylethylamine (10% by
volume in acetonitrile) and is incubated for 20 min at 37°C. The sample is then dried under nitrogen and reconstituted in 50 µl of methanol. It is applied to a silica TLC plate and developed to the top in a solvent system of ethyl acetate-methanol (98/2, v/v). The methyloxime, PFB ester derivative of 6-keto-PGF₁α (5 pg) is chromatographed as a TLC standard on a separate lane and visualized with 10% phosphomolybdic acid after heating. The 6-keto-PGF₁α derivative is used as a TLC standard rather than derivatized 2,3-dinor-6-keto-PGF₁α to avoid any contamination of the biological samples. Using this solvent system, the 2 methyloxime isomers of derivatized 6-keto-PGF₁α are visualized with an \( R_f \) = 0.25 for the upper band and \( R_f \) = 0.21 for the lower band. Derivatized 2,3-dinor-6-keto-PGF₁α migrates in a nearly identical manner with an \( R_f \) = 0.25 for the upper band and \( R_f \) = 0.22 for the lower band. The compounds in the sample migrating 1 cm on either side of the point midway between the two methyloxime isomers of derivatized 6-keto-PGF₁α are scraped from the silica and eluted with 1 ml of ethyl acetate. The sample is then dried under nitrogen and converted to a trimethylsilyl (TMS) ether derivative by addition of 20 µl BSTFA and 10 µl dimethylformamide. The samples are incubated at 37°C for 10 min and dried under nitrogen. The residue is redissolved for GC-MS analysis in 10 µl of undecane which has been stored over a bed of calcium hydride.

GC/NICI-MS is carried out on an Agilent 5973 Inert Mass Selective Detector that is coupled with an Agilent 6890n Network GC system that is interfaced with an Agilent computer. The GC is performed using a 15 m, 0.25 mm film thickness, DB-1701 fused silica capillary column (J and W Scientific, Folsom CA). The column temperature is programmed from 190° to 300°C at 20°C per minute. The major ion generated in the NICI mass spectra of the methyloxime, PFB ester, TMS ether derivative of 2,3-dinor-6-keto-PGF₁α is \( m/z \) 586 which represents the M - 181 (M - CH,C,F,) carboxylate anion. For the deuterated standard, the corresponding ion is \( m/z \) 590. Quantification of endogenous 2,3-dinor-6-keto-PGF₁α in a urine sample is accomplished by selected-ion monitoring analysis of the ratio of intensities of \( m/z \) 586 to \( m/z \) 590. The interday variability for each assay is < 10%. The precision for the assay is ±5% while the accuracy for this assay is 98%.

2.9.3 Measurement of urinary 11-dehydro-TxB₂

For analysis, to 3 ml of urine is added 3.0 ng of [²H₄]-11-dehydro-TxB₂ (Cayman Chemical, Ann Arbor, MI) internal standard. The solution is adjusted to pH 3 with 1N HCl. The sample is then applied to a C-18 Sep-Pak cartridge that has been prewashed with 5 ml methanol
and 5 ml 0.01N HCl. The cartridge is then washed with 10 ml 0.01N HCl, followed by 10 ml 20% methanol in 0.01N HCl, and the analyte is then eluted with 10 ml ethyl acetate. The eluate is dried under nitrogen. The residue is re-dissolved in methanol:water (1:16, v/v) and then adjusted to a pH of 2.5. The sample is allowed to stand for 30 minutes at room temperature. The analyte is extracted from this solution using 1 ml of ethyl acetate that is then transferred to a microcentrifuge tube and dried under nitrogen. The sample is reconstituted in 50 μl ethyl acetate and then purified on a silica TLC plate to 13 cm using a solvent of ethyl acetate:acetone:acetic acid (97:3:2, v/v/v). PGD₂ is chromatographed on a separate lane as a reference standard and visualized with 10% phosphomolybdic acid in ethanol by heating. The Rᵣ of PGD₂ in this solvent system is 0.40. Compounds migrating in the region 1 cm below the PGD₂ standard to 1.0 cm above the standard are scraped from the TLC plate, extracted with 1 ml ethyl acetate, and dried under nitrogen. Compounds are then converted to the PFB esters by the addition of 40 μl of a 10% solution of pentafluorobenzyl bromide in acetonitrile and 20 μl of a solution of 10% diisopropylethanolamine in acetonitrile and allowed to incubate for 30 min at 37°C. Reagents are dried under nitrogen and the residue reconstituted in 50 μl ethyl acetate and chromatographed on a silica TLC plate to 13 cm in a solvent system of ethyl acetate:heptane (60:40, v/v). The PFB ester PGD₂ is chromatographed on a separate lane as a reference standard and visualized with 10% phosphomolybdic acid in ethanol by heating. The Rᵣ of PGD₂ PFB ester in this solvent system is 0.23. Compounds migrating in the region 1.3 cm below the PGF₂α standard to 0.7 cm above the standard are scraped from the TLC plate, extracted with 1 ml ethyl acetate, and dried under nitrogen. Following TLC purification, compounds are converted to trimethylsilyl (TMS) ether derivatives by addition of 20 μl N,O-bis(trimethylsilyl)trifluoroacetamide and 10 μl dimethylformamide. The sample is incubated at 37°C for 10 minutes and then dried under nitrogen. The residue is re-dissolved for GC/MS analysis in 20 μl undecane that has been stored over a bed of calcium hydride.

GC/NICI-MS is carried out on an Agilent 5973 Inert Mass Selective Detector that is coupled with an Agilent 6890n Network GC system (Agilent Labs, Torrance, CA) that is interfaced with an Agilent computer. GC is performed using a 15 m, 0.25 um film thickness, DB-1701 fused silica capillary column (J and W Scientific, Folsom CA). The column temperature is programmed from 190°C to 300°C at 20°C per minute. The major ion generated in the NICI mass spectrum of the PFB ester, TMS ether derivative of 11-dehydro-TxB₂ is the m/z 511 carboxylate anion [M-181 (M-CH₂C₆F₅)]. The corresponding ion generated by the [²H₄]- 11-dehydro-TxB₂ internal standard is m/z 515. Levels of endogenous
11-dehydro-TxB₂ in a biological sample are calculated from the ratio of intensities of the ions m/z 511 to m/z 515. Employing this assay, the lower limit of detection of 11-dehydro-TxB₂ is in the range of 10pg using an internal standard with a blank of 3 parts per thousand. The precision of this assay in biological fluids is ±7% and the accuracy 90%.
3.1 Introduction

In section 1.5 I have outlined the importance of quantification of peripheral vascular dysfunction to evaluate response in therapeutic trials in RP. The episodic nature of RP attacks has led to a dependence on self-report assessment of the frequency, duration and severity of RP symptoms. These tools are subjective, sensitive to placebo effect, time consuming, require a prolonged period of assessment and influenced by seasonal variation in environmental temperature. Furthermore, the frequency, duration and severity of RP attacks are influenced by the effectiveness of coping strategies adopted by patients to avoid the conditions responsible for RP attacks and shorten the duration attacks when they occur.

A major limiting factor of studies assessing efficacy in therapeutic studies of Raynaud’s phenomenon is the lack of validated objective outcome tools for the assessment of digital vascular function. Currently, only patient self-reports and physician global assessments of RP severity are recommended by OMERACT (see section 4.1.2 for further details) for use in therapeutic trials of Raynaud’s phenomenon (Merkel et al., 2003). Of the methods that have been evaluated to attempt to objectively assess digital vascular function, the most widely assessed has been IRT. IRT has the advantage of providing sensitive, quantifiable and responsive data regarding vascular function. Thermographic protocols for RP assessment often incorporate a local cold stress test (CST) to allow dynamic assessment of vascular function, whilst attempting to simulate the conditions responsible for an attack of RP in vivo. Despite the many trials that have incorporated IRT, no consensus has emerged on the preferred thermographic protocol and/or endpoints for use in clinical trials of RP.
3.2 Objectives

In this chapter, I shall report the findings of a systematic review of all the previous therapeutic trials of RP that have incorporated IRT as an endpoint. I shall describe and critique the various thermographic protocols and endpoints adopted and highlight potential areas for future research.

3.3 Methods

3.3.1 Study selection

To ensure comprehensive study identification, our search criteria extended to all articles reporting the use of IRT in the context of RP and SSc-spectrum disease. Initial searches indicated this was necessary to capture all studies using IRT as an outcome measure, despite identifying a large number of primarily diagnostic or unrelated studies (e.g. relating to morphoea). A systematic search of the literature, limited to the English language and articles published between 1975 and the present day, was performed using the MEDLINE®, EMBase®, AMED® databases accessed via the NHS National Health Resources on March 9th 2011. The search terms used were: (Thermography) AND (CREST OR Scleroderma OR systemic sclerosis OR Raynaud's). For completion, an additional search was undertaken using PubMed® on 21st October 2011. The following keywords and medical subject heading (MeSH) terms were used: (Thermography [all fields] OR Thermal [all fields]) AND (Raynaud's [all fields] OR CREST [all fields] OR sclerosis, progressive systemic [all fields] OR scleroderma [all fields]). These 2 separate searches were undertaken to ensure all relevant articles were identified during the search phase of the study. An additional hand search for relevant articles cited in these papers was also performed.

The study selection process comprised two rounds. The first was based on the titles and abstracts of the studies identified following the preliminary searches. Diagnostic studies, those investigating conditions other than RP or SSc (such as morphoea) or studies of paediatric disease were excluded. Similarly, review articles, editorials, comments, conference abstracts and individual case reports were excluded from analysis unless they specifically reported the findings of a clinical trial. Studies incorporating methods other than non-contact IRT for the measurement of cutaneous temperature (e.g. contact thermistors)
were also excluded. The full text of each relevant article identified in the first round was retrieved and reviewed. Studies fulfilling the above exclusion criteria, or those lacking sufficient information to facilitate further analysis were subsequently excluded.

3.3.2 Data extraction
One investigator (Dr John D. Pauling, JDP) was responsible for selecting and reviewing articles based on title and abstract. The full text of studies selected for further consideration were reviewed by 2 investigators (JDP and Dr Jacqueline A. Shipley, JAS). Data was systematically extracted using a pre-designed proforma to record year of publication, study design, study size, patient characteristics, intervention, clinical endpoints and outcome, together with thermographic endpoints and outcome. In addition, each study was scored independently by 2 investigators (JDP and JAS) for methodological quality using the well-described Jadad scoring method (scale 0-5) (Jadad et al., 1996). Where agreement was not present following independent assessment, the full text was re-reviewed by both assessors and consensus reached. In particular, studies were only awarded a point for describing withdrawals and dropouts if this was explicitly stated in the text, even if the results presented would suggest no patients had dropped out of the trial. In accordance with previous studies, Jadad scores of 0-2 and 3-5 were considered low and high quality respectively.

3.3.3 Study analysis
An analysis of all studies was undertaken to describe their key characteristics in terms of study design, intervention and clinical/thermographic parameters used. Relatively small study numbers and significant variation in study design, therapeutic intervention, imaging protocols and thermographic endpoints precluded any useful attempt at statistical pooling of results as part of a formal meta analysis. Nonetheless, some indication of the utility of specific thermographic parameters can be derived from evaluating agreement between the outcomes of clinical and thermographic endpoints of digital vascular function following intervention. A secondary analysis was therefore undertaken focussing on agreement between clinical and thermographic outcomes within studies. Studies failing to adequately report the outcome of clinical and/or thermographic endpoints, or those with small sample sizes (n<13), were excluded from the secondary analysis to ensure this targeted the larger, better described studies with greater informative value.
3.4 Results

3.4.1 Article selection

A flowchart summarising the outcome of the search process used to identify publications for final analysis is presented in Figure 3.1. Of the 294 articles identified from the original searches, 37 were deemed appropriate for full text analysis. Of these publications, a further 6 were excluded on review of the full text; four due to the use of contact thermocouples rather than IRT (Belch et al., 1981, Ranieri et al., 1993, Arosio et al., 1991), one review article (Wouda, 1987), and one which failed to report the methods or outcome of thermal imaging (Lukac J, 1991). We could not obtain the manuscript for one study using either our institutional subscriptions or inter-library loans service (which included access to all journals held within the British Library) (Merla et al., 2002). Two additional publications were identified following a subsequent hand search prompted by citations identified in the full-text of other selected articles and were included in subsequent analysis. This resulted in a full-text review of 32 studies. Twenty studies were excluded from the secondary analysis (evaluating agreement between clinical and thermographic endpoints) due to small sample size (n ≤ 13) and/or an insufficient description of clinical and/or thermographic endpoints (Yamaoka, 1987, Wesseling et al., 1981, Stratton et al., 1997, Shawket et al., 1991, Selenko-Gebauer et al., 2006, Ring and Bacon, 1977, Rademaker et al., 1989, Meloni et al., 1987, Martin et al., 1981, Maeda et al., 1998, Kyle et al., 1985, Kyle et al., 1992, Klimiuk et al., 1989b, Katoh et al., 1992, Kan et al., 2002, Ishigatsubo et al., 2010, Gush et al., 1987, Cooke et al., 1997, Balogh et al., 2002, al-Awami et al., 2001). The key characteristics and findings of the 32 studies included in the analysis are summarised in Table 3.1.

3.4.2 Study population

The 32 publications identified following the literature search represent the study of 654 patients with Raynaud’s phenomenon, the majority of whom had a diagnosis of SSc (326, 49.8%) or primary RP (230, 35.2%). The remaining patients (98, 15%) had RP associated with other conditions, typically other rheumatic diseases such as rheumatoid arthritis, mixed connective tissue disease and systemic lupus erythematosis.
Many studies incorporated a heterogeneous mix of RP patients, and these studies did not generally undertake sub-analyses that specifically compared disease groups. Other studies used more homogeneous groups of either primary RP (e.g. (Schlager et al., 2011, Hirschl et al., 2004, Hirschl et al., 2002)) or SSc (e.g. (Herrick et al., 2000)). IRT did not appear to perform better in one disease group than the other although the small number of studies incorporating a homogenous disease cohort, and the range of interventions, precluded any useful comparison along these lines.
**Table 3.1. Summary table of the studies reporting the use of infrared thermography (IRT) as an endpoint in clinical trials of Raynaud’s phenomenon.**

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study design</th>
<th>Jadad Score</th>
<th>Intervention</th>
<th>Clinical endpoint</th>
<th>IRT protocol</th>
<th>IRT endpoint</th>
<th>Thermographic Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ring, 1977</td>
<td>Open-label</td>
<td>0</td>
<td>Inositol nicotinate 3g daily for &gt;2 months</td>
<td>Not assessed</td>
<td>IRT at baseline, week 1, week 3, week 7 and 9 months</td>
<td>Thermographic index of dorsal aspect of three middle fingers and dorsum of hands</td>
<td>Trend for improvement after 7 weeks treatment</td>
</tr>
<tr>
<td>Clifford, 1980 (Clifford et al., 1980)</td>
<td>Open label</td>
<td>0</td>
<td>26/2/18</td>
<td>72 hours IV PG-E1</td>
<td>IRT at baseline, during, immediately after and at 2 and 6 weeks post treatment</td>
<td>Average T values from spot measurements with an infra red radiometer of 6 areas of dorsum of fingers and 5 areas of dorsum of hands</td>
<td>Significant increase in hand/finger T during, after, at 2 weeks, and at 6 weeks</td>
</tr>
<tr>
<td>Martin, 1981</td>
<td>Single-blind cross over trial</td>
<td>0</td>
<td>12/0/12</td>
<td>3 day treatment period with PG-E1 vs. placebo</td>
<td>One hand subjected to CST with assessments before and at 4 and 10 minutes post-CST (20°C for 60s)</td>
<td>IRT assessments at baseline, daily during treatment period and at 2 weeks post treatment</td>
<td>No change in response to CST</td>
</tr>
<tr>
<td>Wesselingh, 1981</td>
<td>Double-blind randomised placebo-controlled cross-over</td>
<td>3</td>
<td>7/4/2</td>
<td>Acute effects of sublingual and oral isoxsuprined vs. placebo</td>
<td>IRT at baseline and during 20 minutes cooling (hands placed on cooling plate at 6°C) and every 5 minutes during subsequent 30 minutes re-warming</td>
<td>% change in T over cross-section of all fingers of left hand during cooling after treatment</td>
<td>Significantly decreased cooling following sublingual and oral therapy compared with placebo</td>
</tr>
<tr>
<td>Ring, 1981</td>
<td>Open-label</td>
<td>1</td>
<td>Inositol nicotinate 1g qds for 36 weeks</td>
<td>Not assessed</td>
<td>IRT at baseline and during 20 minutes cooling (hands placed on cooling plate at 6°C) and every 5 minutes during subsequent 30 minutes re-warming</td>
<td>% change in T of fingers – dorsum of hands</td>
<td>Significant reduction in the magnitude of the post-CST TG following treatment. No change in baseline TG.</td>
</tr>
<tr>
<td>Dowd, 1982</td>
<td>Open-label</td>
<td>1</td>
<td>25/0/25</td>
<td>IV prostaglandin over 72 hours</td>
<td>Significant improvement in pain, numbness, and reported duration of attacks post-treatment</td>
<td>IRT at 1, 0, 1, 3, 5, 12, 24 &amp; 36 weeks, IRT pre and 10 minutes post CST (20°C for 60s).</td>
<td>Tg (T of fingers – dorsum of hands) at baseline and 10 minutes post-CST</td>
</tr>
<tr>
<td>Kyle, 1985</td>
<td>Open-label</td>
<td>1</td>
<td>10/0/6</td>
<td>3 consecutive day infusions of PG-E1 (12 week assessment period)</td>
<td>Pain (10-VAS)</td>
<td>IRT at baseline and 2 week intervals following treatment.</td>
<td>Significant increase in T of hands and fingers during, immediately after and at 2 weeks post infusion using IRT.</td>
</tr>
</tbody>
</table>

DU, digital ulceration; CREST, calcinosis, Raynaud’s, Esophageal dysmotility, Sclerodactyly, Telangiectasia; CST, cold stress test; PRP, primary Raynaud’s phenomenon; SSc, systemic sclerosis; RP, Raynaud’s phenomenon; T, temperature; VAS, Visual Analogue Scale; PG-E1, Prostaglandin E1; SHAQ, Scleroderma Health Assessment Questionnaire; PG-I2, Prostaglandin I2; CGRP, Calcitonin Gene Related Peptide; Ax, assessment; TG, Thermal Gradient; NS, non-significant; LLLT, low level laser therapy; 5-HT, 5-hydroxytryptamine; DDD, dorsal-distal difference
<table>
<thead>
<tr>
<th>Reference</th>
<th>Design</th>
<th>Intervention</th>
<th>Follow-up</th>
<th>Follow-up</th>
<th>Outcome Measures</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gush, 1987</td>
<td>Placebo-controlled double-blind cross-over study</td>
<td>Infusions of iloprost or placebo over 6 hours on 3 consecutive days</td>
<td>Frequency of RP attacks</td>
<td>Duration of RP attacks</td>
<td>Number, duration and severity of RP attacks</td>
<td>Significant improvement in frequency and severity of attacks following iloprost compared to placebo.</td>
</tr>
<tr>
<td>Yamaoka, 1987</td>
<td>Open-label</td>
<td>Infusions of iloprost or placebo over 6 hours on 3 consecutive days</td>
<td>Frequency of RP attacks</td>
<td>Duration of RP attacks</td>
<td>Number, duration and severity of RP attacks</td>
<td>Significant improvement in frequency and severity of attacks following iloprost compared to placebo.</td>
</tr>
<tr>
<td>Meloni, 1987</td>
<td>Double-blind, placebo-controlled randomised group comparison study</td>
<td>Infusions of iloprost or placebo over 6 hours on 3 consecutive days</td>
<td>Frequency of RP attacks</td>
<td>Duration of RP attacks</td>
<td>Number, duration and severity of RP attacks</td>
<td>Significant improvement in frequency and severity of attacks following iloprost compared to placebo.</td>
</tr>
<tr>
<td>McHugh, 1988</td>
<td>Open-label</td>
<td>Infusions of iloprost or placebo over 6 hours on 3 consecutive days</td>
<td>Frequency of RP attacks</td>
<td>Duration of RP attacks</td>
<td>Number, duration and severity of RP attacks</td>
<td>Significant improvement in frequency and severity of attacks following iloprost compared to placebo.</td>
</tr>
<tr>
<td>Rademaker, 1989</td>
<td>Double-blind, placebo-controlled randomised group comparison study</td>
<td>Infusions of iloprost or placebo over 6 hours on 3 consecutive days</td>
<td>Frequency of RP attacks</td>
<td>Duration of RP attacks</td>
<td>Number, duration and severity of RP attacks</td>
<td>Significant improvement in frequency and severity of attacks following iloprost compared to placebo.</td>
</tr>
<tr>
<td>Klimiuk, 1989</td>
<td>Open-label</td>
<td>Infusions of iloprost or placebo over 6 hours on 3 consecutive days</td>
<td>Frequency of RP attacks</td>
<td>Duration of RP attacks</td>
<td>Number, duration and severity of RP attacks</td>
<td>Significant improvement in frequency and severity of attacks following iloprost compared to placebo.</td>
</tr>
<tr>
<td>Shawkett, 1991</td>
<td>Double-blind cross-over</td>
<td>Infusions of iloprost or placebo over 6 hours on 3 consecutive days</td>
<td>Frequency of RP attacks</td>
<td>Duration of RP attacks</td>
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</tr>
<tr>
<td>Kyle, 1992</td>
<td>Double-blind cross-over</td>
<td>Infusions of iloprost or placebo over 6 hours on 3 consecutive days</td>
<td>Frequency of RP attacks</td>
<td>Duration of RP attacks</td>
<td>Number, duration and severity of RP attacks</td>
<td>Significant improvement in frequency and severity of attacks following iloprost compared to placebo.</td>
</tr>
<tr>
<td>Katoh, 1992</td>
<td>Open-label</td>
<td>Infusions of iloprost or placebo over 6 hours on 3 consecutive days</td>
<td>Frequency of RP attacks</td>
<td>Duration of RP attacks</td>
<td>Number, duration and severity of RP attacks</td>
<td>Significant improvement in frequency and severity of attacks following iloprost compared to placebo.</td>
</tr>
<tr>
<td>Year</td>
<td>Design</td>
<td>Intervention/Method</td>
<td>Outcome Measures</td>
<td>Details</td>
<td></td>
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<tr>
<td>Teh, 1995; Teoh et al., 1995 (Teoh et al., 1995)</td>
<td>Randomised d. double-blind, placebo-controlled, cross-over study</td>
<td>3 10 days transdermal GTN (0.2mg/hr) vs. placebo</td>
<td>Frequency of RP attacks</td>
<td>Improvement in frequency and severity of RP attacks</td>
<td></td>
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<tr>
<td>Hirschl, 1997 (Alroy et al., 1997)</td>
<td>Retrospective re-study</td>
<td>Digital sympathectomy</td>
<td>Frequency of RP attacks</td>
<td>Improvement in frequency and severity of RP attacks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cooke, 1997</td>
<td>Open-label</td>
<td>Autologous blood transfusions following ex vivo heating, ozonation and UV light therapy</td>
<td>Subjective improvement in severity and duration of attacks</td>
<td>Improvement persisting for at least 3 months post treatment reported</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maeda, 1998 (Maeda et al., 1998)</td>
<td>Open-label</td>
<td>Acute effects of electrical acupuncture</td>
<td>Not assessed</td>
<td>IRT assessments at baseline, 10, 45 and 60 minutes following electrical acupuncture</td>
<td>Mean hand T</td>
<td>Non significant increase in all patients immediately post-therapy</td>
</tr>
<tr>
<td>Dzidzio, 1999</td>
<td>Open-label, randomised parallel group study</td>
<td>1 Losartan 50mg od vs. nifedipine 20mg bd 6 weeks</td>
<td>Frequency and severity of RP attacks (0-10 VAS)</td>
<td>Significant reduction in frequency and severity of attacks following losartan only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herrick, 2000</td>
<td>Randomised placebo-controlled double-blind cross-over study</td>
<td>3 10 week combination antioxidants and allopurinol vs. placebo</td>
<td>Frequency of RP attacks Duration of RP attacks Subjective report of RP severity (better, same or worse)</td>
<td>No improvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coleiro, 2001</td>
<td>Open-label randomised cross-over study</td>
<td>2 6-week treatment periods with fluoxetine 20mg OD vs. nifedipine LA 40mg OD 6 weeks</td>
<td>Frequency and severity of RP attacks (0-10 VAS)</td>
<td>Sig reduction in frequency and severity of attacks following fluoxetine only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aliawi, 2001</td>
<td>Open-label</td>
<td>10 sessions of LLLT over 6 weeks</td>
<td>Frequency and severity of RP attacks (0-10 VAS)</td>
<td>Significant improvement in VAS scores at 6 weeks and 3 months Baseline and 6 weeks and 3 months a) basal fingertip skin T b) immediately after 1 minute warm challenge (immersion of gloved hands in water at 39°C), c) measurements immediately after CST (20°C for 60s). Recovery temperatures were measured 10 and 20 minutes later.</td>
<td>Recovery T after cold exposure Significant improvement at 6 weeks reported in all 40 patients but data not presented</td>
<td></td>
</tr>
<tr>
<td>Hirsch, 2002</td>
<td>Randomised double-blind, placebo-controlled, cross-over study</td>
<td>3 LLLT vs placebo (5 sessions per week for 3 weeks)</td>
<td>Frequency of RP attacks</td>
<td>Improvement in intensity but not frequency of RP attacks</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Mean T at baseline, 15°C for 60s</th>
<th>Improvement in mean change over 15 minutes post-CST</th>
<th>No improvement in recovery 10 minutes after cold challenge following other drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author, Year</td>
<td>Study Design</td>
<td>Patient Characteristics</td>
<td>Intervention</td>
</tr>
<tr>
<td>-------------</td>
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</tr>
<tr>
<td>Balogh, 2002</td>
<td>Open-label</td>
<td>7/7/0 Adventitial stripping of radial and ulnar arteries</td>
<td>Questionnaire 1-year post surgery. Qualitative assessment of pain, frequency and duration of RP attacks. QoL 0-10 VAS. Questionnaire assessment of ulcers, use of gloves and fridge/cold tolerance</td>
</tr>
<tr>
<td>Kan, 2002</td>
<td>Open label</td>
<td>25/0/25 Nitro-glycerine (NTG) patch applied to one wrist</td>
<td>Nil</td>
</tr>
<tr>
<td>Al-Awami, 2004</td>
<td>Randomised, double-blind, placebo-controlled</td>
<td>47/18/5 10 sessions of LLLT vs. sham laser placebo</td>
<td>Frequency of RP attacks</td>
</tr>
<tr>
<td>Hirsch, 2004</td>
<td>Double-blind randomised, placebo-controlled cross-over</td>
<td>48/48/0 3 weeks LLLT vs. placebo</td>
<td>Frequency and intensity (5 point VAS) of RP attacks</td>
</tr>
<tr>
<td>Selenko-Gebauer, 2006</td>
<td>Open label</td>
<td>3/0/2 Bosentan 62.5mg bd weeks 0-4, 125mg bd weeks 5-12</td>
<td>Frequency, duration and severity (1-3) of RP attacks. The SHAQ and pain VAS (0-10 VAS)</td>
</tr>
<tr>
<td>Ishigatsu, 2010</td>
<td>Open label</td>
<td>8/0/8 Autologous transplantation of bone-marrow-derived cells</td>
<td>Pain VAS (0-10) and ulcer size</td>
</tr>
<tr>
<td>Shlager, 2011</td>
<td>Open-label</td>
<td>28/28/0 Auricular electroacupuncture e, 6 sessions over 6 weeks</td>
<td>Frequency (diary monitoring) and severity (0-10 VAS) of RP attacks</td>
</tr>
</tbody>
</table>
3.4.3 Study size

Study size was generally small with a median participant number of 15.5 (inter-quartile range 8.75 to 26.75). Fifty percent of the studies consisted of greater than 15 subjects whilst ten studies (31.3%) consisted of fewer than 10 subjects.

3.4.4 Study design

A variety of methodological approaches were used, although the majority of studies were open-label in design (19/32, 59.4%). There were 10 (31.3%) randomised double-blind, placebo-controlled (or cross-over studies), 2 single-blind studies and one retrospective study.

3.4.5 Study quality

The majority of studies (22/32, 68.8%) would be considered of low quality according to the Jadad score (0-2). Seven studies had a Jadad score of 3 and three had a Jadad score of 4.

3.4.6 Date of publication

The studies are evenly distributed in terms of date of publication with 1 study in the 1970’s, 12 studies in the 1980s, 8 studies in 1990s and 11 studies since 2000.

3.4.7 Intervention

A large variety of interventions had been assessed including pharmacological treatments (22/32, 68.7%), complementary therapies (6/32, 18.7%), surgical intervention (2/32, 6.3%) and autologous blood product transfusions (2/32, 6.3%). The most commonly evaluated pharmacological agent was exogenous prostanoid therapy (9/32, 28.1%). Most classes of pharmaceutical drug commonly used in the management of Raynaud’s phenomenon have been evaluated thermographically in clinical trials, one notable exception being phosphodiesterase inhibition.

3.4.8 Technical aspects

As anticipated, a wide variety of brands and models of infrared camera were used in the studies, precluding any evaluation of study quality on the basis of equipment used.

3.4.9 Clinical endpoints

The majority of studies incorporated clinical as well as thermographic endpoints. Many early studies adopted categorical descriptions from patients regarding RP severity following
intervention (e.g. much better, better, no change, worse etc.) or visual analogue scales (VAS) scores assessing pain levels associated with RP attacks (Martin et al., 1981, Clifford et al., 1980). More recent studies have incorporated quantitative assessments of RP severity using VAS and RP diaries recording attack frequency, duration and severity. Interestingly, none of the studies have used the recommended Raynaud’s Condition Score (RCS), originally proposed in 1998 and recommended for use in clinical trials of RP since 2003 (Merkel et al., 2003). Modified versions of the RCS, collecting information on the frequency and duration of RP attacks have been used, presumably for practical reasons relating to study design.

3.4.10 Thermographic protocol

The majority of studies incorporated a period of acclimatisation in a quiet temperature-controlled laboratory prior to thermographic assessment. Temperature measurement of the dorsum of the hands was undertaken in most studies although measurement of the volar aspect of the fingers has also been described (Schlager et al., 2011). The majority of thermographic protocols incorporated a CST (22/32, 68.8%), usually achieved through the submersion of gloved hands into a water bath. The conditions of the CST varied, although the most commonly used protocol involved a cold challenge of 20°C for 60s (14/32, 43.8%). The use of cooled contact plates to promote vasoconstriction has also been evaluated (Wesseling et al., 1981). Several studies incorporated an additional period of digital warming prior to evaluation of the CST (Kyle et al., 1992, Kyle et al., 1985, Shawket et al., 1991, al-Awami et al., 2001, al-Awami et al., 2004).

3.4.11 Thermographic endpoints

Two principle methods have emerged for the reporting of thermographic endpoints; absolute temperature values of the hands and/or fingers from distinct regions of interest, and/or thermal gradients derived from either the difference between the mean temperatures of the dorsum of the hand and of the fingers, or the longitudinal thermal gradient along individual digits. These parameters could be derived from baseline assessment alone although thermographic protocols incorporating a local cold challenge were also able to report additional thermographic parameters describing responses to cold exposure. These included absolute temperature values before and after cold challenge (Clifford et al., 1980, Dowd et al., 1982, Rademaker et al., 1989, Dziadzio et al., 1999, Coleiro et al., 2001, al-Awami et al., 2001, al-Awami et al., 2004, Schlager et al., 2011), percent recovery indices such as maximum percent recovery during 10 minutes re-warming period post-CST (Coleiro et al., 2001), or longitudinal thermal gradients at specific time-points following cold challenge
If sufficient thermographic assessments are undertaken following the cold challenge, attempts can be made to quantify the characteristics of the re-warming curve using parameters such as the lag-time to re-warming and maximum percent recovery (Herrick et al., 2000, Teh et al., 1995). Additional thermographic parameters derived from the re-warming curve characteristics such as area under the curve and maximum gradient of re-warming that have been successfully applied in diagnostic studies have not yet been evaluated as endpoints in therapeutic trials of RP. A few studies, notable for their small sample sizes, used qualitative assessment of the appearances of the thermal images alone to attempt to demonstrate response to therapy (Selenko-Gebauer et al., 2006, Ishigatsubo et al., 2010, Balogh et al., 2002).

3.4.12 Agreement between clinical and thermographic endpoints

Improvement in both clinical and thermographic endpoints

The majority of studies (18/32, 56.3%) reported improvements in both clinical and thermographic outcome measures following intervention. Nine of these studies involved small patient numbers (n ≤ 13) (Stratton et al., 1997, Selenko-Gebauer et al., 2006, Meloni et al., 1987, Martin et al., 1981, Klimiuk et al., 1989b, Katoh et al., 1992, Cooke et al., 1997, Balogh et al., 2002, Kyle et al., 1985). A further two studies failed to substantiate evidence for thermographic improvement with supportive data (Rademaker et al., 1989, al-Awami et al., 2001). The extent to which one can critically appraise the thermographic parameters used in these 11 studies is questionable.

The remaining 7 studies are worth further consideration. Three of these studies (1 blinded) evaluated the use of prostanoids such as prostacyclin (PG-I₂) and prostaglandin E₂ (PG-E₂) in studies of greater than 20 patients (McHugh et al., 1988, Dowd et al., 1982, Clifford et al., 1980). These par-enterally administered agents are amongst the most potent vasodilators and recommended for use in severe RP or for the emergency management of ischaemic complications in SSc (Kowal-Bielecka et al., 2009). Clinical endpoints in these studies included pain VAS, subjective categorical variables (better, same, worse), documenting ulcerative complications, and the frequency, duration and severity of RP attacks. Two studies used the average temperature of hands/fingers at baseline to demonstrate improved peripheral vascular function following therapy, but failed to demonstrate any treatment effect in the parameters following the CST (Dowd et al., 1982, Clifford et al., 1980). In contrast,
McHugh et al. did not identify improvements in baseline thermal imaging following iloprost therapy but did identify a reduced thermal gradient (dorsum of fingers – dorsum of hands) 10 minutes post-CST indicative of a suppressed vasospastic response to cold exposure (McHugh et al., 1988). The remaining 4 studies evaluated a range of interventions including inositol nicotinate, serotonin re-uptake inhibition and low level laser therapy (LLLT) (Ring et al., 1981, Hirschl et al., 2002, Coleiro et al., 2001, al-Awami et al., 2004). Ring et al. identified significant improvements in VAS scores for coldness, pain and numbness VAS scores, and a significant reduction in the estimated duration of vasospastic incidents post-treatment (Ring et al., 1981). This was accompanied by a significant reduction in the magnitude of the thermal gradient 10 minutes post-CST (the same endpoint used in the aforementioned study by McHugh et al.) (McHugh et al., 1988, Ring et al., 1981). Coleiro et al. used an open-label randomised cross over study design to demonstrate a reduction in the frequency and severity of RP attacks following fluoxetine therapy (Coleiro et al., 2001). This was accompanied by a significant improvement in percent re-warming 10 minutes post-cold challenge. There was no improvement in baseline hand temperature following treatment (Coleiro et al., 2001). A double-blind study of LLLT undertaken by Hirschl et al. demonstrated improvement in the intensity of RP attacks accompanied by an improvement in the mean longitudinal temperature gradient across all fingers at 20 minutes post-CST (Hirschl et al., 2002). The number of digits with a pathological thermal gradient of < -1°C 10 minutes post-CST did not improve following LLLT (Hirschl et al., 2002). A similarly designed study, also evaluating LLLT, demonstrated significant improvements in the frequency and severity of RP attacks in both the LLLT and placebo arms (al-Awami et al., 2004). In this study a corresponding improvement in the fingertip skin temperature post-cold challenge was demonstrated following LLLT but not placebo (al-Awami et al., 2004).

**Improvement in clinical endpoints alone**

Six studies (6/32, 18.8%) identified improvements in clinical endpoints that were not accompanied by improvements in thermographic parameters (Yamaoka, 1987, Dziadzio et al., 1999, Hirschl et al., 2004, Kyle et al., 1992, Schlager et al., 2011, Teh et al., 1995). Two studies were excluded from further analysis due to small study numbers (n<13) (Yamaoka, 1987, Kyle et al., 1992). Of the remaining studies, two were double-blind RCTs, one evaluating transdermal Glycerine Tri-Nitrate (Teh et al., 1995), and the other LLLT (Hirschl et al., 2004). Thermographic endpoints in these studies included distal-dorsal gradients, number of digits with a DDD of < -1°C, characteristics of re-warming curves and mean fingertip temperature following cold challenge. Interestingly, the study evaluating LLLT was
undertaken by the same group that had demonstrated improvements of both clinical and thermographic parameters in an earlier similarly designed study involving fewer patients (Hirschl et al., 2002, Hirschl et al., 2004). Of the remaining 2 studies, one was an open label study without a control group (auricular electroacupuncture) and the other an open-label study with a control arm consisting of an established therapy (losartan vs. nifedipine). In each of these studies, it is possible the beneficial effect of the investigational treatment (electroacupuncture and losartan) may have arisen from patient’s expectations concerning the treatment rather than from the treatment itself (Dziadzio et al., 1999, Schlager et al., 2011).

The remaining studies

Six studies failed to report any clinical endpoints, often when evaluating the acute digital vascular effects of therapeutic intervention (Gush et al., 1987, Kan et al., 2002, Maeda et al., 1998, Ring and Bacon, 1977, Shawket et al., 1991, Wesseling et al., 1981). Another small study failed to undertake statistical analysis of clinical or thermographic outcomes, instead providing pictorial examples of ulcer healing and figures presenting the evolution of ulcer size and pain VAS for each patient during the study period (Ishigatsubo et al., 2010). A well-designed study by Herrick et al., failed to identify improvements in either clinical or thermographic endpoints, possibly reflecting true inefficacy of the intervention (combination antioxidants and allopurinol) (Herrick et al., 2000). No studies reported an improvement in thermographic parameters without also demonstrating an improvement in clinical endpoints.

3.5 Discussion

The present paper is the first to systematically review the previous therapeutic studies of Raynaud’s phenomenon that have incorporated IRT as an outcome measure. Comparison between studies is limited by variation in study design, intervention, IRT protocols and endpoints, precluding any attempt at formal meta-analysis. Nonetheless, we have been able to provide a detailed description of the various methods incorporated in previous studies that can help influence the design of future work. No single method has emerged as the preferred thermographic parameter for use in clinical trials. We have attempted to highlight promising thermographic parameters by reporting those studies in which agreement was present between subjective clinical endpoints and thermographic parameters. Thermographic parameters including absolute temperature measurements at baseline, longitudinal thermal
gradients and percent re-warming following cold challenge have been used in studies reporting agreement between clinical and thermographic endpoints. The major limitation of considering the value of thermographic endpoints based upon their correlation with patient self-report is that objective and subjective assessments may provide differing information on digital vascular function in patients with RP.

IRT, along with other non-invasive microvascular imaging modalities, is not currently included in the proposed core set of outcome measures of vascular dysfunction in clinical trials of RP and SSc, although the urgent need for objective biomarkers of vascular function has been highlighted (Merkel et al., 2003). The outcome measures currently recommended for use in clinical trials are patient self-reports or physician global assessments. There are obvious limitations to a reliance on subjective endpoints. Patient self-reports of RP severity, usually taking the form of a diary of frequency, duration and severity of attacks carry several limitations. They are subjective, time consuming, poorly completed, and influenced by seasonal variation in environmental temperature and strategies adopted by patients to avoid conditions necessary for an attack of RP. It is our experience that patients with profound peripheral vascular dysfunction, as is commonly seen in SSc, have a tendency to under-report RP severity in self-reports. This may be the result of habituation and difficulty discerning discreet RP attacks from chronic basal vascular compromise associated with progressive structural microvascular dysfunction. Furthermore, psychosomatic testing has revealed distinct personality traits between patients with primary and secondary RP which could influence self-report measures of RP severity between the 2 groups (Bayle et al., 1990). The extent of placebo effect noted in previous therapeutic studies highlights a major disadvantage of using self-reports when assessing RP severity. Large randomised controlled studies incorporating diary cards have reported statistically significant improvements in the Raynaud’s Condition Score (RCS), duration and frequency of RP attacks of up to 60% in placebo arms (Black et al., 1998, Belch et al., 1995, Nguyen et al., 2010). “Diary fatigue” may contribute to a lower rate of self-report as studies progress. Whatever the cause, it becomes increasingly difficult to identify differences in between-group comparisons in the presence of such stark responses following placebo therapy.

Non-invasive microvascular assessment tools, such as IRT, have the advantage of providing a rapid, quantitative, objective measure of digital vascular dysfunction upon which to guide diagnostic and therapeutic decisions. Any placebo effect might be expected to be lower using
objective microvascular imaging tools compared with self-reports. Furthermore, the use of standardised microvascular imaging protocols, incorporating an acclimatisation period prior to assessment, may help negate the influence of seasonal variation in environmental temperature and the coping strategies adopted by patients to avoid cold, which influence self-reports. Several other non-invasive microvascular imaging techniques such as laser Doppler, plethysmography, digital artery pressure have been used in therapeutic trials of RP. We have not attempted to consider such methods in this chapter as it would have greatly extended the scope of the work, rendering it increasingly difficult to compare and contrast previous work.

3.6 Conclusions

To date, no single thermographic parameter has emerged as a preferred endpoint for clinical trials. The ongoing evaluation of microvascular imaging tools such as IRT in clinical trials of RP, alongside recommended self-report tools such as the RCS, is necessary noting the potential advantages of objective microvascular imaging tools over self-report. Such work needs to define the true nature of what each outcome measure actually informs us regarding digital vascular function. The creation of a consensus panel of experts to form recommendations on a standardised approach to the use of IRT in future clinical trials of RP is needed. This would encourage the use of high quality thermographic protocols and endpoints, and facilitate easier comparison of the outcomes of individual therapeutic studies of RP.
Chapter 4 The Validity and Reliability of LSCI the dynamic assessment of digital vascular function in healthy controls

"In physical science the first essential step in the direction of learning any subject is to find principles of numerical reckoning and practicable methods for measuring some quality connected with it. I often say that when you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely in your thoughts advanced to the state of Science, whatever the matter may be."

Lord Kelvin. "Electrical Units of Measurement", 1883

4.1 Introduction

A review of the background to LSCI and previous studies evaluating the use of LSCI in the assessment of cutaneous perfusion was presented in section 1.5.4. To date, no studies have evaluated the use of LSCI in the assessment of digital vascular responses to local cold challenge. Furthermore, no studies have directly compared the use of LSCI with IRT in the dynamic assessment of digital vascular function. There has also been relatively little work evaluating the repeatability of LSCI in the assessment of digital vascular function. In this chapter we shall describe preliminary work undertaken to evaluate the validity and reliability of LSCI in the assessment of digital vascular function in healthy controls. This preliminary work has helped influence the design of subsequent work in patients with primary RP and SSc that will be reported in subsequent chapters.

To better appreciate the context of the work undertaken in this chapter, I will first take the opportunity to discuss the process of validating outcome measures, using established assessment tools used in SSc as illustrative examples. I shall briefly describe the role of
OMERACT and the Scleroderma Clinical Trials Consortium (SCTC) in evaluating outcome measures for use in clinical trials of rheumatic diseases.

### 4.1.1 Validation and reliability testing of novel measurement tools

All measurement techniques use a practical test (an operationalization) to assess a theory or concept (a construct) (Trochim, 2006). A commonly used example is the use of height or weight to assess the size of an object. Whilst these 2 operationalizations clearly provide different information regarding the construct, in many instances there will be good correlation between the 2 techniques and each can help an independent observer derive conceptual meaning from the values obtained. If a new tool were developed to assess an objects size, we would want to be sure of the validity and reliability of that test before we would accept the results of using such a device with confidence. If the test results were to correlate well with other tests of the construct (such as height and weight), then we would derive some confidence of the validity of that measure. Should the new tool provide consistent readings on each occasion, we would be reassured of the reliability of the test.

### Validity

Methodological literature routinely makes reference to the different forms of “validity” in the context of outcome assessment tools. These terms create confusion and I will define the various terms and methods utilised here, using established methods for the assessment SSc as examples.

When considering RP, a basic construct might be defined as the greater the vascular insufficiency, the more intrusive the ischaemic symptoms experienced by the individual. In this instance, an operationalization such as the RCS diary should reflect the subjective experience of RP symptoms experienced by a subject. Face validity is a component of construct validity which considers whether the operationalization taken “on face value” appears to relate to your construct (Trochim, 2006). The RCS diary achieves this by considering the average daily frequency, duration and impact of RP symptoms in sufferers. If, for example, the RCS diary had ignored such considerations and instead evaluated neck pain, immediate concerns would have been raised regarding its face validity. Face validity is not easily objectively assessed making it a weak but important component of a construct’s validity (Trochim, 2006). Content validity assesses the operationalization against relevant content domain for a particular construct. For example, a novel patient questionnaire for the
diagnosis of Raynaud’s phenomenon would appear to lack content validity if it failed to consider the essential clinical attributes of an RP attack such as “episodes relating to cold exposure” or “the presence of digital cutaneous colour change”.

Criterion-related validity assesses the performance of an operationalization against specific criteria and is commonly divided into 4 domains; predictive, concurrent, convergent and discriminant validity. Predictive validity concerns the ability of an operationalization to predict what we would expect it to predict from our theory (Trochim, 2006). For example, we might theorise that severe digital ischaemia in SSc leads to digital ulceration. We could test the predictive validity of the RCS diary by comparing the daily frequency, duration and impact of RP attacks (assessed using the RCS diary) in SSc patients with and without DU. Concurrent validity assesses the ability of an operationalization to distinguish between groups. For example, we would expect the modified RSS to provide higher scores in patients with lcSSc compared to dcSSc despite each group falling within the same disease spectrum. The results of discriminatory testing such as this are more powerful if they can distinguish between groups that are otherwise very similar (such is the case in the example provided above) (Trochim, 2006). Convergent validity relates to the ability of an operationalization to provide similar information (converge upon) to an alternative operationalization measuring the same concept. For example, the RCS might be expected to correlate with other self-report measures of RP severity such as the Scleroderma HAQ RP VAS. Discriminant validity is the opposite of convergent validity and assesses the ability of an operationalization to provide information that is not similar to (divergent from) that obtained from other operationalizations that you would expect to provide different information based on your construct (Trochim, 2006). For example, you might hope that the RCS diary was not influenced by psychological factors such as depression. This could be evaluated by assessing correlation between anxiety/depression scores and the RCS diary (a poor correlation in this instance would demonstrate good discriminant validity).

Reliability
The reliability of an operationalization relates to the consistency or repeatability of a particular measurement. A measure is reliable if it provides the same result each time. For example, if the RCS could only provide information on digital vascular function, it would be expected that RP severity at 2 time-points would be repeatable, providing no external factors such as therapeutic intervention had influenced digital vascular function. The reliability of a test is
influenced by error, which is typically classified as either random or systematic error. Random error describes factors that randomly affect values obtained using an operationalization and should not have consistent effects across a sample group. Random error increases the variability of data creating unwanted “noise” (Trochim, 2006). Random error in the context of the RCS diary might include the variability introduced by patients when documenting duration of RP attacks. Identical RP attacks of 7 minutes duration may be accurately recorded as 7 minutes, rounded up to 10 minutes or rounded down to 5 minutes. A multitude of factors may influence the behaviour of respondents and across a large sample group this will increase variability of the data but should not affect the outcome across the group. Systematic error affects results in a more predictable manner and alters both the result and variability of the test (Trochim, 2006). For example, if seasonal variation in environmental temperatures affects the outcome of the RCS diary, then systematic error would influence the results of a therapeutic study using the RCS diary that recruited all patients during the winter and assessed efficacy during the summer.

Reliability is assessed by calculating the covariance of 2 observations of the same measure. A perfectly reliable measure would provide a correlation coefficient of 1 whereas a perfectly unreliable measure would give a score of zero. A reliability coefficient of 0.8 suggests that 80% of the score relates to an ability of the measures to provide a true score and 20% error (Trochim, 2006). When a test involves independent observers, the inter-observer reliability provides information on the variation of assessments between observers. Similarly, it is important to consider the intra-observer reliability (the variation in assessment undertaken by the same observer on 2 occasions), particularly when there is a subjective component. An example is the use of the mRSS (section 2.3.4) to assess skin thickening in SSc. The intra-observer reliability is within 4 (of 51) whereas the inter-observer reliability is lower with variation of ≥ 6 points (Furst, 2008). The test-retest reliability (or repeatability) assesses the reliability of an operationalization to provide the same assessment of a construct on 2 separate occasions. The result is influenced heavily by the interval between assessments, particularly in physiological studies in humans. The aforementioned example of the potential impact on seasonal variation on the outcome of the RCS diary is a good example of potential systematic error affecting repeatability when the interval between assessments is extended.
4.1.2 Evaluating outcome measures for use in therapeutic trials of SSc

OMERACT (Outcome MEasures in Rheumatoid Arthritis Clinical Trials) was developed in 1992 to better define a core set of outcome measures for clinical trials of rheumatoid arthritis (RA). OMERACT is a network of rheumatologists, epidemiologists and statisticians whose chief aim is to support the use of valid measures of response in clinical trials. The original initiative in RA provided the template for subsequent OMERACT groups to consider outcome measures for other rheumatic diseases such as SSc (Merkel et al., 2003, Furst, 2008, Furst et al., 2007). Central to the process of assessing outcome measures is the OMERACT filter which evaluates many of the key components of validation described above under the auspices of 3 main themes; truth, discrimination and feasibility. “Truth” questions the ability of an outcome tool to measure what is intended and encompasses aspects of face, content, construct and criterion validity. “Discrimination” assesses the ability of an outcome measure to discriminate between disease states at a single time point and identify changes over time, and encompasses issues surrounding reliability and responsiveness to change. “Feasibility” considers the utility of the tool within constraints set by time, affordability and interpretation. The work of OMERACT has been extended by the SCTC who have proposed a core set of outcome measures for clinical trials of SSc (Khanna et al., 2008). To date, no objective non-invasive microvascular imaging tools have been recommended for use in clinical trials of SSc, chiefly due to the paucity of work establishing the validity, reliability and feasibility of such techniques.

4.2 Objectives

The chief objective of the work undertaken in this chapter was to evaluate the validity and reliability of LSCI in the dynamic assessment of digital vascular perfusion. To this end, the specific objectives were to assess the convergent validity, repeatability and responsiveness to change of LSCI by evaluating: 1) the correlation between IRT and LSCI at baseline assessment, and following a standardised cold challenge, 2) the reproducibility of the two techniques, 3) the spatial resolution of each technique in assessing cutaneous perfusion across different regions of the digits, and 4) responsiveness of each technique to a reduction in ambient room temperature.
4.3 Subjects and Methods

4.3.1 Study population

Members of staff from the RNHRD and BIRD, aged between 18-75 years, were approached for potential inclusion as healthy controls in the study. Potential participants were issued with a participant information sheet (PIS, Appendix 1) and given the opportunity to discuss the study prior to enrollment. Subjects were excluded if they fulfilled proposed criteria for RP (Brennan et al., 1993), had a history of conditions associated with secondary RP or illnesses that might influence digital vascular function such as diabetes or peripheral vascular disease. Subjects with controlled hypertension were eligible if they had remained on a stable dose of vasodilator medication for the previous calendar month. All participants provided informed written consent (Appendix 2) and the study received prior approval from the Bath Research Ethics Committee (see section 2.2.1 for further details).

4.3.2 Study Design

Participants attended on 3 occasions at approximate weekly intervals (median 8 days, interquartile range 7-15 days). The temperature of the room was maintained at 23°C (+/- 0.5°C) throughout assessments 1 and 2 to assess repeatability. Assessment 3 was undertaken at a lower room temperature of 18°C (+/- 0.5°C) to evaluate vascular responsiveness to physiological stimulus. At each visit participants underwent simultaneous assessment of digital vascular function using IRT and LSCI at baseline and following a standardised cold challenge test, as described in section 2.4.

4.3.2 Image analysis

IRT and LSCI images were analysed as described in section 2.4.4. An example of characteristic IRT and LSCI images before and after cold challenge highlighting the ROIs chosen for assessment is presented in Figure 4.1.

The vascular endpoints chosen in this work included mean perfusion (skin temperature using IRT and arbitrary flux values obtained using LSCI) during baseline assessment (abbreviated hereafter as B), immediately following cold challenge (t0) and maximum measurement during re-warming (Max). The characteristics of the re-warming/reperfusion curves were used to evaluate the maximum % recovery (R%max, calculated as [Max - t0]/[B – t0] x100) and the maximum gradient of re-warming (Gmax). For illustrative purposes, an example of a re-
warming curve generated using IRT, highlighting each of the endpoints used, is presented in Figure 2.4. An example of a typical re-perfusion curve generated using LSCI is presented in Figure 4.2.
Figure 4.1. Example images taken using Infrared thermography (IRT) and Laser Speckle Contrast Image (LSCI).
A) IRT image baseline with colour scale; B) IRT immediately following cold challenge; C) Corresponding LSCI at baseline (red areas correspond to regions of high flux/perfusion, blue regions indicate low flux/perfusion); D) LSCI immediately following cold challenge. The ROIs used for the purposes of this work are numbered and demonstrated on image C. The temperature scale for thermal images is placed next to the images on the right side. A colour scale and associated flux values for LSCI can be found in Figure 2.4.
4.3.3 Statistical analysis

All data is presented as median values and interquartile ratio, unless otherwise stated. The interquartile ratio (calculated as interquartile range / median) was used to facilitate comparison of the variation of data between the 2 assessment tools. Correlation between LSCI and IRT was assessed using Spearman’s rank correlation coefficient ($r_s$). Comparison between different ROIs for both IRT and LSCI utilised the Wilcoxon signed-rank test. Reproducibility (between visits 1 and 2) was assessed using intra-class correlation co-efficient (ICC) to allow comparison between imaging techniques (Shrout and Fleiss, 1979). Various recommendations have been made concerning the interpretation of ICC values but ranges which have previously been adopted have suggested an ICC value of <0.4 generally indicates poor reproducibility, 0.4-0.75 represents fair to good reproducibility and >0.75 indicates excellent reproducibility (Sampat et al., 2006). Changes in response to reduction in ambient room temperature (visit 3 vs. visit 1), was assessed using Wilcoxon signed-rank test, and the use of Cohen’s $d$ effect sizes to facilitate comparison of the magnitude of response between assessment tools. All data was analysed using SPSS version 17.0 and a $p$ value of <0.05 was considered statistically significant.

Figure 4.2. An illustrative example of a typical reperfusion curve at baseline and following cold challenge (15°C for 60s) at ROI 3 using laser speckle contrast imaging (LSCI).
4.4 Results

4.4.1 Subjects
Fifteen healthy volunteers (1 male) from within our institution were recruited to the study. One female subject had a necessary change to hypertensive medication between visits 1 and 2 requiring withdrawal from the study. The mean age of the remaining subjects was 44.3 years (+/- SD 11.9 years). There was one previous and one current smoker (who refrained from smoking on the morning of assessments). Two subjects remained on stable doses of vasodilator therapy (irbesartan and atenolol respectively) during the study for underlying hypertension. Of the 13 females, 8 were pre-menopausal (4 of whom were taking hormonal contraception) and 5 post-menopausal.

4.4.2 Descriptive data from IRT and LSCI
Data generated from each ROI using IRT and LSCI for visit 1 is presented in Table 4.1. There was greater variation between subjects in the data generated with LSCI compared to IRT, as reflected in the considerably higher interquartile ratios (IQRs 0.03-0.62 vs. 0.25-1.57 for IRT and LSCI respectively). IQRs were also greater for dynamic parameters (i.e. Gmax and R%max), suggesting greater variability in data generated from dynamic parameters (i.e. the re-warming/reperfusion curve characteristics) compared to the absolute measurements (e.g. B and t0). Another notable finding was the higher R%max using LSCI in comparison with the more damped IRT (~125% vs. 102%) for which the post-ischaemic hyperaemic phase is less easily translated into an increase in surface skin temperature during reperfusion compared to baseline assessment.
Table 4.1. Infrared thermography (IRT) and Laser Speckle Contrast Imaging (LSCI) data for visit 1 (23°C ambient temperature).

All values expressed as median (interquartile ratio). ROI, Region Of Interest; B, baseline assessment at 23°C; t 0, assessments immediately following 60s cold challenge at 15°C; Max, maximum surface temperature / flux during 15 minute re-warming period; R%max, maximum percent recovery during reperfusion following cold challenge; Gmax, maximum gradient of re-warming/reperfusion.

† p<0.05 vs. ROI 2 for IRT, † p=0.001 vs. ROI 3 for IRT
‡ p=0.04 vs. ROI 2 for LSCI, ** p<0.005 vs. ROI 2 for LSCI, ¶ p=0.048 vs. ROI 3 for LSCI

<table>
<thead>
<tr>
<th></th>
<th>n=14</th>
<th>B</th>
<th>t0</th>
<th>Max</th>
<th>R%max (%)</th>
<th>Gmax</th>
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<tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ROI 1</td>
<td>14</td>
<td>34.1 (0.04) †</td>
<td>22.6 (0.05) ‡</td>
<td>34.4 (0.03) †</td>
<td>101.8 (0.06) †</td>
<td>4.67 (0.41) †</td>
</tr>
<tr>
<td>ROI 2</td>
<td></td>
<td>33.4 (0.05)</td>
<td>22.1 (0.04)</td>
<td>34 (0.05)</td>
<td>103 (0.08)</td>
<td>3.91 (0.44)</td>
</tr>
<tr>
<td>ROI 3</td>
<td></td>
<td>33.3 (0.09)</td>
<td>21.4 (0.04)</td>
<td>34.2 (0.07)</td>
<td>102.1 (0.12)</td>
<td>5.67 (0.62)</td>
</tr>
<tr>
<td>LSCI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROI 1</td>
<td>14</td>
<td>658 (0.25) **</td>
<td>389 (0.37) **</td>
<td>753 (0.25) **</td>
<td>125 (0.51) ¶</td>
<td>215 (0.62) **</td>
</tr>
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<td>ROI 2</td>
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<td>126 (0.76)</td>
<td>94 (0.59)</td>
<td>155 (1.01)</td>
<td>153 (0.69)</td>
<td>36 (1.57)</td>
</tr>
<tr>
<td>ROI 3</td>
<td></td>
<td>748 (0.36) **</td>
<td>301(0.74) **</td>
<td>883 (0.38) **</td>
<td>116 (0.43)</td>
<td>305 (1.45) **</td>
</tr>
</tbody>
</table>

4.4.3 Correlation between LSCI and IRT

Correlation coefficients for pooled data of all assessments, and individual regions of interest, are presented in table 4.2. For pooled data, moderate correlations existed between IRT and LSCI for absolute measurements at baseline and following cold challenge, and the re-warming curve characteristics ($r_s$ values 0.53 to 0.65, $P<0.00001$). Secondary analysis comparing correlation between IRT and LSCI for different ROI identified greater correlation (moderate to high) at ROIs 1 and 3 (the fingertips) compared with the more proximal ROI 2 where there was low or no correlation between IRT and LSCI, with the exception of recovery max % ($r_s$ 0.61, $p<0.05$). Similar correlation between IRT and LSCI was present at 10 and 15 minutes following cold challenge (absolute data presented in Table 5.4).
Table 4.2. Correlation between IRT and LSCI for pooled data from all visits and all regions of interest (ROI), and for individual ROI (visits 1 and 2).

B, baseline assessment at 23°C; t 0, assessments immediately following 60s cold challenge at 15°C; Max, maximum surface temperature / flux during 15 minute re-warming period; R%max, maximum percent recovery during reperfusion following cold challenge; Gmax, maximum gradient of re-warming/reperfusion. All values expressed as r.

* p<0.05; † p<0.005; ‡ p<0.0005; *** p<0.00005

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>t0</th>
<th>Max</th>
<th>R%Max</th>
<th>Gmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>All ROI, visits 1-3 (n=126)</td>
<td>0.53 ***</td>
<td>0.53 ***</td>
<td>0.59 ***</td>
<td>0.5 ***</td>
<td>0.65 ***</td>
</tr>
<tr>
<td>ROI 1. Visits 1 &amp; 2 (n=28)</td>
<td>0.58 †</td>
<td>0.81 ***</td>
<td>0.72 ***</td>
<td>0.69 ‡</td>
<td>0.61 †</td>
</tr>
<tr>
<td>ROI 2. Visits 1 &amp; 2 (n=28)</td>
<td>0.13</td>
<td>0.18</td>
<td>0.38 †</td>
<td>0.61 †</td>
<td>0.21</td>
</tr>
<tr>
<td>ROI 3. Visits 1 &amp; 2 (n=28)</td>
<td>0.58 †</td>
<td>0.64 ‡</td>
<td>0.65 ‡</td>
<td>0.45 †</td>
<td>0.65 ‡</td>
</tr>
</tbody>
</table>

4.4.4 Reproducibility

Data generated at visit 2 is presented in Table 4.3.

Table 4.3. Infrared thermography (IRT) and Laser Speckle Contrast Imaging (LSCI) data for visit 2 (23°C ambient temperature).

IRT and LSCI data expressed in °C and arbitrary flux units respectively unless stated. All values expressed as median (interquartile ratio). B, baseline assessment at 23°C; t 0, assessments immediately following 60s cold challenge at 15°C; Max, maximum surface temperature / flux during 15 minute re-warming period; R%max, maximum percent recovery during reperfusion following cold challenge; Gmax, maximum gradient of re-warming/reperfusion.

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>t0</th>
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<th>R%Max (%)</th>
<th>Gmax (°C/min or fu/min)</th>
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<td>n=14</td>
<td></td>
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<td></td>
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<tr>
<td>IRT (°C unless stated)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ROI 1</td>
<td>34.5 (0.07)</td>
<td>22.8 (0.29)</td>
<td>34.8 (0.06)</td>
<td>102.1 (0.08)</td>
<td>3.91 (0.52)</td>
</tr>
<tr>
<td>ROI 2</td>
<td>34 (0.08)</td>
<td>22.6 (0.10)</td>
<td>34.5 (0.09)</td>
<td>101.9 (0.1)</td>
<td>3.74 (0.61)</td>
</tr>
<tr>
<td>ROI 3</td>
<td>33.6 (0.05)</td>
<td>22.1 (0.23)</td>
<td>34.5 (0.08)</td>
<td>102.2 (0.17)</td>
<td>4.61 (0.83)</td>
</tr>
<tr>
<td>LSCI (arbitrary flux units [fu] unless stated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROI 1</td>
<td>681 (0.29)</td>
<td>298 (1.07)</td>
<td>743 (0.47)</td>
<td>114 (0.66)</td>
<td>149 (1.53)</td>
</tr>
<tr>
<td>ROI 2</td>
<td>133 (0.72)</td>
<td>82 (0.32)</td>
<td>154 (0.66)</td>
<td>113 (0.91)</td>
<td>39 (0.79)</td>
</tr>
<tr>
<td>ROI 3</td>
<td>908 (0.27)</td>
<td>270 (0.77)</td>
<td>881 (0.32)</td>
<td>116 (0.63)</td>
<td>320 (0.64)</td>
</tr>
</tbody>
</table>
The ICC values derived from comparing assessments 1 and 2 are presented in Table 4.4. Reproducibility of absolute measurements using IRT between visits 1 and 2 was generally excellent, as indicated by the high ICC values (all >0.68, table 3). ICC values for dynamic assessments using IRT were lower but remained good or excellent (ICCs ~0.7). Reproducibility of LSCI was similarly excellent at ROIs 1 and 3 (i.e. the fingertips), but reproducibility was generally lower for the R%max (ICC ~0.5 for LSCI cf. ~0.7 for IRT). Reproducibility was excellent at ROI 2 at baseline for both IRT and LSCI (ICC~0.85), although there was possibly some loss of reproducibility using LSCI at ROI 2 following cold challenge when compared to IRT. This was particularly evident for the % recovery indices with good reproducibility for IRT compared with poor to fair reproducibility for LSCI at ROI 2 (ICCs 0.67 vs. 0.45 respectively).

Table 4.4 Reproducibility of IRT and LSCI between visits 1 and 2 for each region of interest.

All data expressed as intra-class correlation coefficients (ICC). B, baseline assessment at 23°C; t 0, assessments immediately following 60s cold challenge at 15°C; Max, maximum surface temperature / flux during 15 minute re-warming period; R%max, maximum percent recovery during reperfusion following cold challenge; Gmax, maximum gradient of re-warming/reperfusion.

<table>
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<tr>
<th>n=14</th>
<th>B</th>
<th>t0</th>
<th>Max</th>
<th>R%max (%)</th>
<th>Gmax (°C/min or fu/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRT</td>
<td>ROI 1</td>
<td>0.83</td>
<td>0.84</td>
<td>0.79</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>ROI 2</td>
<td>0.83</td>
<td>0.74</td>
<td>0.81</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>ROI 3</td>
<td>0.96</td>
<td>0.68</td>
<td>0.92</td>
<td>0.80</td>
</tr>
<tr>
<td>LSCI</td>
<td>ROI 1</td>
<td>0.89</td>
<td>0.85</td>
<td>0.88</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>ROI 2</td>
<td>0.88</td>
<td>0.64</td>
<td>0.65</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>ROI 3</td>
<td>0.84</td>
<td>0.65</td>
<td>0.95</td>
<td>0.50</td>
</tr>
</tbody>
</table>

4.4.5 Variation between regions of interest

There were generally no differences between measurements at ROIs 1 and 3 (dorsal and volar aspects of fingertips) using either IRT or LSCI (Table 4.1). Where present, the magnitude of any differences was modest, only achieving statistical significance immediately after cold challenge for IRT (median 22.6°C vs. 21.4°C, P=0.001) and for Maximum flux following local cold challenge for LSCI (median 753fu vs. 883fu, P=0.048, Table 4.1). This
suggests similar perfusion at the dorsal and palmer aspect of the fingertips, and would not support a methodological preference for assessing digital vascular perfusion at either site.

Significant differences in LSCI flux values between ROI 2 and those measured at ROIs 1 and 3 were demonstrated for all static assessments and Gmax ($P<0.005$ for all comparisons, Table 4.1). In contrast, the ability of IRT to identify differences in surface skin temperature at ROI 2 were limited to baseline at ROI1 ($P=0.001$), maximum temperature at ROI1 following cold challenge ($P=0.006$) and Gmax at ROIs 1 and 3 ($P$ values of 0.04 and 0.03 respectively). The reduced capacity of IRT in distinguishing variation in perfusion across different regions of the digit may account for the poor correlation between LSCI and IRT at ROI 2 discussed previously. The R%max did not differ significantly between ROIs for IRT. There was a higher R%max at ROI 2 compared with ROI 1 for LSCI (153% vs. 125%, $P=0.04$) possibly caused by lower baseline flux at ROI2 allowing modest increases in perfusion during re-warming to be translated into more exaggerated relative changes.

4.4.6 Response to reduction in ambient room temperature

Data generated from each ROI using IRT and LSCI for visit 3 (reduced ambient room temperature) are presented in Table 4.5.

The effect sizes of the differences between visits 1 and 3 are presented in Table 4.6. There were significant reductions in surface skin temperature between visits 1 and 3 for all absolute IRT endpoints (and Gmax at ROIs 2 and 3) with correspondingly high effect sizes (e.g. the temperature at ROI 1 at baseline reduced from 34.1 to 30.2, $p=0.001$, ES -1.17. Table 4.6). In contrast, IRT assessment of R%max did not respond to a reduction in ambient room temperature.
### Table 4.5. Infrared thermography (IRT) and Laser Speckle Contrast Imaging (LSCI) results for visit 3 (ambient room temperature 18°C).

All values expressed as median (interquartile ratio). ROI, Region of Interest; B, baseline assessment at 23°C; t0, assessments immediately following 60s cold challenge at 15°C; Max, maximum surface temperature / flux during 15 minute re-warming period; R%max, maximum percent recovery during reperfusion following cold challenge; Gmax, maximum gradient of re-warming/reperfusion.

† \( p<0.05 \), ‡ \( p<0.005 \) vs. visit 1

<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
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<td><strong>ROI 1</strong></td>
<td>30.2 (0.34) ‡</td>
<td>20.9 (0.17) †</td>
<td>31.6 (0.36) ‡</td>
<td>100 (0.77)</td>
</tr>
<tr>
<td></td>
<td><strong>ROI 2</strong></td>
<td>28 (0.32) ‡</td>
<td>20.2 (0.14) †</td>
<td>30.8 (0.32) †</td>
<td>101.7 (0.64)</td>
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<td></td>
<td><strong>ROI 3</strong></td>
<td>30.4 (0.38) ‡</td>
<td>19.9 (0.16) †</td>
<td>28 (0.52) ‡</td>
<td>99.9 (0.78)</td>
</tr>
<tr>
<td><strong>LSCI</strong> (arbitrary flux units [fu] unless stated)</td>
<td><strong>ROI 1</strong></td>
<td>537 (0.89) ‡</td>
<td>193 (1.23)</td>
<td>530 (1.15) †</td>
<td>80 (2.03) †</td>
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<tr>
<td></td>
<td><strong>ROI 2</strong></td>
<td>81 (0.70) ‡</td>
<td>49 (0.5) †</td>
<td>100 (0.83) ‡</td>
<td>93 (1.42)</td>
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<td></td>
<td><strong>ROI 3</strong></td>
<td>531 (0.94) ‡</td>
<td>170 (1.32) †</td>
<td>328 (1.79) ‡</td>
<td>108 (1.30)</td>
</tr>
</tbody>
</table>

Similarly, LSCI identified significant reductions in the majority of absolute flux values in response to reduction in ambient room temperature (Table 4.5) and when failing to achieve statistical significance, strong trends were present (e.g. ROI1 at t0, 389fu for visit 1 vs. 193fu for visit 3, \( p=0.09 \)). This indicates LSCI was capable of measuring vascular responses elicited by the reduced ambient temperature.
Table 4.6. Responsiveness to total body cooling (visit 1 vs. visit 3) presented as Cohen’s d effect sizes.

IRT, infrared thermography; LSCI, laser speckle contrast imaging; ROI, region of interest; B, baseline assessment at 23°C; t 0, assessments immediately following 60s cold challenge at 15°C; Max, maximum surface temperature / flux during 15 minute re-warming period; R%max, maximum percent recovery during reperfusion following cold challenge; Gmax, maximum gradient of re-warming/reperfusion.

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<td>ROI 3</td>
<td>-0.82</td>
<td>-0.55</td>
<td>-1.14</td>
<td>-0.76</td>
<td>-0.89</td>
</tr>
</tbody>
</table>

The magnitude of the response measured using LSCI was lower than that identified using IRT, as reflected in the lower corresponding effect sizes (e.g. in contrast to the earlier example using IRT, LSCI flux at ROI 1 at baseline only reduced from 657.9 to 536.6, p=0.001, ES -0.85, Tables 4.5 and 4.6). In contrast with IRT, a significant change in the R%max was identified using LSCI at ROI1 (125% vs. 80%, p=0.03, ES -0.43)

4.5 Discussion

The present study is the first to make direct comparison between LSCI with IRT in the dynamic assessment of digital microvascular function. Most of the previously reported studies of LSCI have evaluated microvascular function in the retina or brain cortex of animals (Briers, 2006). Only recently has attention diverted to its potential application in the assessment of the cutaneous circulation, and little work has focussed on peripheral vascular function (section 1.5.4).
We have identified moderate to strong correlation between absolute assessments made with LSCI and IRT at the fingertips (ROI 1 and 3) at baseline and following cold challenge. It is important to note that the high coefficients of correlation should be interpreted as indicating a close relationship between the 2 techniques, but can not evaluate agreement since the 2 techniques do not measure the same parameter (Bland and Altman, 1986).

Correlation between the 2 techniques is weaker for dynamic parameters generated during rewarming (R%max and Gmax), and little or no correlation between LSCI and IRT was identified from measurements overlying the dorsal aspect of the middle phalanx (ROI 2). Differences in the capacity of the 2 techniques to identify important variation in cutaneous perfusion within different regions of the digit are likely to account for these findings. LSCI identified significantly lower perfusion measured at the middle phalanx (ROI 2) when compared to the fingertips (ROI 1 and 3). These findings may be the result of a lower density of thermoregulatory AVAs within the non-glabrous skin overlying ROI2 (section 1.5.1). These findings are of interest as it has been proposed that LSCI lacks the penetration of IRT and conventional laser Doppler, possibly only allowing assessment of perfusion within the superficial nutritive bed. Our findings suggest LSCI, at the wavelengths used by the MoorFLPI (775nm), may indeed be capable of identifying variation in tissue perfusion defined by the presence and function of AVAs. This may be due to the influence of AVA function on perfusion in superficial nutritive vessels or alternatively, it is possible the penetration of LSCI is greater than previously thought. In contrast, IRT measurements of skin temperature (which are influenced by thermal conduction from adjacent deep and superficial tissue) appears to lack the capacity to differentiate perfusion between AVA-rich and AVA-poor areas of the skin.

The relative paucity of significant differences between ROIs 1 and 3 (using both techniques) suggest cutaneous perfusion and vascular responses within the palmer and dorsal aspects of the fingertips is similar, and would not support a preference for either the palmer or dorsal aspects of the fingertips when assessing digital vascular function. Participants found it more comfortable and easier to maintain their hand in a static position when they had their forearms pronated, which may provide an advantage to using the dorsal aspect of the hands to assess digital vascular perfusion.

Reproducibility of LSCI has previously been evaluated with conflicting results. One study identified superior reproducibility of cutaneous perfusion at the forearm with LSCI compared
with both LDPI and LDF (ICCs 0.66 vs. 0.51 and 0.28 respectively) (Roustit et al., 2010b). In contrast, Murray et al. identified poor reproducibility using LSCI (ICC 0.15) although repeat assessments were only undertaken in a small number of subjects (n=5) (Murray et al., 2009). We have demonstrated generally excellent reproducibility of absolute measurements of both techniques, particularly at baseline. Reproducibility was generally better for the IRT, although differences were not stark and this finding was not entirely unexpected considering the highly damped nature of thermographic assessment. This issue raises a particular limitation with LSCI. In contrast to IRT, LSCI is responsive to physiological variation in autonomic function e.g. deep breathing, in addition to movement artefact. We attempted to address these limitations by setting the image processing time constant to 1 second (reducing variability secondary to the cardiac cycle) and by using a vacuum-stiffened beanbag to reduce movement artefact. Reproducibility was poorer using parameters derived from the re-warming curve characteristics (e.g. R%max) than absolute measurements for both techniques, but particularly with LSCI (see below for further discussion).

Our final objective compared responsiveness of each technique to a reduction in ambient room temperature. There was a significant reduction in absolute temperatures obtained using IRT at baseline, and following cold challenge, in response to reduction in ambient room temperature with corresponding high effect sizes. The R%max using IRT, meanwhile, failed to respond to a reduction in ambient room temperature highlighting a potential pitfall in the use of this parameter in therapeutic studies of digital vascular perfusion. The response of LSCI to reduction in ambient room temperature was generally less pronounced than IRT, although there was still strong evidence of increased vascular tone following cooling. Responsiveness of LSCI to reduced ambient room temperature at ROI 2 appears greater than at the fingertips on initial review of effect sizes (Table 4.6). These findings require further consideration, as it seems unlikely that regions characterised by such low perfusion could represent the optimum site for the assessment of vascular responsiveness to therapeutic or physiological intervention. The apparent greater responsiveness of IRT must also be interpreted with some caution owing to potential non-vascular effects of conductive and convective heat transfer from the surface of the digits (discussed in section 1.5.3). This consideration is highly pertinent for the many previous thermographic studies of digital perfusion that have incorporated a local cold challenge without questioning the potential contribution of environmental heat transfer in dictating the subsequent response to cold exposure. As discussed in chapter 3, the application of IRT to assess digital vascular function
in therapeutic studies of RP has been largely disappointing. Further work comparing IRT and LSCI responses to locally applied vasoactive mediators is needed to establish whether LSCI offers greater potential in this field.

We have identified potential disadvantages of parameters generated from reperfusion curve characteristics such as the R%max and Gmax when compared to absolute measurements in the assessment of digital vascular function. These indices may fail to identify differences in vascular function apparent from analysis of the absolute values. For example, a relatively smaller degree of re-warming / reperfusion may be sufficient to achieve 100% recovery in a subject with poorly perfused digits at baseline which respond modestly to cold challenge, whereas incomplete re-warming/reperfusion may be apparent in subjects with high perfusion at baseline, and marked reduction in response to cold exposure, who fail to re-achieve baseline values despite achieving near maximal vasodilatation.

There were limitations to this work that must be considered. Despite its small size, it was well conducted and has helped identify associations and trends that has influenced the design of subsequent larger work evaluating the use of LSCI in primary RP and SSc that will be described in the following chapters. Our cohort was somewhat heterogeneous with only a single male volunteer and one current smoker. Similarly, there were differences in female hormone status amongst the volunteers that may have influenced microvascular reactivity. We do not feel the primary objectives of the study were negatively influenced by failing to manufacture a more homogeneous cohort through adoption of stricter eligibility criteria. Indeed, the decision to recruit consecutive willing healthy participants was made in an attempt to provide a representative real-life experience of the use of LSCI in clinical practice. The chief limitation was the extent to which the findings can be generalised to the population of interest (patients with RP and SSc). Further validation work of LSCI within these patient populations will form a major component of the next chapter.

### 4.6 Conclusions

This study has contributed to a growing body of work exploring the potential application of LSCI as a novel non-invasive microvascular assessment tool for the dynamic assessment of digital microvascular function. We are the first to compare LSCI with IRT (the historical “gold standard” in microvascular imaging terms) in the dynamic assessment of digital vascular perfusion. The highly damped IRT provides lower inter-individual variation and greater
reproducibility, which may have contributed to the successful application of IRT in the classification of disease states (Anderson et al., 2007), however the reduced spatial and temporal resolution of IRT might have limited the use of IRT in therapeutic trials of RP (Merkel et al., 2003, Herrick and Clark, 1998). Additional work is needed to evaluate the potential for LSCI to measure cutaneous AVA responses and autonomic function, which could be of major value in the assessment of cutaneous vascular disorders such as RP.
Chapter 5 Evaluating vascular outcome measures in Raynaud’s phenomenon and systemic sclerosis

5.1 Introduction

I have described the various subjective and objective vascular outcome measures used in the assessment of digital vascular function in RP and SSc in section 1.5 and elsewhere. I have largely restricted this review to validated self-report assessments, IRT and laser-derived techniques. There are additional established (e.g. digital plethysmography) and novel (e.g. optical coherence tomography) techniques for the assessment of vascular function, which have not been considered within the remit of this thesis. Whilst researching these techniques, I have often found it curious that whilst much work has compared two distinct imaging modalities, few studies have directly compared the outcomes of subjective and objective microvascular imaging tools. It would be unwise to assume that these 2 approaches provide similar information on peripheral vascular function. This is highlighted in the outcome of studies exploring the peripheral vascular effects of ERA therapy in SSc. ERA therapy has been shown to reduce the burden of DU in SSc (Korn et al., 2004) and studies of ERA therapy incorporating objective assessment of peripheral vascular function (LDF, LDPI and IRT) have consistently demonstrated improvements in digital blood flow following ERA therapy (Rosato et al., 2010, Selenko-Gebauer et al., 2006, Dunne et al., 2006). And yet, despite anecdotal evidence of improved RP symptoms, studies incorporating self-report assessment of RP activity in patients with SSc (including the RCS diary) have so far failed to demonstrate any improvement following ERA therapy (Rosato et al., 2010, Nguyen et al., 2010). Such findings lead one to question the value of self-reports in the assessment of digital vascular function. To consider the validity of patient self-reports, one should first consider the exercises that led to their validation and widespread use.

Validation of the RCS diary

The RCS diary was originally developed for use in 2 concurrent RCTs evaluating oral iloprost in RP (Wigley et al., 1998, Black et al., 1998). The RCS diary evaluates the frequency, duration and impact of RP attacks over a 2-week period. Each day, patients record the number and duration in minutes of each RP attack (Appendix 7). Patients also record a
separate RCS, which is a daily self-report assessment of the impact of RP symptoms using 0-10 ordinal scale. Patients are encouraged to consider the number, duration, severity and impact on functional capacity when completing the RCS. The RCS (and the frequency and duration of daily RP attacks derived from the diary) has been used successfully in multicentre clinical trials and is the recommended outcome measure for use in clinical trials of SSc (Merkel et al., 2002, Merkel et al., 2003) (Appendix 7 for example of day sheet from RCS diary). Formal validation of the RCS diary was undertaken subsequently using clinical trial data from the study reported by Wigley et al. (Merkel et al., 2002). This validation exercise identified associations between the presence of DU and the overall RCS score, but not the frequency and duration of RP attacks. The RCS diary-related variables demonstrated moderate effect sizes (0.33-0.60) following oral iloprost (Merkel et al., 2002). Reproducibility was evaluated by examining the variability of the RCS diary parameters during the relatively stable post-intervention period, comparing assessments at weeks 6 and 12. Reliability (calculated as the standard deviation of the differences in comparison with the baseline range) fell within 4.6% and 10.3% of the baseline range (Merkel et al., 2002). The portability of the diary led to a high acceptance and completion rate amongst patients, supporting the feasibility of the outcome measure. This validation work gained subsequent support from OMERACT and the RCS diary was included in the core set of outcome measures of peripheral vascular function in clinical trials of SSc (Merkel et al., 2003). Using Dephi and Nominal Group techniques, the Scleroderma Clinical Trials Consortium (SCTC) have also included the RCS diary in a provisional core set of response measures for clinical trials of systemic sclerosis (Khanna et al., 2008).

Further examination of the published findings of the original clinical trials reveals some additional interesting findings. For example, whilst Black et al. did not report the date range of recruitment, they did report an increase in the mean minimum and maximum daily temperatures of +5°C and +8°C at 12 weeks follow-up (Black et al., 1998). It is likely this may have accounted for some of the improvement observed in RCS diary parameters following intervention although this was not explicitly stated. There was differing interpretation of the placebo effect across the 2 studies. Black et al. reported an acceptable placebo effect of ~15-20%, in comparison to a physician global assessment response to placebo of ~40-50% (Black et al., 1998). In contrast, Wigley et al. expressed concern at the extent of the placebo response with 41.7% of participants reporting a 50% improvement in the duration of RP
attacks, and 24.5% of participants reporting >50% improvements in the frequency of RP attacks and RCS score, following placebo intervention (Wigley et al., 1998).

The RCS diary is recommended for use in therapeutic trials of RP, and yet little attention has focussed on determinants of the RCS diary. For example, previous studies have not compared the use of the RCS diary in primary RP and SSc patients, or compared the results of the RCS diary with other patient self-reports of peripheral vascular function e.g. the SHAQ RP VAS. Moreover, previous studies have not specifically explored the influence of age, gender and seasonal variation on the outcome of the RCS diary parameters. Of particular interest, no previous studies have investigated the relationship between the RCS diary parameters and objective assessment of digital vascular function.

**5.2 Aims and Objectives**

The aims of this chapter can be broadly split into 2 parts. Part 1 will evaluate factors that influence the outcome of subjective (RCS diary) and objective (using IRT and LSCI) assessment of peripheral vascular function in RP and SSc (and the relationship between subjective and objective methods of assessing peripheral vascular function). Part 2 shall extend the validation work of LSCI commenced in the previous chapter and evaluate the discriminatory capacity of IRT and LSCI in differentiating between primary RP and SSc. The specific objectives of each of these 2 parts are outlined below:

**Part 1 Evaluation of factors that influence the outcome of subjective and objective assessment of peripheral vascular function in RP and SSc**

1) Inter-relationship of individual components of RCS diary in primary RP and SSc and differences between disease groups

2) Relationship between individual components of RCS diary and outcomes of SHAQ in patients with SSc

3) Influence of age, gender, vasodilatory therapy and season on parameters of RCS diary in patients with primary RP and SSc

4) Influence of age, gender, vasodilatory therapy and season on outcome of objective assessment of peripheral vascular function using IRT and LSCI in patients with primary RP and SSc
5) Correlation between subjective (RCS diary) and objective (IRT and LSCI) assessment of digital vascular function in primary RP and SSc

Part 2 Further validation work of LSCI in the assessment of digital vascular function in primary RP and SSc and evaluation of the discriminatory capacity of IRT and LSCI in differentiating between primary RP and SSc

1) Correlation between IRT and LSCI in the assessment of digital vascular function in primary RP and SSc

2) Reproducibility of IRT and LSCI in the assessment of digital vascular function in primary RP and SSc

3) Discriminatory capacity of IRT and LSCI to differentiate between perfusion at distinct ROI (differing anatomically by the presence or absence of AVAs)

4) Discriminatory capacity of IRT and LSCI to differentiate between HC, primary and SSc

5.3 Subjects and Methods

5.3.1 Patient identification and screening

Patients with primary RP and SSc were targeted as outlined in section 2.2.3. A total of 180 patients were sent an information pack containing a PIS and study interest form. One hundred and two (56.7%) patients returned the study interest form. Of those 70 (68.6%) patients expressed an initial interest in taking part in the study. Five patients later withdrew their interest following further discussion (e.g. unable to commit to study visits), 3 did not respond to requests to arrange screening and 1 failed to attend a scheduled screening visit. A further 11 potential participants were excluded prior to screening (6 due to preliminary pre-screening discussion which established they did not fulfill inclusion criteria, 3 patients who were acutely unwell or had recently received new treatments for their SSc, 1 patient who was pregnant and 1 who was already taking part in an interventional study). The remaining 50 patients attended a screening assessment between December 2010 and February 2012. Six patients were excluded who did not fulfill inclusion criteria (for either primary RP (n=3) or SSc (n=3)). All participants provided informed written consent and the study received prior approval from the South West 3 Research Ethics Committee (Reference 10/H0106/69).
5.3.2 Eligibility criteria

Inclusion criteria
All patients were aged between 18-75 years, with a diagnosis of either SSc or primary RP. Patients with SSc fulfilled either the ARA criteria and/or the LeRoy and Medsger criteria for early SSc (Masi, 1980, LeRoy and Medsger, 2001). In brief, the ARA criteria require the presence of either 1 major or 2 minor criteria. The major criterion is skin thickening, tightening and induration affecting any part of the body proximal to the metacarpophalangeal (MCP) and metatarsophalangeal (MTP) joints. Minor criteria include sclerodactyly (skin thickening restricted to distal to the MCP and MTP joints), digital pitting scars (secondary to digital ischaemia) and bi-basilar pulmonary fibrosis (Masi, 1980). These original criteria attracted criticism as they restricted classification to patients with established disease. LeRoy and Medsger subsequently proposed additional classification criteria to capture patients with early disease (LeRoy and Medsger, 2001). The LeRoy and Medsger criteria incorporated microvascular imaging modalities such as nailfold capillaroscopy (NC) and the presence of auto-antibodies, to identify patients with SSc prior to the emergence of cutaneous disease and/or other organ specific manifestations of SSc such as ILD. In brief, patients require either objective evidence of RP (either direct observation of any 2 of pallor, cyanosis or suffusion, or objective evidence of delayed recovery following cold challenge e.g. using IRT), in addition to either SSc-selective autoantibodies and/or characteristic abnormalities on NC. Alternatively, if there is only subjective history of RP (requiring 2 distinct colour changes) then both SSc-selective autoantibodies and NC abnormalities are required to fulfill the classification criteria (LeRoy and Medsger, 2001).

Primary RP was defined as at least 2 episodes of fingertip localized notable blue and/or sequential blue and white discoloration, in conjunction with pain upon cold exposure or emotional stress within one week of examination and a previous negative anti-nuclear autoantibody reactivity by immunofluorescence on HEp-2 cell substrate (using serum diluted to >1:160).

Exclusion criteria
Subjects were excluded from all aspects of the study if they were pregnant or breastfeeding (women of child bearing age will be required to practice a medically acceptable method of birth control throughout the study period). Patients who had undergone surgical
sympathectomy in the last 12 months, or had received new medications used for the treatment of RP or SSc in the 2 months preceding the trial period were excluded (this included the recent administration of selective serotonin reuptake inhibitor even if used for other licensed indications e.g. depression due to their vasoactive effects (Coleiro et al., 2001)). Vasodilators used for the treatment of hypertension were required to be maintained at a constant dose throughout the study period.

5.3.3 Study design and endpoints

Participants attended on 2 occasions, exactly 2 weeks apart. At the first visit information was collected from the participant and their medical records relating to age, gender, smoking history, medication usage and clinical phenotype (section 2.3.1). Patients with SSc were also asked to complete the SHAQ (section 2.3.2). At each visit participants underwent simultaneous assessment of digital vascular function using both IRT and LSCI at baseline and following the standardised cold challenge test described previously (section 2.4). The temperature of the microvascular imaging suite was maintained at 23°C (+/- 0.5°C) for each assessment. The conditions of the assessment were exactly the same as for assessments 1 and 2 in the healthy control group described in Chapter 4, to facilitate direct comparison between results of the separate studies. IRT and LSCI images were analysed using the regions of interest (ROIs) described in section 2.4.4. In view of the advantages of absolute assessments over parameters derived from the re-warming curve characteristics identified in Chapter 4, the vascular endpoints chosen for this work included mean perfusion (skin temperature using IRT and arbitrary flux values obtained using LSCI) during baseline assessment (abbreviated hereafter as B), immediately following cold challenge (t0) and at 5-minute intervals for a period of 15 minutes following cold challenge (t5, t10 and t15 respectively). The endpoints derived from the characteristics of the re-warming/reperfusion curves such as Max, R%max and Gmax described in 4.3.2 were not assessed in this work due to the perceived limitations of these endpoints identified and discussed previously (section 4.5). In addition, a secondary analysis of thermographic data included calculating the longitudinal thermal gradient (a modified version of the “distal dorsal difference”). This was calculated by subtracting the temperature of the skin overlying the middle finger MCP from the temperature at ROI1 (a negative value indicating lower temperature at the fingertips and peripheral microvascular compromise).

At the end of the cold challenge at assessment 1, patients were issued with a 2 week RCS diary and given tuition on its completion (section 2.3.3). Patients returned the diary and
underwent an identical cold challenge assessment at week 2, which signified the end of this component of the study.

To explore the effect of season on vascular outcomes, patients were subsequently divided according to date of enrollment into study (April to September vs. October to March) into 2 groups relating to Spring/Summer and Autumn/Winter.

5.3.4 Statistical analysis

All data is presented as median values (and interquartile range [IQR]) unless otherwise stated. Correlation between LSCI and IRT was assessed using Spearman’s rank correlation coefficient ($r_s$). Between group comparisons of unpaired data used the Mann Whitney U test. Comparison of paired data (e.g. comparison between different ROIs for both IRT and LSCI) used the Wilcoxon signed-rank test. Reproducibility (between assessments 1 and 2) was assessed using intra-class correlation co-efficient (ICC) to allow comparison between imaging techniques (Shrout and Fleiss, 1979). Various recommendations have been made concerning the interpretation of ICC values but ranges which have previously been adopted have suggested an ICC value of <0.4 generally indicates poor reproducibility, 0.4-0.75 represents fair to good reproducibility and >0.75 indicates excellent reproducibility (Sampat et al., 2006).

All data was analysed using SPSS version 18.0. All tests were two-tailed and a $p$ value of <0.05 was considered statistically significant.

Despite the large number of individual comparisons, no Bonferroni adjustment correction factor was applied to the analyses. Where relevant, I have highlighted statistically significant associations which may have occurred as a result of type I error and I have guarded against over-interpretation of such findings.
5.4 Results

5.4.1 Patient demographics

Forty-three patients (34 female) were enrolled into the study. There were 25 patients with SSc and 18 patients with primary RP. The baseline demographics of the cohort are presented in Table 5.1.

Table 5.1 Baseline demographics of patients

* Mann-Whitney U test used for comparing distribution across groups and Fisher's exact test to compare frequencies of categorical data between groups

<table>
<thead>
<tr>
<th></th>
<th>Primary RP, n=18</th>
<th>SSc, n=25</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years, median (interquartile range)</td>
<td>51.7 (19.3)</td>
<td>59.2 (19.8)</td>
<td>0.03</td>
</tr>
<tr>
<td>Age at RP onset in years, median (interquartile range)</td>
<td>20 (24)</td>
<td>35 (19)</td>
<td>0.02</td>
</tr>
<tr>
<td>Age at diagnosis in years, median (interquartile range)</td>
<td>43 (6)</td>
<td>45 (19)</td>
<td>0.61</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>4 (22.2)</td>
<td>5 (20)</td>
<td>1.00</td>
</tr>
<tr>
<td>Female</td>
<td>14 (77.8)</td>
<td>20 (80)</td>
<td>1.00</td>
</tr>
<tr>
<td>Vasodilatory therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any, n (%)</td>
<td>5 (27.8)</td>
<td>12 (48)</td>
<td>0.22</td>
</tr>
<tr>
<td>Calcium antagonist</td>
<td>3 (16.7)</td>
<td>6 (24)</td>
<td>0.71</td>
</tr>
<tr>
<td>ACEi</td>
<td>0 (0)</td>
<td>7 (28)</td>
<td>0.03</td>
</tr>
<tr>
<td>SSRI</td>
<td>2 (11.1)</td>
<td>1 (4)</td>
<td>0.56</td>
</tr>
<tr>
<td>ERA</td>
<td>0 (0)</td>
<td>2 (8)</td>
<td>0.50</td>
</tr>
<tr>
<td>Smoking history, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>10 (55.6)</td>
<td>13 (52)</td>
<td>1.00</td>
</tr>
<tr>
<td>Ex</td>
<td>7 (38.9)</td>
<td>9 (36)</td>
<td>1.00</td>
</tr>
<tr>
<td>Current</td>
<td>1 (5.5)</td>
<td>3 (12)</td>
<td>0.63</td>
</tr>
<tr>
<td>Season enrolled, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autumn/Winter</td>
<td>10 (55.6)</td>
<td>13 (52)</td>
<td>1.00</td>
</tr>
<tr>
<td>Spring/Summer</td>
<td>8 (44.4)</td>
<td>12 (48)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

There were significant differences between patients with primary RP and SSc for age (51.7 vs. 59.2 years, p=0.03), age at RP onset (20 vs. 35 years, p=0.02) and use of ACEi (0% vs. 28%, p=0.03). As expected, there were no patients with primary RP taking ERA. The overall proportion of patients taking vasodilator therapy was similar in both SSc and primary RP (12/25 and 5/18 respectively, p=0.22). One patient used nifedipine (a calcium channel antagonist) on an as needed basis but did not require treatment in the weeks leading up to, or during, their participation in the study. For the purposes of the analysis it was therefore
assumed they were not receiving nifedipine therapy. There were no differences in gender or smoking history between groups. The distribution of patients with primary RP and SSc was similar for patients enrolled in Spring/Summer and Autumn/Winter. Two patients (1 with primary RP and 1 with SSc) did not attend for a second cold challenge assessment preventing the assessment of reproducibility in these participants. The disease characteristics of the patients with SSc are presented in Figure 5.1.

**Figure 5.1 Clinical phenotype of patients with SSc.**

lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc; lSSc, limited SSc; DU, digital ulceration; DP, digital pitting; GI, gastrointestinal; PAH, pulmonary arterial hypertension; ILD, interstitial lung disease; SRC, scleroderma renal crisis.

![Clinical features diagram](image_url)

The majority of patients had lcSSc (22/25, 88%) and its associated clinical features. For example, digital ischaemic lesions were common (DU or DP were present in 12/25, 48%). As expected there were relatively few patients with associated PAH or ILD (Figure 5.1). The majority of patients carried anti-centromere autoantibodies (ACA, n=15, 34.9%) followed by anti-Scl-70 (n=4, 9.3%), anti-U3-RNP (n=3, 7%) with the remaining 3 patients carrying anti-U1-RNP, anti-Th and uncharacterized ANA respectively. Four (9.3%) patients failed to adequately complete or return the RCS diary (2 males with SSc, 1 male with primary RP and 1 female with SSc). No microvascular imaging data was available for the left hand of one patient with primary RP due to shoulder discomfort preventing him maintaining his left arm in a supinated position for the duration of the study. No data was available from the right middle finger of one patient with SSc because of previous amputation for critical ischaemic complications. The 4th (ring) finger was used as an alternative for vascular assessments for this subject as discussed previously (Section 2.4.4).
5.4.2 Inter-relationship of individual components of RCS diary in primary RP and SSc and the differences in RCS outcomes between primary RP and SSc

Across the whole cohort of primary RP and SSc the median RCS score was 2.00 (IQR 2.6). The median daily duration and frequency of RP attacks was 22.1 (46.9) minutes and 1.54 (2.13) attacks respectively. There was high correlation between the individual components of the RCS diary (Spearman’s Rho 0.62-0.78, p<0.001 for all comparisons, Table 5.2). There were no significant differences in the individual parameters of the RCS diary between patients with primary RP and SSc (Figure 5.2).

Table 5.2 Associations between individual components of RCS diary and SHAQ patient self reports

<table>
<thead>
<tr>
<th></th>
<th>RCS</th>
<th>Dur</th>
<th>Freq</th>
<th>Pain VAS</th>
<th>Resp VAS</th>
<th>GI VAS</th>
<th>RP VAS</th>
<th>DU VAS</th>
<th>Global VAS</th>
<th>HAQ-DI</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCS</td>
<td>0.62§</td>
<td>0.71§</td>
<td></td>
<td>0.53‡</td>
<td>0.58‡</td>
<td>0.61‡</td>
<td>0.83§</td>
<td>0.46‡</td>
<td>0.69§</td>
<td>0.51‡</td>
</tr>
<tr>
<td>Dur</td>
<td>0.78§</td>
<td></td>
<td></td>
<td>0.23‡</td>
<td>0.64‡</td>
<td>0.48</td>
<td>0.58‡</td>
<td>0.26</td>
<td>0.47‡</td>
<td>0.31</td>
</tr>
<tr>
<td>Freq</td>
<td></td>
<td></td>
<td></td>
<td>0.26</td>
<td>0.55‡</td>
<td>0.34</td>
<td>0.54‡</td>
<td>0.32</td>
<td>0.46‡</td>
<td>0.27</td>
</tr>
</tbody>
</table>

All associations are expressed as Spearman’s Rho. n=39 for internal associations of the RCS diary (pooled data for primary RP and SSc). n=23 for associations between RCS diary and SHAQ in patients with SSc. § p<0.001  † p<0.005  ‡ p<0.05
Figure 5.2 Differences in RCS diary outcomes between patients with SSc and primary RP

n=22 for SSc and n=17 for primary RP. A) RCS score, B) median daily duration of RP attacks (minutes) and C) median daily frequency of RP attacks. The bold lines indicate the median value and boxplots indicate IQR. The whiskers reveal range of data points that sit within 1.5(IQR) of the 1st and 3rd quartiles respectively and equals the range in most instances. Outliers are depicted as circles if their value sits between 1.5(IQR) and 3(IQR) of the quartile. Outliers are depicted as stars if their value is 3(IQR) from the adjacent quartile.
Median daily frequency 2.04 vs. 1.36
for SSc vs. primary RP, p=0.47
5.4.3 Relationship between individual components of RCS diary and outcomes of SHAQ in patients with SSc

There was moderate correlation between the RCS score and the HAQ-DI in patients with SSc (Rho 0.51, p=0.013, Table 5.2, Figure 5.3). There was no correlation between the HAQ-DI and either the frequency or duration of RP attacks reported using the RCS diary (Table 5.2). There were significant correlations between the RCS score and each of the organ-specific VAS from the SHAQ. The strength of these relationships varied between good and excellent (Spearman’s rho between 0.46-0.83, p<0.05 for all associations, Table 5.2).

**Figure 5.3 The relationship between the RCS score and disability (HAQ-DI) in SSc (n=23)**

For example, there was moderate to high correlation between each individual component of the RCS diary and the SHAQ RP Visual Analogue Scale (VAS, Rho 0.54-0.83, p<0.05). Associations between individual parameters of the RCS diary and the SHAQ DU VAS meanwhile were generally weak and/or non-significant in patients with SSc (Spearman’s Rho 0.26-0.46, Table 5.2). A documented history of DU and/or DP (n=12) in patients with SSc was not associated with significant differences in the RCS score (median 2.3 vs. 0.83, p=0.47), duration (37.9 vs. 19.3 minutes, p=1.00) or frequency (2.6 vs. 0.86, p=0.50) of RP attacks, compared with patients with SSc without a history of DU/DP (n=13).
5.4.4 Influence of age, gender, vasodilatory therapy and season on parameters of RCS diary in patients with primary RP and SSc

Males were significantly more likely to have failed to complete the diary adequately than females (3/9, 33.3% vs. 33/34, 97.1%, p=0.024). Males reported a significantly lower frequency of RP attacks (median daily frequency 0.82 vs. 1.93, p=0.031, Figure 5.4). The median daily duration of RP attacks (13.75 vs. 26.14 minutes, p=0.33) and RCS score (1.06 vs. 2.00, p=0.38) were lower in males than females, but did not achieve statistical significance.

Figure 5.4 Impact of gender on mean daily frequency of RP attacks

Males (n=6) vs. females (n=33). The bold lines indicate the median value and boxplots indicate IQR. The whiskers reveal range of data points that sit within 1.5(IQR) of the 1st and 3rd quartiles respectively and equals the range unless stated.

There was no correlation between age and the RCS score (rho -0.03, p=0.86), frequency of RP attacks (rho -0.08, p=0.65) or duration of RP attacks (rho 0.1, p=0.54). The RCS score was higher in those patients receiving vasodilator therapy although this trend failed to achieve statistical significance (median 2.14 vs. 1.57, p=0.058). This may reflect patients with more intrusive symptoms seeking therapy. Vasodilator therapy did have any effect on frequency or duration of RP attacks.
The RCS scores were lower in the Spring/Summer (median scores 1.57 vs. 2.29, p=0.024, Figure 5.5). The daily frequency of RP attacks did not differ between Autumn/Winter and Spring/Summer (median 1.54 vs. 1.21 respectively, p=0.673). There was a strong trend for a higher daily duration of RP attacks during Autumn/Winter but this association failed to achieve statistical significance (median daily duration 37.69 vs. 19.29 minutes, p=0.152).

**Figure 5.5 Impact of season on mean daily RCS score in patients with primary RP and SSc**

n=39. The bold lines indicate the median value and boxplots indicate IQR. The whiskers reveal range of data points that sit within 1.5(IQR) of the 1st and 3rd quartiles respectively and equals the range in most instances. Outliers are depicted as circles if their value sits between 1.5(IQR) and 3(IQR) of the quartile. Outliers are depicted as stars if their value is 3(IQR) from the adjacent quartile.

5.4.5 Influence of age, gender, vasodilatory therapy and season on outcome of objective assessment of peripheral vascular function using IRT and LSCI in patients with primary RP and SSc

I identified a number of statistically significant negative correlations between age and perfusion of the digits assessed using both LSCI and IRT. The association between advancing age and lower cutaneous perfusion was most evident at the palmer aspect of the digit (ROI3, Table 5.3). The Spearman Rho values were generally low (all $r_s<0.43$) and the
relationship between age and digital cutaneous perfusion in RP should be re-evaluated in a larger number of patients to confirm this possible trend.

Digital perfusion assessed using IRT was significantly higher in males compared to females for the majority of assessments at each ROI, at both baseline and during re-warming (Figure 5.6 provides an example using ROI2 at baseline and following cold challenge). Similarly, the majority of assessments of digital perfusion undertaken using LSCI revealed significantly higher perfusion in males compared to females (one notable exception being perfusion assessed using LSCI at ROI1 at baseline [median for males 437.9 vs. 345.9 fu for females, p=0.353]). Where failing to achieve statistical significance, strong trends were typically present (e.g. at ROI3 at baseline [median perfusion 758.2 vs. 491.6, p=0.065], and similar trends for ROI1 at t5, t10 and t15 [p values between 0.07 and 0.13]).

**Table 5.3 Correlation between age and perfusion at ROI3 assessed using IRT and LSCI at baseline and following cold challenge.**

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>t0</th>
<th>t5</th>
<th>t10</th>
<th>t15</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LSCI</strong></td>
<td>( r_s )</td>
<td>-.369</td>
<td>-.196</td>
<td>-.343</td>
<td>-.381</td>
</tr>
<tr>
<td></td>
<td>( p ) value</td>
<td>0.016</td>
<td>0.224</td>
<td>0.026</td>
<td>0.013</td>
</tr>
<tr>
<td><strong>IRT</strong></td>
<td>( r_s )</td>
<td>-.366</td>
<td>-.411</td>
<td>-.360</td>
<td>-.415</td>
</tr>
<tr>
<td></td>
<td>( p ) value</td>
<td>0.017</td>
<td>0.008</td>
<td>0.019</td>
<td>0.006</td>
</tr>
</tbody>
</table>
Figure 5.6 Influence of gender on IRT assessment of skin temperature at ROI2 at baseline and following cold challenge

B, baseline assessment at 23°C; t0, assessments immediately following 60s cold challenge at 15°C; t5, perfusion 5 minutes post cold challenge etc. The bold lines indicate the median value and boxplots indicate IQR. The whiskers reveal range of data points that sit within 1.5(IQR) of the 1st and 3rd quartiles respectively and equals the range in most instances. Outliers are depicted as circles if their value sits between 1.5(IQR) and 3(IQR) of the quartile. Outliers are depicted as stars if their value is 3(IQR) from the adjacent quartile.

In contrast to self-report assessment of digital vascular function, there was no association between vasodilator use and objective assessment of cutaneous perfusion using either IRT or LSCI.

With the exception of perfusion at ROI1 immediately following cold stress (249 fu [277.5] in Autumn/Winter vs. 173 fu [108] in Spring/Summer, p=0.043), there were no consistent associations between season and digital vascular function assessed using LSCI. The paradoxical higher perfusion identified during colder seasons in this comparison suggests this was a statistical anomaly (possibly related to the large number of individual comparisons undertaken). Similarly, there were no associations between season and skin temperature at baseline and/or following cold challenge for IRT assessments of the dorsal aspect of the finger at ROIs 1 and 2. In contrast, the surface skin temperature was consistently significantly
higher at ROI3 during Spring/Summer compared with Autumn/Winter, at baseline and at t5, t10 and t15 following cold challenge (Figure 5.7, \( p<0.05 \) for each comparison). The reasons for these findings are not immediately evident but may include differences in core temperature and homeostasis in warmer months.

**Figure 5.7 Influence of seasonal variation on skin temperature at the palmer aspect of the left middle finger pulp (ROI3) assessed using IRT.**

B, baseline assessment at 23°C; t 0, assessments immediately following 60s cold challenge at 15°C; t5, perfusion 5 minutes post cold challenge etc. The bold lines indicate the median value and boxplots indicate IQR. The whiskers reveal range of data points that sit within 1.5(IQR) of the 1\textsuperscript{st} and 3\textsuperscript{rd} quartiles respectively and equals the range in most instances. Individual outliers are numbered and depicted as circles if their value sits between 1.5(IQR) and 3(IQR) of the quartile.
5.4.6 Correlation between RCS diary parameters and objective assessment of digital vascular function using IRT and LSCI in primary RP and SSc

There were no correlations identified between any of the parameters of the RCS score diary and non-invasive microvascular imaging assessment using LSCI or IRT assessment at any ROI, at baseline and/or following cold challenge (Table 5.4 for examples of correlation coefficients between individual RCS diary parameters and LSCI assessment of perfusion at ROI1 before and following cold challenge).

Table 5.4 Correlation between individual parameters of the RCS diary and objective assessment of digital microvascular function using LSCI at ROI 1, at baseline and following cold challenge, for both primary RP and SSc.

B, baseline assessment at 23°C; t 0, assessments immediately following 60s cold challenge at 15°C; t5, perfusion 5 minutes post cold challenge etc. All values expressed as Spearman's rho ($r_s$), $p>0.05$ for all comparisons.

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>t0</th>
<th>t5</th>
<th>t10</th>
<th>t15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of RP attacks</td>
<td>0.05</td>
<td>-0.2</td>
<td>0.01</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>Frequency of RP attacks</td>
<td>0.02</td>
<td>-0.19</td>
<td>-0.08</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Raynaud's Condition Score</td>
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<td>-0.01</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
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PART 2
Further validation work of LSCI in the assessment of digital vascular function in primary RP and SSc and evaluation of the discriminatory capacity of IRT and LSCI in differentiating between primary RP and SSc

Descriptive data for both IRT and LSCI for each ROI at baseline and following cold challenge are presented in Table 5.5. This table incorporates data from healthy controls reported in Chapter 4. The following analyses use pooled data from primary RP and SSc unless otherwise stated.

Table 5.5 Infrared thermography (IRT) and Laser Speckle Contrast Imaging (LSCI) data for patients with SSc and primary RP at assessment 1.

All values expressed as median (interquartile range). ROI, Region Of Interest; na, not assessed; B, baseline assessment at 23°C; t0, assessments immediately following cold challenge; t5, perfusion 5 minutes post cold challenge etc. na, not available

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>t0</th>
<th>t5</th>
<th>t10</th>
<th>t15</th>
</tr>
</thead>
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<tr>
<td><strong>IRT (°C)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROI 1</td>
<td>29.8 (5.5) $</td>
<td>21.5 (2.6) $</td>
<td>23.7 (7.1) $</td>
<td>25.4 (9.2) $</td>
<td>27 (9.4) $</td>
</tr>
<tr>
<td>ROI 2</td>
<td>30.8 (7.3) ¶</td>
<td>21.4 (2.9) ¶</td>
<td>24.4 (7.9) ¶</td>
<td>25 (9.7) ¶</td>
<td>26.7 (11.1) ¶</td>
</tr>
<tr>
<td>ROI 3</td>
<td>28.8 (6.4) $</td>
<td>20.6 (3.0) $</td>
<td>22.4 (6.2) $</td>
<td>24 (8.8) $</td>
<td>25.9 (9.4) $</td>
</tr>
<tr>
<td>Primary RP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROI 1</td>
<td>31.4 (8.0) ¶</td>
<td>21.1 (3.2) ¶</td>
<td>25.1 (9.5) ¶ $</td>
<td>31.4 (11) ¶</td>
<td>32.4 (10.8) ¶</td>
</tr>
<tr>
<td>ROI 2</td>
<td>30.8 (6.6) ¶</td>
<td>21.5 (3.5) ¶</td>
<td>25.2 (7.2) ¶</td>
<td>30.4 (10.5) ¶</td>
<td>31.7 (10.6) ¶</td>
</tr>
<tr>
<td>ROI 3</td>
<td>29.5 (6.7) ¶</td>
<td>20.7 (3.1) ¶</td>
<td>23.5 (9.6) ¶</td>
<td>30.5 (10.5) ¶</td>
<td>32 (10) ¶</td>
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<td>Healthy Control</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROI 1</td>
<td>34.1 (1.6) $ ^\ddagger$</td>
<td>22.6 (1.42) na</td>
<td>34.1 (3.5) $ ^\ddagger$</td>
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<tr>
<td>ROI 2</td>
<td>33.4 (2.2) $ ^\ddagger$</td>
<td>22.1 (1.0) na</td>
<td>33.7 (4.3) $ ^\ddagger$</td>
<td>33.7 (2.3) $ ^\ddagger$</td>
<td></td>
</tr>
<tr>
<td>ROI 3</td>
<td>33.3 (3.3) $ ^\ddagger$</td>
<td>21.4 (1.0) na</td>
<td>33.7 (5.6) $ ^\ddagger$</td>
<td>33.8 (3.9) $ ^\ddagger$</td>
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<tr>
<td><strong>LSCI (fu)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROI 1</td>
<td>364 (299) ¶</td>
<td>188 (177) ¶</td>
<td>205 (282) ¶</td>
<td>243 (257) ¶</td>
<td>298 (288) ¶</td>
</tr>
<tr>
<td>ROI 2</td>
<td>235 (306) *</td>
<td>79 (84)</td>
<td>100 (269)</td>
<td>132 (391)</td>
<td>128 (364)</td>
</tr>
<tr>
<td>ROI 3</td>
<td>632 (757) ¶</td>
<td>213 (424) ¶</td>
<td>320 (642) ¶</td>
<td>422 (955) ¶</td>
<td>479 (770) ¶</td>
</tr>
<tr>
<td>Primary RP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROI 1</td>
<td>372 (378) ¶</td>
<td>179 (128) ¶</td>
<td>274 (346) ¶</td>
<td>452 (430) ¶</td>
<td>488 (446) ¶</td>
</tr>
<tr>
<td>ROI 2</td>
<td>124 (93) $ ^\ddagger$</td>
<td>81 (61) $ ^\ddagger$</td>
<td>81 (81) $ ^\ddagger$</td>
<td>104 (161)</td>
<td>120 (128)</td>
</tr>
<tr>
<td>ROI 3</td>
<td>536 (483) ¶</td>
<td>202 (135) ¶</td>
<td>261 (577) ¶</td>
<td>484 (604) ¶</td>
<td>487 (672) ¶</td>
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<td>Healthy Control</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROI 1</td>
<td>658 (185) $ ^\ddagger$</td>
<td>389 (198) $ ^\ddagger$ na</td>
<td>723 (233) $ ^\ddagger$</td>
<td>646 (350) $ ^\ddagger$</td>
<td></td>
</tr>
<tr>
<td>ROI 2</td>
<td>126 (114) $ ^\ddagger$</td>
<td>94 (64) na</td>
<td>134 (111)</td>
<td>125 (99)</td>
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<tr>
<td>ROI 3</td>
<td>748 (314) $ ^\ddagger$</td>
<td>301 (271) $ ^\ddagger$ na</td>
<td>748 (429)</td>
<td>647 (657)</td>
<td></td>
</tr>
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5.4.7 Correlation between IRT and LSCI in the assessment of digital vascular function in primary RP and SSc

Using pooled data from primary RP and SSc, there was moderate to excellent correlation between assessments of digital vascular function using IRT and LSCI at all ROIs at baseline and at all time-points following cold challenge (Table 5.6). The lowest correlations were generally identified immediately post-cold challenge although $r_s$ values were still suggestive of close agreement between the 2 techniques ($t_0$, Spearman’s Rho values 0.577-65).

Table 5.6. Correlation between IRT and LSCI for each ROI at baseline and following cold challenge for both primary RP and SSc.

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>t0</th>
<th>t5</th>
<th>t10</th>
<th>t15</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROI 1</td>
<td>0.725</td>
<td>0.615</td>
<td>0.813</td>
<td>0.843</td>
<td>0.748</td>
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<tr>
<td>ROI 2</td>
<td>0.702</td>
<td>0.577</td>
<td>0.837</td>
<td>0.827</td>
<td>0.809</td>
</tr>
<tr>
<td>ROI 3</td>
<td>0.806</td>
<td>0.650</td>
<td>0.819</td>
<td>0.823</td>
<td>0.829</td>
</tr>
</tbody>
</table>

n=43 (unless stated)

5.4.8 Reproducibility of IRT and LSCI in the assessment of digital vascular function in primary RP and SSc

The outcome of reproducibility analysis using pooled data for patients with both primary RP and SSc is presented in Table 5.7. The ICC values were generally lower than we had identified in HC in the previous chapter (Table 4.4). Nonetheless, the majority of ICC values fell with the 0.55-0.7 range suggesting good to excellent reproducibility of the 2 techniques. Reproducibility was generally good but lower than had been found in healthy controls (table 4.4). Reproducibility of LSCI was comparable, if not superior, to IRT. The highest ICC values were found immediately following cold challenge using LSCI at ROIs 1 and 3 (0.86 and 0.79 respectively). This suggests a reproducible nadir of digital perfusion to the intensity of the cold challenge irrespective of other factors influencing vascular function on the day of assessment. The lowest ICC values were recorded for LSCI at 10 and 15 minutes post cold challenge (e.g. 0.3 for ROI2 at 15 minutes and 0.34 for ROI at 10 minutes). This may reflect
greater variation in digital vascular responses during re-warming in comparison to more stable perfusion at baseline (following 20 minute acclimatization) and immediately following cold challenge (as discussed above).

**Table 5.7. Reproducibility of IRT and LSCI for each ROI at baseline and following cold challenge for both primary RP and SSc.**

All data expressed as intra-class correlation coefficients (confidence intervals). B, baseline assessment at 23°C; t0, assessment immediately following cold challenge; t5, 5 minutes post-cold challenge etc. All values expressed as ICC (with 95% confidence intervals) n=41 (unless stated)

<table>
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<th>ROI</th>
<th>Imaging method</th>
<th>B</th>
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<th>t5</th>
<th>t10</th>
<th>t15</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IRT</td>
<td>0.51 (0.24-0.71)</td>
<td>0.61 (0.36-0.78)</td>
<td>0.52 (0.25-0.72)</td>
<td>0.5 (0.23-0.7)</td>
<td>0.54 (0.28-0.73)</td>
</tr>
<tr>
<td></td>
<td>LSCI</td>
<td>0.62 (0.39-0.78)</td>
<td>0.86 (0.75-0.92)</td>
<td>0.52 (0.25-0.71)</td>
<td>0.34 (0.04-0.58)</td>
<td>0.47 (0.19-0.68)</td>
</tr>
<tr>
<td>ROI 2</td>
<td>IRT’</td>
<td>0.51 (0.24-0.71)</td>
<td>0.56 (0.3-0.75)</td>
<td>0.54 (0.28-0.73)</td>
<td>0.54 (0.28-0.73)</td>
<td>0.55 (0.28-0.73)</td>
</tr>
<tr>
<td></td>
<td>LSCI’</td>
<td>0.63 (0.4-0.78)</td>
<td>0.59 (0.33-0.76)</td>
<td>0.65 (0.43-0.79)</td>
<td>0.54 (0.28-0.73)</td>
<td>0.3 (0-0.55)</td>
</tr>
<tr>
<td>ROI 3</td>
<td>IRT’’</td>
<td>0.59 (0.33-0.76)</td>
<td>0.6 (0.35-0.77)</td>
<td>0.55 (0.28-0.73)</td>
<td>0.63 (0.4-0.79)</td>
<td>0.7 (0.5-0.83)</td>
</tr>
<tr>
<td></td>
<td>LSCI’’</td>
<td>0.55 (0.29-0.73)</td>
<td>0.79 (0.63-0.89)</td>
<td>0.5 (0.22-0.7)</td>
<td>0.42 (0.13-0.65)</td>
<td>0.55 (0.29-0.73)</td>
</tr>
</tbody>
</table>

n=40  n=39

**5.4.9 Discriminatory capacity IRT and LSCI to differentiate between perfusion at distinct ROI (differing anatomically by the presence or absence of AVAs)**

**LSCI**

LSCI perfusion values were significantly higher at ROIs 1 and 3 compared with ROI 2 for all time-points in patients with primary RP (Table 5.5), as was demonstrated in healthy controls in the last chapter. This trend was less pronounced in patients with SSc, indicating greater similarity in perfusion between glabrous and non-glabrous regions, possibly secondary to AVA dysfunction in patients with SSc. Perfusion was also significantly lower at ROI1 compared with ROI3 at baseline and 10 and 15 minutes post cold challenge in patients with SSc, but not primary RP. One possible explanation is that capillary dropout and avascular regions at the nailfold in patients with established SSc (such as those typically found on nailfold capillaroscopy) leads to lower perfusion at the dorsal aspect of the fingertip compared with the palmer aspect in SSc. Patients with SSc experience ulcerative complications on the dorsal and palmer aspects of the fingers to an equal extent, leading to uncertainty regarding the possible clinical implications of our findings.
IRT

In contrast to healthy controls, patients with SSc had significantly lower skin temperatures at the distal ends of the fingers (ROIs 1 and 3) compared to proximal regions (e.g. ROI 2) at both baseline and for the majority of assessments post cold challenge (Table 5.5). This probably underpins the success of the longitudinal thermal gradient in the diagnostic assessment of RP (Schuhfried et al., 2000, Clark et al., 1999, Anderson et al., 2007). Where present, significant differences between ROIs 1 and 3 with skin temperature at ROI 2 (e.g. baseline assessment for ROI 1) were usually the result of a negative distal dorsal difference secondary to lower perfusion at distal regions (as opposed to higher perfusion identified in normal subjects). Differences in surface skin temperature between ROI 2 and both ROIs 1 and 3 in primary RP were less marked (and only following cold challenge), but when present also revealed a negative thermal gradient. In contrast to LSCI, there was little difference between perfusion assessed using IRT at ROIs 1 and 3 in patients with SSc. In primary RP, however, skin temperature was significantly lower at ROI 3 at baseline, t0 and t5 compared with ROI 1 despite similar perfusion between these sites at the same time-points using LSCI. Potential reasons for these findings are not clear.

5.4.10 Discriminatory capacity of IRT and LSCI to differentiate between HC, primary and SSc

There were significant differences in digital perfusion assessed using IRT between healthy controls (Table 5.5) and both primary RP and SSc at all ROIs at baseline and at 10 and 15 minutes post-cold challenge. The discriminatory capacity of IRT was lost at t0, perhaps suggesting the nadir in skin temperature immediately post cold challenge is similar irrespective of disease states. The discriminatory capacity of LSCI differs and provides an interesting insight into the strengths and limitations of the 2 techniques. LSCI could also discriminate between healthy controls and both primary RP and SSc at baseline and during reperfusion at ROI 1. The discriminatory capacity of LSCI to distinguish disease states at ROI 3 was limited to identifying differences between healthy controls and primary RP but, interestingly, not SSc. This was due to the unexpectedly higher perfusion at ROI3 in patients with SSc compared with primary RP. As had been noted with primary RP, patients with SSc had unexpectedly higher perfusion at ROI2 at baseline compared to healthy controls (235 vs. 126, p=0.035). Another interesting observation using LSCI, when comparing healthy controls with primary RP and SSc, was the improved discriminatory capacity of perfusion values at t0 within glabrous regions (ROIs 1 and 3). Despite similar digital skin temperatures at t0 across

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disease groups, flux values were significantly greater in healthy controls compared with both SSc and primary RP at ROI1, and compared with primary RP at ROI3.

Neither IRT or LSCI was consistently able to discriminate between primary RP and SSc at any ROI, either at baseline or following cold challenge (Figure 5.8 for example of LSCI data, Table 5.5 for descriptive data). The one exception was perfusion assessed using LSCI at ROI 2 at baseline, which, paradoxically, was significantly greater in SSc compared with primary RP (235fu vs. 124fu, p=0.007) and may have occurred by chance. Digital skin temperature assessed using IRT was generally lower in SSc but differences failed to achieve statistical significance.
Figure 5.8 Differences in LSCI flux values between primary RP and SSc, at baseline and following cold challenge.

RMP = ROI1; RMMP = ROI2 and LMP = ROI3. B, baseline assessment at 23°C; t0, assessments immediately following 60s cold challenge at 15°C; t5, perfusion 5 minutes post cold challenge etc. The bold lines indicate the median value and boxplots indicate IQR. The whiskers reveal range of data points that sit within 1.5(IQR) of the 1st and 3rd quartiles respectively and equals the range in most instances. Individual outliers are numbered and depicted as circles if their value sits between 1.5(IQR) and 3(IQR) of the quartile. Outliers are depicted as stars if their value is 3(IQR) from the adjacent quartile. p>0.05 for all comparisons.
Significant differences between primary RP and SSc were achieved following a secondary analysis of thermographic data when the longitudinal thermal gradient (a modified version of the “distal dorsal difference”) was calculated by subtracting the temperature of the skin overlying the middle finger MCP with the temperature at ROI1 (Figure 5.9). The limited field of view prevented a similar assessment of longitudinal perfusion gradients from being undertaken using LSCI.
Figure 5.9 The discriminatory capacity of the longitudinal thermal gradient at baseline and following cold challenge in differentiating between disease states in primary RP and SSc.

The longitudinal thermal gradient was calculated by subtracting the temperature overlying the dorsal aspect of the right middle MCP from the temperature from ROI 1. B, baseline assessment at 23°C; t0, assessments immediately following 60s cold challenge at 15°C; t5, perfusion 5 minutes post cold challenge etc. The bold lines indicate the median value and boxplots indicate IQR. The whiskers reveal range of data points that sit within 1.5(IQR) of the 1\textsuperscript{st} and 3\textsuperscript{rd} quartiles respectively and equals the range in most instances. Individual outliers are numbered and depicted as circles if their value sits between 1.5(IQR) and 3(IQR) of the quartile.

* p<0.05 for primary RP vs. SSc
5.5 Discussion

To my knowledge, this is the first study to simultaneously evaluate subjective and objective assessments of digital vascular function in RP and SSc. Whilst many studies have evaluated the convergent validity of novel techniques using other established non-invasive microvascular imaging modalities, it is surprising that more work has not attempted to elucidate determinants of self-report assessment of RP severity and agreement with objective assessments of digital vascular function. In this study, I have highlighted some of the pitfalls of relying upon patient self-report for the assessment of digital vascular function in patients with RP, in addition to building on our previous validation work of LSCI for this purpose.

5.5.1 Limitations of self-report assessment of RP activity in primary RP and SSc

I have raised specific personal concerns regarding self-report in previous chapters including diary fatigue, habituation, and the influence of season and coping strategies adopted by patients. Many of these concerns were brought to my attention by patients themselves when coaching them on RCS diary completion as part of this study.

I have been able to explore some of these limitations further in this work. For example, there is a significant failure rate for completion of the diary (~10%) despite careful tuition of patients on completion of the diary at the outset of the study. This issue must be considered when calculating sample sizes for studies incorporating the RCS diary. I explored the impact of seasonal variation in weather and as expected, I have demonstrated a lower burden of RP symptoms (assessed using the RCS score) during Spring/Summer compared with Autumn/Winter. This effect of season on self-report assessment of RP severity could be more robustly investigated by undertaking paired analysis of RCS diary parameters within the same patients 6 months apart. I considered whether our results could be due to selection bias caused by more severely affected patients being recruited early in the study (December 2010). I do not think this was the case. Similar numbers of patients with primary RP and SSc were recruited in Autumn/Winter and Spring/Summer. Many patients who expressed an interest in the study shortly after our written approach in November 2010 were not screened until mid 2011 due to either personal preference and/or delayed recruitment due to the time-limiting effects of the study design. Furthermore, patients identified at a later stage of the recruitment window were also recruited in Autumn/Winter months (Oct 2011-Feb 2012).
There was no such association between season and digital perfusion before and following a standardized cold challenge assessed using LSCI, which appears to be a major advantage of LSCI. There appeared to be an association between season and skin temperature at the palmer aspect of the digital pulp assessed using IRT despite a 20 minute acclimatization period designed to negate the effects of external temperature on peripheral vascular function. The explanation for this finding is unclear but may include subtle differences in core temperature affected by season that a 20-minute period of acclimatization period cannot eradicate. This did not appear to influence skin temperature at other ROIs, which would perhaps favour the use of dorsal digital assessment of vascular function (e.g. ROI 1). The inability of some patients to sit for the duration of the cold challenge test with their forearm suppiinated to allow palmer assessment, and the tendency for the fingers to involuntarily flex during assessments, provides additional support for future imaging studies to concentrate on the dorsal aspect of the hands during microvascular assessment. There have been conflicting results on whether objective assessments of vascular function are influenced by season (Klein-Weigel et al., 2003, Gardner-Medwin et al., 2001). Seasonal differences in perfusion of the digits assessed using LDF in patients with primary RP has previously been noted (Gardner-Medwin et al., 2001). Evaluation of impact of season was not a primary objective of my study and my study design was not the preferred method to explore this hypothesis. Nonetheless, my findings support those of previous studies that have identified the impact of seasonal variation in self-report assessment of RP symptoms (Watson et al., 1999). Additional work is required to establish whether the impact of season on objective assessment of digital vascular function can be adequately attenuated by an acclimatization period and/or pre-test procedures (e.g. local warming) as this would represent an important advantage of objective assessment over self-report in future therapeutic trials of RP.

Gender appears to influence RP symptoms with males experiencing significantly fewer attacks of RP. This may reflect gender-related differences in completing the diary although objective assessment using IRT and LSCI suggests these findings genuinely reflect greater perfusion of the digits in males compared with females. There is a well-established female predominance for RP, possibly related to differences in temperature homeostasis and the influence of sex hormones (Greenstein et al., 1996). No information was collected regarding the sex-hormone status of females participating in the study to explore this further. It would be important to consider the influence of gender should one consider using absolute values
obtained using subjective and objective assessment of digital vascular function as diagnostic aids or to guide treatment need or response.

The RCS diary parameters lacked the discriminant capacity to differentiate between primary RP and SSc, although the strongly held conviction of most clinicians would argue the degree of vascular dysfunction in SSc is greater than in primary RP. Similarly, the RCS diary failed to differentiate between patients with SSc with a history of DU/DP and those without. There was close agreement between RCS diary parameters and other self-report measures of RP activity such as the SHAQ RP VAS. Strong associations were also found between the RCS diary parameters and self-report assessment of other organ manifestations that can not easily be explained on the basis of disease pathogenesis and associations (e.g. RCS score and SHAQ GI VAS, Spearman’s Rho 0.61, Table 5.2). Whilst these associations could be genuine, I have encountered this phenomenon in previous work exploring fatigue, quality of life and disability in SSc and suspect it reflects patient-specific health beliefs with some patients consistently scoring highly in self-report assessment of unrelated aspects of their health and vice versa (Strickland et al., 2012).

Finally, I have demonstrated close agreement between IRT and LSCI in the dynamic assessment of digital vascular function in RP and SSc, but poor correlation between these techniques and patient self-report assessment of RP severity using the RCS diary. To my knowledge, this is the first study to establish this. It seems subjective and objective assessments provide differing information on digital vascular function. This may account for the lack of efficacy of novel potent vasodilatory therapies (such as ERAs) in clinical trials of SSc, which have relied upon patient self-reports of RP severity. Use of subjective self-report may be appropriate if the sole purpose of such studies is to examine symptomatic efficacy of such treatments (e.g. in primary RP) but may fail to identify the potentially disease modifying effect that substantial improvements in digital perfusion may exert on ischaemic complications and tissue fibrosis in SSc. In this way, the limitations of self-report measures such as the RCS diary may be restricting access for patients to effective therapies in the management of SSc.
5.5.2 Further validation of LSCI and IRT in the assessment of digital vascular function in RP/SSc

I have extended the validation work for LSCI and IRT undertaken in Chapter 4 to the assessment of digital vascular function in patients with primary RP and SSc.

Once again, I was able to demonstrate close agreement between IRT and LSCI in the assessment of digital perfusion before and following cold challenge. Reproducibility of both techniques was good to excellent but lower than in healthy controls. I was surprised to see comparable, if not superior reproducibility with LSCI compared with IRT. I had expected the greater sensitivity of LSCI to have a negative effect on reliability studies but this was not the case. I was not surprised to see lower ICC values in our patient groups compared with healthy controls as subjects with healthy circulation would not be expected to demonstrate significant variation in digital perfusion under standardized assessment conditions. Nonetheless, reproducibility of both IRT and LSCI remained good to excellent in RP/SSc. Relatively little work has considered the reproducibility of the RCS diary. One might expect it to be excellent over the short term but the influence of seasonal variation highlighted by this work would be expected to negatively affect reliability over longer periods. Objective assessment of vascular function under standardized conditions may fare better in this regard but this needs to be demonstrated in future studies.

Both IRT and LSCI successfully differentiated between healthy controls and disease states. Neither IRT nor LSCI, however, could differentiate between primary RP and SSc using the ROIs I chose for assessment. In contrast, by adopting the thermographic DDD, I improved the discriminatory capacity of IRT and it is possible the discriminatory capacity of LSCI could be improved by optimizing the imaging endpoints chosen (perhaps by evaluating flux gradients or through composite scores derived from simultaneous assessment of multiple digits). It would be preferable to assess perfusion in multiple digits. I was unable to achieve this in this study due to the restricted field of view of LSCI and our determination to compare perfusion at the volar and dorsal aspect of the fingers. I have previously identified a superior discriminatory capacity by using multiple digits over single digits when using IRT to differentiate between primary RP and SSc (Pauling et al., 2011b). It would be feasible to assess perfusion of all the fingers (excluding thumbs) of one hand using LSCI, which may improve the discriminatory capacity of LSCI.
I was interested to note the higher perfusion in patients with SSc and a history of DU/DP. This may be explained by greater use of vasodilatory therapy and/or the higher proportion of males in this group. It is possible that male patients with SSc have a lower threshold for developing digital ischaemic complications than women. Alternatively, DU/DP associated with localised hypoperfusion may occur in association with normal, or even increased, perfusion of surrounding tissues. Asymmetry in digital perfusion is typical of SSc and I have only assessed digital perfusion at small regions of interest within individual digits. Additional work incorporating a larger number of patients is needed to explore these possibilities further.

This work has considered the application of LSCI to evaluate cutaneous perfusion in AVA-rich and AVA-poor regions of the digits. The discriminatory capacity of LSCI in differentiating between glabrous and non-glabrous regions was reduced in SSc in comparison with primary RP and healthy controls. This was not unexpected and reflects greater similarity in perfusion between ROIs due to vascular dysfunction and lower perfusion within glabrous regions. IRT did identify differences in perfusion at ROIs 1 and 3 when compared with ROI 2, but in contrast to healthy controls, this reflected lower perfusion within the distal glabrous ROIs, presumably secondary to AVA dysfunction.

I have not considered the possible impact of sclerodermatous skin on flux values caused by alterations in tissue absorption and reflection following tissue re-modelling (section 1.5.3). We are planning further work using LSCI to compare perfusion within areas of sclerodermatous skin, unaffected skin, healed DU/DP and active ulceration.

5.6 Conclusions

In this chapter, I have highlighted challenges in the assessment of digital vascular function in primary RP and SSc. I will discuss the implications of my findings for the future assessment of digital vascular function in RP/SSc, both in clinical practice and therapeutic trials in Chapter 8. It is imperative we develop better methods for assessing peripheral vascular function in RP to facilitate improved access to treatment, and better assessment of treatment efficacy, in view of the high cost and restricted availability of novel vasoactive drugs, such as ERA, in the treatment of SSc.
Chapter 6 Platelet function, eicosanoid biosynthesis and oxidative stress in primary Raynaud’s phenomenon and systemic sclerosis

Among red and white cells small corpuscles are visible, having at the beginning the appearance of very delicate and pale erythrocytes. They rapidly modify their shape, becoming spinous and adhering to the glass...showing a tendency to stick to other similar corpuscles and to form aggregates....

Hayem M.G. (1878),

6.1 Introduction

In Chapter 1, I reviewed the significant body of evidence that supports enhanced platelet activation in RP and SSc. Many of these studies incorporated heterogeneous populations of patients with both primary and secondary RP. Whilst SSc formed the large majority of patients with secondary RP studied, many studies enrolled patients with other rheumatic diseases, often characterised by severe RP, including mixed connective tissue disease (MCTD) and systemic lupus erythematosus (SLE). Few studies to date have specifically evaluated platelet function in well-characterised cohorts of primary RP and SSc. In this chapter, I will report the findings of work evaluating platelet function, eicosanoid synthesis and oxidative stress in primary RP and SSc. I shall first provide a description of the various methods available for assessing platelet function, which have influenced the design of this work. In addition, I shall describe the inter-play between platelet function, eicosanoid synthesis and oxidative stress. I shall specifically describe the biosynthesis, biological function and pathogenic significance in SSc of a recently discovered family of compounds that are important biomarkers of oxidative stress in vivo (F₂-isoprostanes).

6.1.1 Assessment and Quantification of Platelet Function
Despite the large number of diseases in which excessive platelet activation occurs, there is no definitive test of platelet function to facilitate targeted therapy and assessment of treatment efficacy. Methods for evaluating platelet function are typically labour intensive, expensive and require a degree of technical expertise, which has limited their use, both in therapeutic trials and clinical practice. The various laboratory methods available for the assessment and quantification of platelet activation and their respective merits and limitations are summarized in Table 6.1.

Table 6.1. Methods for the quantification and assessment of platelet function in humans.

<table>
<thead>
<tr>
<th>Method</th>
<th>Strengths</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical assessment</td>
<td>Cheap and readily available</td>
<td>Only associated with major platelet dysfunction e.g. ITP</td>
</tr>
<tr>
<td>Platelet count and morphology</td>
<td>Cheap and readily available</td>
<td>False positives e.g pseudothrombocytopenia 2° to platelet agglutinins</td>
</tr>
<tr>
<td>Bleeding time</td>
<td>Cheap and readily available</td>
<td>Invasive, non-specific, major variability</td>
</tr>
<tr>
<td>Bone Marrow examination</td>
<td>Exclusion of myeloproliferative disease</td>
<td>Invasive and expensive</td>
</tr>
<tr>
<td>Platelet aggregometry (e.g. to agonists ADP, AA, collagen, adrenaline)</td>
<td>Physiological assessment of platelet function e.g. reversible and irreversible aggregation</td>
<td>Time consuming, labour intensive, Non-physiological ex vivo and in vitro analysis</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>Assessment of platelet structure and function e.g. P-selectin expression</td>
<td>Expensive and limited to specialist centres</td>
</tr>
<tr>
<td>Biomarkers (plasma or urine) of platelet activation e.g. vWF, βTG, PF-4, TxB, soluble P-selectin</td>
<td>Cheap commercially available ELISAs</td>
<td>Not always specific to platelet function (e.g. endothelium derived vWF). Affected by anticoagulant (e.g. PF-4 and heparin) and renal function (e.g. βTG)</td>
</tr>
</tbody>
</table>

MPV, mean platelet volume; PDW, platelet distribution width; ITP, idiopathic thrombocytopenic purpura; ADP, adenosine diphosphate; AA, arachidonic acid; βTG, β-thromboglobulin; PF-4, Platelet factor 4; TXB, Thromboxane B2; vWF, von Willebrand’s factor; ELISAs, Enzyme Linked Immunosorbert Assays.

Adapted from references (Gurney et al., 2002, Kottke-Marchant and Corcoran, 2002) and elsewhere.

Platelet number, structure and the bleeding time
The formation of platelets follows cytoplasmic fragmentation from megakaryocytes and is under the control of humoral agents such as thrombopoietin. The normal platelet count is between 150 and 350x10⁹/L (Kamath et al., 2001). An elevated platelet count (thrombocythaemia) can be associated with an increased thrombotic tendency whereas a reduction in the platelet count (thrombocytopenia) typically causes increased bleeding phenomena. Quantification of the platelet count is the crudest method for detecting major platelet abnormalities but is often normal in primary disorders of platelet function such as Glanzmann's thrombasthenia (abnormality in gpIIb/IIIa receptors). For disorders associated with normal thrombocytosis, quantification of the bleeding time was the first widely available method for detecting inherent platelet dysfunction. The platelet count and bleeding time are less useful in those conditions associated with, but not defined by, platelet dysfunction e.g. atherosclerosis. In such conditions platelet structure may still provide evidence of platelet function. Resting platelets are smooth discoid structures with a diameter of 1-2μm and a mean cell volume of 5-6 fl (Kamath et al., 2001). The mean platelet volume (MPV) is incorporated in automated Full Blood Count (FBC) analysis and is an often overlooked parameter despite providing useful information regarding in vivo platelet activation. Activated platelets transform from discoid structures to swollen spheres upon activation, increasing their apparent size. An increase in MPV has been associated with macrovascular diseases such as hypertension, acute myocardial infarction and diabetes (Coban et al., 2005, Park et al., 2002, Nadar et al., 2004). The MPV also increases ex vivo in response to collagen and hypothermia induced platelet activation (Park et al., 2002). The platelet distribution width (PDW) is another measure of platelet size that may provide evidence of platelet activation. For example, the PDW is elevated in diabetics following acute coronary events (Hendra et al., 1988).

Light Transmission Aggregometry

Light transmission aggregometry (LTA) or platelet aggregometry (PA) was developed by Born in the early 1960's and quickly regarded as the “gold standard” for platelet function testing (Born and Cross, 1963, Born, 1962). The method is simple and has been described in further detail previously (section 2.7). LTA is not a direct measure of physiological platelet function as it tests platelet activation under low shear stress, in free solution (within platelet rich plasma) and does not therefore accurately simulate in vivo haemostasis. Other limitations

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*Gustav V. R. Born (b1921- ) is the son of the physicist and Nobel Prize winner Max Born (1882-1970) and uncle of the singer Olivia Newton-John. I had the pleasure of meeting Gustav Born at the 2011 UK platelet meeting in Cardiff where he recounted tales of spending time with his father's close friend and colleague Albert Einstein before fleeing Nazi Germany with his family prior to the outbreak of WW2.
of LTA relate to its labour intensity and logistical difficulties such as the time constraints following blood collection.

**Platelet membrane glycoproteins**

The outermost layer of the platelet (the glycocalyx) expresses glycoproteins following activation. For example, P-selectin translocates from the intracellular granule membrane to receptors on the glycocalyx following activation. Increased membrane expression of P-selectin identified using flow cytometry is a useful marker of platelet activation (Kamath et al., 2001). Shed plasma soluble P-selectin levels have also been used successfully as markers of platelet activation and response to anti-thrombotic medication. For example, sP-selectin levels are reduced in hypertensive patients taking aspirin (Nadar et al., 2004, Nadar et al., 2006, Gurney et al., 2002). Further work is required to investigate the use of assays developed to measure other membrane bound and circulating glycoproteins including glycoprotein V, glycoprotein Ib, glycoprotein IIb/IIIa and glycocalicin, which may also allow ex vivo quantification of platelet activation (Gurney et al., 2002).

**Circulating platelet derived mediators**

Platelets release a large array of mediators from intracellular granules and the OCS following activation (Table 1.1). Many of these factors (e.g. ATP) are too ubiquitously expressed to be seriously considered useful biomarkers of platelet activation (Gurney et al., 2002). Other biomarkers are stored but not produced in platelets and hence circulating levels do not specific relate to platelet activation (Kamath et al., 2001). For example, serotonin is chiefly manufactured in enterochromaffin cells within the gastrointestinal tract. Other granule-release products are more platelet-specific and thus form potentially attractive biomarkers for platelet activation. Many early studies evaluated plasma levels of platelet factor 4 (PF-4) and β-Thromboglobulin (β-TG) as biomarkers of platelet activation. These classical α-granule proteins are both members of the C-X-C subfamily of chemokines and derived from proteolysis of platelet basic protein (Klinger, 1997). One potential consideration where SSc is concerned is the effect of renal impairment on circulating levels of β-TG and PF-4. There is also little evidence of any direct pathogenicity of these chemokines, although it is possible they attract leukocytes, and promotes the inflammatory response at areas of endothelial damage and secondary platelet activation. The CD40 ligand is potentially of greater interest due to its potential contribution to both fibrosis and humoral and cell-mediated immune responses in the pathogenesis of SSc (sections 1.4.3 and 1.4.4). Platelets are the major reservoir of circulating sCD40L and plasma levels have been used successfully as
biomarkers of platelet activation. Similarly, whilst TGFβ expression is not exclusive to platelets and is produced by many cells, platelets are a major source of circulating TGFβ. Indeed, TGFβ was originally identified in the alpha granules of platelets (Lev et al., 2007). Plasma levels of TGF-β have been associated with platelet count and aggregability in pre-eclampsia (Peracoli et al., 2008), and it is plausible that platelets are an important source of TGFβ at sites of vascular injury promoting tissue healing but also helping to drive pro-fibrotic signaling and peri-vascular fibrosis in SSc.

**Eicosanoid biosynthesis**

Measurement of metabolites of the arachidonic acid pathway is a more established method of assessing platelet function. Thromboxane A₂ (TXA₂), and its more stable metabolite TXB₂, is the obvious candidate, but others include lipoxygenase (LOX) eicosanoids such as 12-Hydroxyeicosatetraenoic acid (12-HETE) and the leukotrienes. An important recently discovered family of eicosanoids are the F₂-isoprostanes, which provide an attractive link between oxidative stress, platelet function and eicosanoid biosynthesis. F₂-isoprostanes are potentially attractive biomarkers of oxidative stress in SSc and may contribute to pathogenesis. It is the potential contribution of oxidative stress to the pathogenesis of SSc that I shall now divert my attention.

**6.1.2 Oxidative stress and its potential contribution to the pathogenesis of SSc**

**Oxidative stress**

Reactive oxygen species (ROS) are formed as by-products of aerobic metabolism during the intermediate steps required to reduce molecular oxygen to water (Sies, 1997). Examples of ROS are the superoxide anion (-O₂⁻, one electron reduction state of O₂), hydrogen peroxide (H₂O₂) and the hydroxyl radical (-OH). ROS are capable of intense tissue damage, which has led to the evolution of a host of anti-oxidant defence mechanisms, which are equally important as O₂ in the survival of aerobic organisms (Halliwell, 2007). Oxidative stress describes an imbalance between ROS and anti-oxidant defence systems that has the potential to cause tissue damage (Halliwell, 2007). ROS have potentially beneficial roles such as regulation of gene expression and ROS destruction of invading pathogens at sites of inflammation, which might explain the evolutionary acceptance to attenuate, rather than eliminate, the threat of ROS-mediated tissue damage (Halliwell, 2007, Sies, 1997). Anti-oxidant pathways include enzymatic catabolism (e.g. superoxide dismutase and catalase)
and non-enzymatic micronutrients (e.g. glutathione, carotene, vitamin C and E) capable of “scavenging” free radicals from the circulation. Another important scavenger of ROS is the potent vasodilator nitric oxide (NO). Exogenous antioxidant treatment in diseases characterised by increased ROS formation such as diabetes and coronary heart disease, has been shown to enhance endothelium-dependent vasodilatation by potentiating the local action of NO which might otherwise have been consumed (Moncada, 2006).

**Platelet function, eicosanoid formation and oxidative stress: The F<sub>2</sub>-isoprostanes**

Platelets are themselves capable of generating ROS, primarily as a result of arachidonic acid metabolism by COX and the activation of the platelet isoform of NADPH oxidase (Davi and Patrono, 2007). COX products also accelerate the removal of local NO. In addition to ROS formation, platelets are themselves activated in environments of oxidative stress. For example, ROS can induce lipid peroxidation of cell membrane phospholipids leading to the production of compounds such as F<sub>2</sub>-isoprostanes which, like COX products, are potent activators of platelets.

F<sub>2</sub>-isoprostanes are formed following non-enzymatic free-radical oxidation of arachidonic acid (Cracowski et al., 2002b). The process is random and up to four regioisomers (with differing side chains) can form, of which up to 8 isomers can exist for each regioisomer i.e. up to 64 F<sub>2</sub>-isoprostanes in total, but their biological functions are similar. Their name derives from isoprostanes being isomeric to prostaglandins and due to the presence of an F-type cyclopentane (prostane) ring. They are attractive biomarkers of oxidative stress owing to the fact that levels are not influenced by dietary lipid content, they are stable in urine and levels are responsive to changes in oxidative stress following anti-oxidant therapy (Cracowski et al., 2002b).

F<sub>2</sub>-isoprostanes can be measured in serum, plasma and urine. Urinary quantification is the preferred method owing to the potential for *ex vivo* formation and the relatively short half-life in blood products. Indeed, F<sub>2</sub>-isoprostanes were initially discovered as an auto-oxidation product of plasma samples stored at -20°C highlighting the propensity for *ex vivo* lipid oxidation. An important consideration when investigating urinary levels of isoprostanes in SSc is the presence of renal failure as clearance of isoprostanes is reliant on both renal filtration and metabolism. The preferred method for measuring F<sub>2</sub>-isoprostanes is gas chromatography-mass spectroscopy (GC/MS) (Cracowski, 2006). Enzyme linked immunoassays have been developed but report lower levels of F<sub>2</sub>-isoprostanes than for
GC/MS owing to their specificity for a limited number of F₂-isoprostane isomers (Proudfoot et al., 1999).

**Oxidative stress in primary RP and SSc**

Oxidative stress is thought to be an important mechanism of cellular injury contributing to the pathogenesis of systemic sclerosis and a number of factors combine to create a highly oxidative environment (Gabrielli et al., 2008). Repeated hypoxic and reperfusion injury secondary to vascular dysfunction in SSc generate ROS (Simonini et al., 2000). Intravascular factors such as monocytes and platelets are thought to contribute a rich source of ROS in SSc (Sambo et al., 1999). ROS have been implicated in several key pathogenic pathways in SSc including endothelial damage, up-regulation of adhesion molecules, B and T cell activation (King et al., 2006), platelet activation (De la Cruz et al., 1992), fibroblast proliferation (Mulder, 2000, Erre and Passiu, 2009) in addition to fragmentation of auto-antigens and auto-antibody formation (Casciola-Rosen et al., 1997). Endothelial cells are particularly prone to ROS damage owing to a low capacity to produce free radical scavenging enzymes such as catalase (Shingu et al., 1985). These factors are thought to combine to lead to a vicious cycle of tissue ischaemia, oxidative stress, endothelial damage, vasoconstriction and additional tissue ischaemia (Herrick, 2005). Oxidative stress may be of particular importance in the early stages of the disease process, and of greater pathogenic significance in lcSSc versus dcSSc (possibly reflecting the greater degree of vascular dysfunction in lcSSc) (Simonini et al., 1999). Animal models have demonstrated significant increases in smooth muscle ROS production in response to cooling, possibly due to electron leakage to O₂ and decreased efficiency of ROS scavengers at lower temperatures (Bailey et al., 2005). In such models, the administration of exogenous anti-oxidants did not influence alpha adrenoceptor vasoconstriction at 37°C, but significantly reduces vascular responses at 28°C (Bailey et al., 2005). These findings, which have recently been replicated in humans using ascorbic acid, provide an attractive framework linking cold exposure, oxidative stress and vascular dysfunction (Yamazaki, 2010).

Levels of circulating scavengers of ROS e.g. ascorbic acid and selenium are lower in primary RP and SSc compared with healthy controls, particularly in smokers (Herrick et al., 1994). Likewise, levels of native antioxidants such as catalase and superoxide dismutase are also reduced in SSc (Balbir-Gurman et al., 2007).
F2-isoprostanes have a number of biological functions relevant to SSc including vasoconstriction (probably via TxA2 receptor stimulation), mitogenesis, platelet activation, immune cell endothelial adhesion, endothelial apoptosis and collagen production in fibrosing disorders (Erre and Passiu, 2009, Cracowski and Ormezzano, 2004, Comporti et al., 2008). Increased levels of F2-isoprostanes and other markers of lipid peroxidation e.g. malondialdehyde have been demonstrated in SSc (Balbir-Gurman et al., 2007, Cracowski et al., 2002a, Volpe et al., 2008, Cracowski et al., 2001, Stein et al., 1996) but not primary RP (Cracowski et al., 2002a). In addition to inadvertently highlighting the unwanted potential for ex vivo artefactual generation of F2-isoprostanes in serum, Ogawa et al. identified associations between F2-isoprostane levels and ILD and autoantibody formation that warrants further investigation (Ogawa et al., 2006, Cracowski, 2006).

**Use of antioxidants in the management of RP and SSc**

Several studies have evaluated the use of antioxidant therapy in RP and SSc, suggesting some benefit on vascular disease in SSc. An early study of oral N-acetyl cysteine (NAC) therapy failed to achieve benefit over 1 year, although the study was limited by small study numbers (n=11), blunt clinical non-vascular endpoints such as oral aperture and hand spread and the potential reduced bioavailability of oral NAC due to extensive first pass metabolism (Furst et al., 1979). A subsequent open-label study suggested promising improvements in RP severity, digital ulcer count and digital arteriolar perfusion (assessed using plethysmography) following intravenous NAC therapy in patients with SSc (Sambo et al., 2001). Denton et al. reported superior efficacy of probucol compared with low dose nifedipine in reducing Raynaud’s symptoms in patients with both primary RP and SSc (Denton et al., 1999). Herrick et al. meanwhile were unable to demonstrate any improvement in RP severity, thermographic response to cold stress or markers of endothelial dysfunction following treatment with an array of micronutrients, although the potential for carry-over effect following each treatment period hampered data analysis (Herrick et al., 2000). Few studies have investigated F2-isoprostanes levels in therapeutic trials of antioxidants for patients with SSc. A neatly designed RCT investigating treatment with vitamin E in SSc evaluated urinary F2-isoprostanes and LDPI assessment of peripheral vascular function (before and following total body cooling) but was unable to demonstrate any differences between treatment and placebo groups (Cracowski et al., 2005).
Other studies have explored the potential anti-oxidant effects of established vasodilator therapy in SSc. For example, treatment with dihydropyridine calcium channel antagonists (nifedipine or nicardipine) have been shown to reduce markers of oxidative stress (carbonyl residues, advanced oxidation protein products, malondialdehyde and nitrosothiols) in SSc (Allanore et al., 2004). It was not known whether this related to a direct antioxidant effect or secondary to improved vascular perfusion, although subsequent work suggested nifedipine could directly attenuate monocyte ROS production in SSc (Allanore et al., 2005b). There was no reduction in urinary levels of 8-iso-PGF$_{2\alpha}$ (a member of the F$_2$-isoprostane family) after 1 day (n=4) or 3 days (n=10) following a single infusion of iloprost (Volpe et al., 2008). This study enrolled a small number of patients with SSc already incorporated onto a cyclical regime of monthly iloprost infusions as part of their disease management creating significant potential for carry-over effect (Volpe et al., 2008). In contrast, others have demonstrated significant reductions in urinary F2-isoprostane levels following 5 days intravenous iloprost therapy in patients with SSc and post hoc analysis suggested the effect was greatest in those patients with early lcSSc (Erre et al., 2008). Other studies have demonstrated improvements in oxidative status (serum malondialdehyde, and activity of catalase and superoxide dismutase) following iloprost therapy in patients with SSc (Balbir-Gurman et al., 2007).

### 6.2 Aims and Objectives

The principal aim of this chapter is to explore the relationship between platelet function, eicosanoid biosynthesis and oxidative stress in both primary RP and SSc.

The specific objectives of this chapter are to explore:

1. Explore inter-relationship between platelet number, platelet morphology, plasma viscosity, LTA, eicosanoid biosynthesis, plasma biomarkers of platelet activation and peripheral microvascular function (using RCS diary, IRT and LSCI) in patients with primary RP and SSc
2. Evaluate differences in platelet number, platelet morphology, plasma viscosity, LTA, eicosanoid biosynthesis and plasma biomarkers of platelet activation between patients with primary RP and SSc
3. Evaluate the influence of season on platelet number, platelet morphology, plasma viscosity, LTA, eicosanoid biosynthesis and plasma biomarkers of platelet activation in patients with primary RP and SSc
6.3 Subjects and Methods

6.3.1 Patient eligibility and recruitment

The patients were selected from patients recruited to participate in the study reported in Chapter 5 (section 5.3). Patients currently taking anti-platelet agents or NSAIDs were excluded from the analyses undertaken in this chapter due to the expected effects of such treatments on platelet function and eicosanoid biosynthesis. No attempt was made to exclude patients taking anti-oxidant treatments due to the pleiotropic anti-oxidant effects of numerous drugs.

6.3.2 Study design and endpoints

The patients were seen at weeks 0 and 2 for cold challenge assessment and clinical assessment as described in section 5.3.3. Patients were issued with a universal container at their 1st assessment to allow collection of a fasting mid-stream urine collection on the morning of their second assessment (as outlined in section 2.5.5). Patients with SSc completed the SHAQ at their first assessment and all patients were trained on completion of the RCS diary between assessments 1 and 2. At the end of the 2nd assessment patients provided a sample of blood as described in section 2.5.1.

Patient demographics and clinical data

Information was collected from participants (with the help of their medical records) at enrollment regarding clinical phenotype, disease duration, age, gender, smoking history and medication usage (as described in section 2.3.1). Patients were subsequently divided according to season of enrollment into Autumn/Winter (October-March) and Spring/Summer (April-September) as described in section 5.3.3.

Platelet number, structure and haemorrheological endpoints

Automated FBC analysis and PV were evaluated on the day of assessment as described in section 2.6.1. The FBC analysis allowed us to assess the platelet count, MPV, PDW and plateletcrit (PCT).

Light transmission aggregometry

LTA using the agonists ADP (working concentrations of 20µmol/L 10µmol/L, 5µmol/L, and 2.5µmol/L) and AA (working concentrations of 1.64 and 0.82 mmol/L) were undertaken as
described in section 2.7 using plasma prepared as described in section 2.5. All LTA assessments were undertaken within 1 hour of sample collection.

**Circulating plasma markers**
Platelet free plasma was prepared as described in section 2.5.4. Plasma levels of human soluble P-selectin, human soluble CD40 ligand (sCD40L), and human TGF-β were measured using separate commercially available ELISAs (R&D systems) as described in 2.8. Plasma was stored at -80°C for batch analysis.

**Urinary eicosanoid metabolites**
Urinary levels of the stable metabolite of TxA₂ (11-dehydro-TxB₂) and prostacyclin (3-dinor-6-keto-PGF₁₀) were measured in unaltered urine. Urinary F₂-isoprostanes were measured in urine containing BHT antioxidant to avoid artefactual ex vivo lipid peroxidation following collection. The methods for sample collection, preparation and assaying quantifying these eicosanoid metabolites are described in full in sections 2.5.5 and 2.9. Urine was stored at -80°C before being transferred (in dry ice) to the eicosanoid research laboratory at Nashville, Tennessee for batch analysis.

**Vascular outcome measures**
Subjective assessment of digital vascular function was undertaken using the RCS diary completed between assessments 1 and 2 as described in section 2.3.3. Objective assessment of digital vascular function before and after cold challenge was undertaken using LSCI and IRT as described in section 2.4. Image analysis was undertaken as described in section 2.4.4 using the endpoints described in section 5.3.3.

**6.3.3 Statistical analysis**
All data is presented as median values (and interquartile range [IQR]) unless otherwise stated. Correlations were assessed using Spearman’s rank correlation coefficient ($r_s$). Categorical data were compared using the Fisher’s exact test. Between group comparisons of unpaired data used the Mann Whitney U test. All data was analysed using SPSS version 18.0. All tests were 2-tailed and a $p$ value of <0.05 was considered statistically significant. Despite the large number of individual comparisons, no Bonferroni adjustment correction factor was applied to the analyses. Where relevant, I have highlighted significant
associations, which may have occurred as a result of type I error and I have guarded against over-interpretation of the findings.

6.4 Results

6.4.1 Patient recruitment and demographics

Of 43 patients recruited to the microvascular imaging study described in the previous chapter, only 34 were eligible for inclusion in this analysis of eicosanoid biosynthesis and platelet function. Five patients were taking anti-platelet agents at enrollment (n=4 aspirin, n=1 clopidogrel). Two patients were taking regular NSAID therapy. Two patients inadvertently took aspirin during the run-in phase precluding any useful analysis of platelet function in this study. One patient had a supply of meloxicam (an NSAID) for arthritic symptoms but had not required this in the 2 weeks prior to or during the study, and was therefore included in the analysis.

In total, data was available for 34 patients (17 with primary RP and 17 with SSc). The majority of patients with SSc had lcSSc (n=14) with the remaining patients being classified as dcSSc (n=2) and lSSc (n=1). The baseline demographics of patients with primary RP and SSc are presented in Table 6.2.

The age of onset was significantly lower in the primary RP group as expected (20.5 vs. 35 years, p=0.03). The groups were otherwise similar for age, gender, vasodilator therapy use and smoking history. A similar proportion of patients were recruited in Spring/Summer vs. Autumn/Winter. The clinical features of patients with SSc are presented in Figure 6.1.

14 This emerged on direct questioning at assessment 2 in one patient. In the second patient, platelet aggregometry using the agonist AA was highly suspicious of aspirin usage. The patient was contacted and it was clarified that the OTC paracetamol preparation she had been taking also contained aspirin. There was a third patient in whom aspirin use was suspected due to the appearance of the aggregation curves. She had taken an OTC flu-remedy preparation but re-checked its contents and confirmed it had not contained aspirin.
Table 6.2 Baseline demographics of group.

All data expressed as median (IQR) unless otherwise stated. * Mann-Whitney U test for comparing distribution across unpaired groups and Fisher’s exact test to compare frequencies of categorical data

<table>
<thead>
<tr>
<th></th>
<th>Primary RP</th>
<th>Systemic Sclerosis</th>
<th>p value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4</td>
<td>3</td>
<td>1.00</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Age in years (IQR)</td>
<td>49.6 (20)</td>
<td>53.6 (19.9)</td>
<td>0.13</td>
</tr>
<tr>
<td>Age at RP onset in years (IQR)</td>
<td>20.5 (24)</td>
<td>35 (13)</td>
<td>0.03</td>
</tr>
<tr>
<td>Age at diagnosis in years (IQR)</td>
<td>42.5 (6)</td>
<td>45 (16)</td>
<td>0.79</td>
</tr>
<tr>
<td>Smoking (%; unless stated)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>1 (5.9)</td>
<td>2 (11.8)</td>
<td>1.00</td>
</tr>
<tr>
<td>Ex</td>
<td>6 (35.3)</td>
<td>5 (29.4)</td>
<td>1.00</td>
</tr>
<tr>
<td>Mean accrued pack year history (SD)</td>
<td>4.8 (7.6)</td>
<td>9.3 (20)</td>
<td>0.95</td>
</tr>
<tr>
<td>Never</td>
<td>10 (58.8)</td>
<td>10 (58.8)</td>
<td>1.00</td>
</tr>
<tr>
<td>Season enrolled</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autumn/Winter</td>
<td>9 (52.9)</td>
<td>9 (52.9)</td>
<td>1.00</td>
</tr>
<tr>
<td>Spring/Summer</td>
<td>8 (47.1)</td>
<td>8 (47.1)</td>
<td></td>
</tr>
<tr>
<td>Platelet count (IQR)</td>
<td>269.5 (62.5)</td>
<td>257 (87)</td>
<td>1.00</td>
</tr>
<tr>
<td>Vasodilator therapy (%)</td>
<td>4 (23.5)</td>
<td>8 (47.1)</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Figure 6.1 Clinical phenotype of patients with SSc.

lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc; ISSc, limited SSc; DU, digital ulceration; DP, digital pitting; GI, gastrointestinal; PAH, pulmonary arterial hypertension; ILD, interstitial lung disease; SRC, scleroderma renal crisis.
Subjective assessment of digital vascular function using the RCS diary was unavailable for 1 patient with primary RP due to inaccurate completion of the diary and 1 patient with SSc who failed to return the completed diary. No analysis (LTA, FBC/PV or plasma biomarkers) was available for 1 patient (primary RP) in whom venesection was unsuccessful on first attempt and who declined a second attempt of blood collection. No LTA results were available in another patient with primary RP in whom the citrated sample for LTA studies clotted during collection. Plasma biomarkers and haematological indices were available for this participant. The full complement of LTA analyses (all concentrations of ADP and AA) was available for 21/32 (65.6%) of participants. Of the remaining patients, the majority (7/11, 63.6%) only lacked a single concentration analysis (typically due to insufficient PRP [as outlined in section 2.7.5]). In the remaining 4 patients (12.5%), 2 or more concentrations were missing, typically due to insufficient plasma. Urine samples for eicosanoid biosynthesis were available for all participants.

6.4.2 Inter-relationship between platelet number, platelet morphology, plasma viscosity, LTA, eicosanoid biosynthesis, plasma biomarkers of platelet activation and peripheral microvascular function (using RCS diary, IRT and LSCI) in patients with primary RP and SSc

The following analyses use pooled data from patients with primary RP and SSc unless stated.

**Platelet number, structure and haemorrhheological parameters**

There was significant internal correlation between the platelet count and the PCT, MPV and PDW (Table 6.3). As expected, the correlation between MPV and PDW was strong ($r_s 0.94$, $p<0.001$). There was no correlation between platelet number or morphology and either the parameters of the RCS diary or objective assessment of vascular function using LSCI. An interesting relationship between platelet number/structure and thermographic assessment of digital vascular function assessed using the DDD (as opposed to individual ROI assessments) was identified (Table 6.3). A significant negative correlation between platelet count and the DDD immediately following cold challenge (t0) was identified ($r_s -0.478$, $p=0.006$). Significant negative correlations were also identified between the PV and the DDD at baseline and at 5, 10 and 15 minutes post-cold challenge (i.e. indicating a higher PV or platelet count is associated with lower peripheral vascular perfusion, Table 6.3). Significant positive correlations were identified between both the MPV and PDW, and the DDD at
baseline and t0 (with strong trends for a similar relationship at t10 and t15, Table 6.3). These findings suggest the larger the platelets, the better the digital perfusion, which was contrary to our initial hypothesis that increased platelet activation would be associated with more profound vasculopathy (further discussion will follow).

Table 6.3 Correlation between platelet number, structure and PV with the thermographic assessment of digital vascular function in primary RP and SSc

<table>
<thead>
<tr>
<th></th>
<th>DDD B</th>
<th>DDD t0*</th>
<th>DDD t5</th>
<th>DDD t10</th>
<th>DDD t15</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet count</td>
<td>rs</td>
<td>.390</td>
<td>-.076</td>
<td>-.167</td>
<td>-.478</td>
</tr>
<tr>
<td>Sig</td>
<td></td>
<td>.025</td>
<td>.000</td>
<td>.021</td>
<td>.674</td>
</tr>
<tr>
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<td>.352</td>
<td>.006</td>
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<td>.771</td>
<td>.551</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>.274</td>
<td></td>
</tr>
<tr>
<td>PDW</td>
<td>rs</td>
<td>.019</td>
<td>-.134</td>
<td>.495</td>
<td>.504</td>
</tr>
<tr>
<td>Sig</td>
<td></td>
<td>.916</td>
<td>.000</td>
<td>.459</td>
<td>.003</td>
</tr>
<tr>
<td></td>
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<td>.130</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>.020</td>
<td></td>
</tr>
<tr>
<td>PCT</td>
<td>rs</td>
<td>.002</td>
<td>.021</td>
<td>-.007</td>
<td>-.321</td>
</tr>
<tr>
<td>Sig</td>
<td></td>
<td>.993</td>
<td>.906</td>
<td>.969</td>
<td>.073</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>.826</td>
<td></td>
</tr>
<tr>
<td>MPV</td>
<td>rs</td>
<td>-.001</td>
<td>.417</td>
<td>.533</td>
<td>.209</td>
</tr>
<tr>
<td>Sig</td>
<td></td>
<td>.994</td>
<td>.016</td>
<td>.002</td>
<td>.243</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>.098</td>
<td>.072</td>
</tr>
<tr>
<td>PV</td>
<td>rs</td>
<td>-.423</td>
<td>-.074</td>
<td>-.379</td>
<td>-.467</td>
</tr>
<tr>
<td>Sig</td>
<td></td>
<td>.014</td>
<td>.687</td>
<td>.030</td>
<td>.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.001</td>
<td></td>
</tr>
</tbody>
</table>

Light transmission aggregometry studies

There was no relationship between platelet number/morphology and LTA results. Positive correlations were, however, identified between PV and the maximum % aggregation to ADP 20μmol/L ($rs$ 0.441, $p=0.019$), ADP 2.5μmol/L ($rs$ 0.437, $p=0.016$) and between PV and maximum gradient of aggregation for ADP 2.5μmol/L ($rs$ 0.542, $p=0.002$) and AA 1.64mmol/L ($rs$ 0.545, $p=0.006$). These findings suggest a possible link between haemorrhheological factors and intrinsic platelet aggregability. Aggregation to low concentration (2.5-5μmol/L) ADP was significantly lower in males than females. For example, the median maximum % aggregation to 2.5μmol/L ADP was 27 in males vs. 68 in females ($p=0.037$). The median maximum % aggregation to 5μmol/L ADP was 60 in males vs. 77 in females ($p=0.018$).
There were no consistent correlations between the results of LTA and the individual parameters of the RCS diary or objective assessment of digital vascular function using IRT or LSCI at individual ROIs. As with platelet number and structure, an interesting trend was observed when comparison was made between LTA and thermographic assessment of digital vascular function using the DDD (as opposed to individual ROI assessments). Weak, but statistically significant negative correlations, were observed between the DDD at both t10 and t15 post-cold challenge and max % aggregation to ADP concentrations of 20μmol/L and 10μmol/L ($r_s$ between -0.445 to -0.392, p<0.05). These findings suggest more pronounced peripheral vascular responses to cold exposure in patients with greater platelet aggregability.

**Eicosanoid biosynthesis**

There was a moderate correlation between urinary levels of F$_2$-isoprostanes and urinary 11-dehydro-TxB$_2$ ($r_s$ 0.612, p<0.001, Figure 6.2) and between urinary 2,3-dinor-6-keto-PGF$_{1\alpha}$ and 11-dehydro-TxB$_2$ ($r_s$ 0.537, p=0.001, Figure 6.2). There was no correlation however between urinary levels of F$_2$-isoprostanes and 2,3-dinor-6-keto-PGF$_{1\alpha}$ ($r_s$ 0.298, p=0.09, Figure 6.2).

There were consistent moderate negative correlations between urinary F$_2$-isoprostanes levels and platelet aggregometry to a range of ADP concentrations (Table 6.4). In contrast, there was no association between platelet aggregometry using ADP and urinary levels of 2,3-dinor-6-keto-PGF$_{1\alpha}$ and/or 11-dehydro-TxB$_2$. Similarly, there were no associations between eicosanoid biosynthesis and LTA to AA.
Figure 6.2 Inter-relationship between urinary eicosanoid levels in patients with SSc and primary RP

Correlation coefficients reported as Spearman’s rho with associated p values. A) F₂-isoprostanes vs. 11-dehydro-TxB₂, B) 11-dehydro-TxB₂ vs. 2,3-dinor-6-keto-PGF₁α, and C) F₂-isoprostanes vs. 2,3-dinor-6-keto-PGF₁α (n=34)
Table 6.4 Correlation between platelet aggregability to ADP and urinary \( F_2 \)-isoprostane levels in patients with primary RP and SSc
(n=34)

<table>
<thead>
<tr>
<th>Urinary ( F_2 )-isoprostane levels</th>
<th>ADP concentration</th>
<th>20 μmol/L</th>
<th>10 μmol/L</th>
<th>5 μmol/L</th>
<th>2.5 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman’s Rho</td>
<td>Maximum % platelet aggregation</td>
<td>-0.438</td>
<td>-0.476</td>
<td>-0.395</td>
<td>-0.346</td>
</tr>
<tr>
<td>p value</td>
<td>0.020</td>
<td>0.007</td>
<td>0.025</td>
<td>0.061</td>
<td></td>
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<tr>
<td>Spearman’s Rho</td>
<td>% Aggregation after 5 minutes</td>
<td>-0.438</td>
<td>-0.471</td>
<td>-0.395</td>
<td>-0.348</td>
</tr>
<tr>
<td>p value</td>
<td>0.020</td>
<td>0.009</td>
<td>0.025</td>
<td>0.060</td>
<td></td>
</tr>
<tr>
<td>Spearman’s Rho</td>
<td>Maximum gradient of aggregation</td>
<td>-0.261</td>
<td>-0.459</td>
<td>-0.503</td>
<td>-0.292</td>
</tr>
<tr>
<td>p value</td>
<td>0.189</td>
<td>0.011</td>
<td>0.003</td>
<td>0.118</td>
<td></td>
</tr>
</tbody>
</table>

There were some interesting associations between objective assessment of digital vascular function and eicosanoid biosynthesis at baseline and immediately following cold challenge (Table 6.5) using IRT. For example, there were consistent significant negative correlations between IRT assessments (at each ROI before and immediately following cold challenge)
and levels of both \( \text{F}_2 \)-isoprostanes and \( \text{11-dehydro-TxB}_2 \) (Table 6.5). Significant correlations were also identified for some of the corresponding LSCI assessments (most notably ROI3 at baseline, Spearman’s Rho \(-0.489, p=0.004\), Table 6.5). There were fewer significant correlations between 2,3-dinor-6-keto-PGF\(_{1\alpha}\) levels and assessment of digital vascular function using LSCI and IRT (Table 6.5) although possible trends might have been strengthened with larger patient numbers.

There was no correlation between urinary levels of \( \text{F}_2 \)-isoprostanes or \( \text{11-dehydro-TxB}_2 \). And self-report assessment of digital vascular function (using individual components of the RCS diary). Moderate positive correlations were identified meanwhile between urinary levels of the vasodilator 2,3-dinor-6-keto-PGF\(_1\) with duration \( (r_s \ 0.418, p=0.017) \) and frequency \( (r_s \ 0.492, p=0.005) \) of RP attacks using the RCS diary, which are not easily explained. There was no correlation between the overall RCS score and urinary 2,3-dinor-6-keto-PGF\(_{1\alpha}\) levels.

Table 6.5 Relationship between eicosanoid biosynthesis and objective assessment of digital microvascular dysfunction at baseline (B) and immediately following cold challenge (t0).

All values reported as Spearman’s rho correlation coefficients. LSCI, laser speckle contrast imaging; IRT, infrared thermography; \( r_s \), Spearman’s rho; ROI, region of interest. B, baseline; t0, immediately following cold challenge. Significant results highlighted in bold.

<table>
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<th></th>
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<th></th>
<th></th>
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<td></td>
<td></td>
<td>ROI 1</td>
<td></td>
<td></td>
<td>ROI 2</td>
<td></td>
<td></td>
<td>ROI 3</td>
<td></td>
<td></td>
<td>ROI 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>t0</td>
<td>B</td>
<td>B</td>
<td>t0</td>
<td>B</td>
<td>t0</td>
<td>B</td>
<td>t0</td>
<td>B</td>
</tr>
<tr>
<td>( \text{F}_2 )-isoprostane</td>
<td></td>
<td>( r_s )</td>
<td>0.258</td>
<td>0.85</td>
<td>-0.370</td>
<td>0.135</td>
<td>-0.489</td>
<td>0.175</td>
<td>-0.358</td>
<td>0.393</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>( \rho )</td>
<td>0.14</td>
<td>0.63</td>
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<td>0.45</td>
<td>0.004</td>
<td>0.34</td>
<td>0.04</td>
<td>0.024</td>
<td>0.04</td>
</tr>
<tr>
<td>2,3-dinor-6-keto-PGF(_{1\alpha})</td>
<td></td>
<td>( r_s )</td>
<td>-0.075</td>
<td>-0.288</td>
<td>-0.072</td>
<td>-0.057</td>
<td>-0.261</td>
<td>-0.321</td>
<td>-0.342</td>
<td>-0.262</td>
<td>-0.378</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( \rho )</td>
<td>0.67</td>
<td>0.1</td>
<td>0.68</td>
<td>0.75</td>
<td>0.14</td>
<td>0.07</td>
<td>0.05</td>
<td>0.14</td>
<td>0.03</td>
</tr>
<tr>
<td>( 11\text{-dehydro-TxB}_2 )</td>
<td></td>
<td>( r_s )</td>
<td>-0.365</td>
<td>-0.007</td>
<td>-0.269</td>
<td>0.109</td>
<td>-0.448</td>
<td>-0.141</td>
<td>-0.462</td>
<td>-0.373</td>
<td>-0.421</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( \rho )</td>
<td>0.03</td>
<td>0.97</td>
<td>0.13</td>
<td>0.54</td>
<td>0.009</td>
<td>0.44</td>
<td>0.006</td>
<td>0.03</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Plasma biomarkers of platelet activation

There was a significant positive correlation between plasma levels of TGF-\( \beta \) and sCD40L \( (r_s \ 0.448, p=0.009) \). There was no relationship between sP-selectin and levels of either TGF-\( \beta \) or sCD40L.
There were moderately positive correlations between levels of sP-selectin and the platelet count ($r_s 0.497$, $p=0.003$) and PCT ($r_s 0.482$, $p=0.004$). There were no associations between platelet number and/or structure with circulating levels of sCD40L or TGF-β. A positive correlation was identified between PV and levels of sCD40L ($r_s 0.404$, $p=0.02$), which may reflect the importance of sCD40L in adaptive immune function and inflammation.

There was no consistent relationship between the plasma biomarkers and LTA. Significant positive correlations were identified between sCD40L and both 2,3-dinor-6-keto-PGF$_{1α}$ ($r_s 0.449$, $p=0.009$) and 11-dehydro-TxB$_2$ ($r_s 0.436$, $p=0.011$). Plasma TGF-β levels correlated with both F$_2$-isoprostanes ($r_s 0.404$, $p=0.02$) and 11-dehydro-TxB$_2$ ($r_s 0.519$, $p=0.002$). There were no associations between circulating sP-selectin levels and urinary eicosanoid levels.

There was no correlation between plasma biomarker levels and subjective (RCS diary) or objective (IRT and LSCI) assessment of peripheral vascular function.

6.4.3 Evaluate differences in platelet number, platelet morphology, plasma viscosity, LTA, eicosanoid biosynthesis and plasma biomarkers of platelet activation between patients with primary RP and SSc

**Platelet number, platelet morphology and plasma viscosity**

The platelet count did not differ between primary RP and SSc (median 269.5 vs. 257, $p=1.00$). Similarly, there were no differences in platelet structure assessed using the mean platelet volume (MPV, $p=0.326$), platelet distribution width (PDW, $p=0.217$) or plateletcrit (PCT, $p=0.465$) between primary RP and SSc. The plasma viscosity (PV) was higher in SSc than primary RP (median [IQR] 1.7 [0.15] vs. 1.6 [0.11], $p=0.006$). There was a trend for higher platelet counts in patients with SSc with a history of DU (median 301.5 vs. 254, $p=0.078$). The MPV (median 9.5 vs. 10.7fL, $p=0.005$) and PDW (median 10.5 vs. 12.2, $p=0.01$) were significantly lower in patients with SSc with a history of DU. There were similar trends for the platelet count ($p=0.088$) and MPV ($p=0.07$) in patients with SSc and a history of digital pitting. No other associations between clinical phenotype in SSc and platelet number/morphology were identified.
Light transmission aggregometry

Examples of aggregometry traces following addition of varying concentrations of the agonist ADP in a patient with primary RP and a patient with SSc are presented in Figure 6.3.

Platelet aggregation (Maximum % aggregation and % aggregation at 5 minutes) was significantly higher in SSc compared to primary RP to low to medium working concentrations (2.5μmol/L-10μmol/L ADP) of ADP (p<0.05 for all comparisons, Table 6.6). Percent aggregation to high concentration ADP (20μmol/L) and AA (1.64 and 0.82mmol/L) did not differ between groups, possibly representing a supramaximal dosing effect (i.e. these doses precipitated maximal aggregation in both patient groups reducing the discriminatory capacity of the test). The maximum gradient of aggregation did not generally help differentiate between patients groups, with the exception of 1.64mmol/L AA, which was significantly greater in SSc compared with primary RP (41 vs. 36 respectively, p<0.05).
Figure 6.3. Examples of aggregometry curves from a patient with primary RP and a patient with SSc using serial decreasing working concentrations of ADP agonist

1=20 μmol/L; 2=10 μmol/L; 3=5 μmol/L and 4=2.5 μmol/L respectively. Traces in A are derived from a patient with primary RP. Traces in B are derived from a patient with SSc. Note the rapid extensive primary waves of aggregation in the patient with SSc to all concentrations of ADP. At the lowest concentration of 2.5 μmol/L there are distinguishable primary and secondary waves of aggregation (representing initial aggregation to ADP and a secondary wave following platelet granule release). In the patient with primary RP, meanwhile, there is a lower degree of aggregation to lower concentrations of ADP with reversal of the primary wave of aggregation at 2.5 μmol/L due to failure to stimulate granule release and subsequent de-aggregation during the 5-minute period following addition of agonist.
Table 6.6 Outcome of platelet aggregometry to varying concentrations of adenosine diphosphate (ADP) and arachidonic acid (AA) in primary RP and SSc

Max%, maximum % aggregation during 5 minutes post agonist; % 5 mins, % aggregation after 5 minutes post agonist; Gmax, Maximum gradient of aggregation following addition of agonist. 1° RP, primary Raynaud’s phenomenon. All values expressed as median (IQR range).

† p<0.05 vs. primary RP; * p=0.089 vs. primary RP

<table>
<thead>
<tr>
<th>Agonist and concentration</th>
<th>Adenosine diphosphate</th>
<th>Arachidonic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 μmol/L</td>
<td>10 μmol/L</td>
</tr>
<tr>
<td>Max % SSc</td>
<td>80 (9)</td>
<td>79 (7) †</td>
</tr>
<tr>
<td>1° RP</td>
<td>75 (10)</td>
<td>74 (12)</td>
</tr>
<tr>
<td>% 5 mins SSc</td>
<td>80 (9)</td>
<td>79.5 (6) †</td>
</tr>
<tr>
<td>1° RP</td>
<td>75 (10)</td>
<td>74 (12)</td>
</tr>
<tr>
<td>Gmax SSc</td>
<td>40 (11)</td>
<td>39.5 (9)</td>
</tr>
<tr>
<td>1° RP</td>
<td>42 (12)</td>
<td>39.5 (15)</td>
</tr>
</tbody>
</table>

Eicosanoid biosynthesis

Urinary levels of 11-dehydro-TxB2 (medians 425 vs. 382 pg/mg creatinine [Cr], p=0.26), 3-dinor-6-keto-PGF1α (160 vs. 122 pg/mg creatinine, p=0.079) and F2-isoprostanes (1.00 vs. 1.12 ng/mg creatinine Cr, p=0.39) did not differ between SSc and primary RP (Figure 6.4).
Figure 6.4 Eicosanoid biosynthesis in SSc and primary RP

A) levels of urinary 11-dehydro-TxB₂, B) levels of urinary 2,3-dinor-6-keto-PGF₁α, and C) levels of urinary F₂-isoprostanes

The bold lines indicate the median value and boxplots indicate IQR. The whiskers reveal range of data points that sit within 1.5(IQR) of the 1st and 3rd quartiles respectively and equals the range in most instances. Individual outliers are numbered and depicted as circles if their value sits between 1.5(IQR) and 3(IQR) of the quartile. Outliers are depicted as stars if their value is 3(IQR) from the adjacent quartile.
Circulating plasma biomarkers of platelet activation

Circulating sCD40L was higher in SSc compared to primary RP although this trend failed to achieve statistical significance (median 62 vs. 52.3 pg/ml, p=0.087). Levels of TGF-β and sP-selectin did not differ between patients with primary RP and SSc (Table 6.7).

Table 6.7 Circulating plasma biomarkers of platelet activation in primary RP and SSc

All values expressed as median (IQ range). Mann Whitney U test used for between group comparisons.

<table>
<thead>
<tr>
<th></th>
<th>Primary RP</th>
<th>SSc</th>
<th>p value (primary RP vs. SSc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β (pg/mL)</td>
<td>2177.4 (2048.3)</td>
<td>2540.9 (3193.3)</td>
<td>0.488</td>
</tr>
<tr>
<td>sCD40L (pg/mL)</td>
<td>52.3 (20.7)</td>
<td>62 (33.2)</td>
<td>0.087</td>
</tr>
<tr>
<td>sP-selectin (ng/mL)</td>
<td>17.9 (5.5)</td>
<td>18.2 (5.3)</td>
<td>0.423</td>
</tr>
</tbody>
</table>
6.4.4 Evaluation of the influence of season on platelet number, platelet morphology, plasma viscosity, LTA, eicosanoid biosynthesis and plasma biomarkers of platelet activation in patients with primary RP and SSc

There was no relationship between season of enrollment and either PV, platelet number, platelet morphology, LTA studies or circulating plasma biomarkers of platelet activation. Urinary levels of F₂-isoprostanes were significantly greater in patients enrolled in the Autumn/Winter months compared with Spring/Summer (median 1.255 [IQR 0.435] vs. 0.70 [0.51] ng/mg Cr, p=0.001, Figure 6.5A). Similarly, urinary 11-dehydro-TxB₂ levels were also greater in the Autumn/Winter (466.5 (206) vs. 348.5 (133) pg/mg Cr, p=0.025, Figure 6.5B). Urinary levels of 2,3-dinor-6-keto-PGF₁α meanwhile were not influenced by season (133.5 (91) vs. 156.5 (98) pg/mg creatinine, p=0.96).
Figure 6.5 Influence of season on eicosanoid biosynthesis.

A) Influence of season on F₂-isoprostane formation and B) Influence of season on thromboxane formation.

The bold lines indicate the median value and boxplots indicate IQR. The whiskers reveal range of data points that sit within 1.5(IQR) of the 1ˢᵗ and 3ʳᵈ quartiles respectively and equals the range in most instances. Individual outliers are numbered and depicted as circles if their value sits between 1.5(IQR) and 3(IQR) of the quartile. Outliers are depicted as stars if their value is 3(IQR) from the adjacent quartile.
6.5 Discussion

This is the first study to simultaneously explore a range of methods for assessing platelet activation, incorporating assessments of eicosanoid biosynthesis and oxidative stress, in patients with primary RP and SSc. In addition to replicating the findings of previous studies such as those demonstrating increased platelet aggregability in SSc, we have also identified some novel associations that help inform our understanding of the contribution of platelets and oxidative stress in the pathogenesis of SSc. I shall discuss the relevance of these novel findings, in the context of previous work, in further detail here.

6.5.1 Platelet number and morphology in primary RP and SSc

We were unable to identify any differences in platelet count or morphology between primary RP and SSc. A higher total platelet count in whole blood has been identified from patients with the CREST variant of SSc compared with healthy controls (Price et al., 1991). In this study, the MPV and PDW did not differ between SSc and healthy controls (Price et al., 1991). In contrast, a recent study has demonstrated an increased MPV in primary RP compared with healthy controls (Shemirani et al., 2012). We did not assess platelet structure in healthy controls but did not identify any differences in platelet count or platelet morphology between patients with primary RP and SSc.

The relationship between platelet count/morphology and objective evidence of digital vascular dysfunction using both IRT and a history of DU, was contrary to my original hypothesis that an increase in platelet size would provide evidence of in vivo activation of platelets and their contribution to vascular dysfunction in primary RP and SSc. It may be that we harvested “exhausted” platelets (i.e. smaller) from participants with severe vascular disease in whom platelet activation had already occurred. The local cold challenge assessment may have contributed to this (Park et al., 2002). There were 2 reasons for undertaking venesection following the cold challenge. Firstly, we did not want the physical trauma relating to venesection to interfere with the acclimatization period prior to vascular assessment. We also wanted to minimize the period between venesection and sample processing for both LTA studies and the storage of plasma to prevent ex vivo platelet activation. Further work is
needed to explore the determinants of abnormal platelet structure and effects of local cold exposure in larger numbers of patients with primary RP and SSc.

6.5.2 Light Transmission Aggregometry in primary RP and SSc

Many studies have identified increased platelet aggregability in both primary RP and SSc when compared with healthy controls, to a panel of agonists including adrenaline (Hutton et al., 1984), ADP (Wilkinson et al., 1989, Cuenca et al., 1990, Lau et al., 1993), collagen (Goodfield et al., 1988, Wilkinson et al., 1989, Lau et al., 1993, Goodfield et al., 1993), serotonin (5-HT) (Beretta et al., 2008, Biondi and Marasini, 1989) and AA (Reilly et al., 1986). Many of these studies incorporated a heterogeneous mix of patients and only a few studies have specifically compared platelet aggregation in patients with primary RP and SSc and have resulted in conflicting results (Cuenca et al., 1990, Biondi and Marasini, 1989, Wilkinson et al., 1989).

We have demonstrated greater aggregation of platelets to ADP in patients with SSc compared with primary RP, which supports the findings of earlier studies (Cuenca et al., 1990, Biondi and Marasini, 1989). We did not identify differences in LTA to AA, possibly due to a ceiling effect arising from the concentrations we chose for analysis. The discriminatory capacity of ADP in differentiating between primary RP and SSc was better for lower working concentrations of ADP. The single study to solely compare primary RP with healthy controls failed to identify differences in aggregation to the agonists AA, ADP and Platelet Activating Factor (Dowie et al., 1990). We did not collect data on healthy controls to explore this further. We did not extend our work to include functional studies to establish why in vitro platelet aggregation to ADP under low shear stress was higher in SSc compared with primary RP. The underlying cause for this may be due to inherent differences in platelet ADP responses between disease states or different plasma constituents (e.g. thromboxane and thrombin levels) leading to enhanced aggregatory responses to ADP. The trend for higher urinary levels of 3-dinor-6-keto-PGF$_{1\alpha}$ in SSc compared to primary RP suggests dampening of ADP signaling via PGI$_2$ induced cAMP formation was not the cause of increased aggregation to ADP (Figure 1.4). Future functional studies could evaluate the possible contribution of extracellular platelet activators (e.g TxA2) and/or inhibitors (e.g. adenosine). Studies exploring selective blockade of the intracellular signaling pathways down stream of P2Y$_1$ and P2Y$_{12}$ receptors (summarized in Figure 1.4) may help to establish evidence of inherent
differences in platelet function to account for increased aggregation to low concentration ADP in SSc.

The impact of gender on platelet aggregation to agonists including ADP has been noted previously in healthy controls but this is the first study to demonstrate this in patients with RP (Becker et al., 2006, Otahbachi et al., 2010). The association between LTA and PV we identified may be the result of elevated fibrinogen levels in SSc which can influence both platelet aggregability and PV (Meade et al., 1985, Landolfi et al., 1991). Further work would be needed to explore this hypothesis.

The association between platelet aggregation and peripheral vascular function following local cold challenge has not been identified previously. The strength of the association was weak but deserves further consideration as it provides an attractive pathological framework linking platelet dysfunction, cold exposure and digital vascular function in RP, particularly in the context of SSc. The association we have identified also highlights an additional potential advantage of the DDD over absolute assessments at individual ROIs (as was noted in the chapter 5 when exploring the discriminatory capacity of the DDD vs. individual ROIs in differentiating primary RP and SSc).

6.5.3 Eicosanoid biosynthesis in primary RP and SSc

We did not identify any differences in urinary 11-dehydro-TxB\textsubscript{2} levels between primary RP and SSc. No previous studies have compared thromboxane biosynthesis between patients with primary RP compared with SSc. Plasma TxB\textsubscript{2} levels are higher in SSc compared with HC (Herrick et al., 1996, Wilkinson et al., 1989) but studies evaluating plasma levels of TxB\textsubscript{2} in primary RP have not identified differences compared with healthy controls (Dowie et al., 1990). We might therefore have expected to see a difference in thromboxane synthesis between primary RP and SSc in our study. Urinary levels of 2-3 dinor TxB\textsubscript{2} (a stable urinary metabolite of TxB\textsubscript{2}) are elevated in SSc compared with HC, and rise in response to total body cooling (Reilly et al., 1986). We did not explore the effect of total body cooling but have identified higher 11-dehydro-TxB\textsubscript{2} levels in Autumn/Winter compared with Spring/Summer. This has not previously been investigated but provides a tantalizing glimpse into the potential relationship between season and platelet function, as well as potentially highlighting the importance of patients with RP in maintaining their core and peripheral temperatures during
the colder months to avoid excessive platelet activation. Previous work suggests local cooling (forearm and hand) does not have the same influence on TxA\(_2\) biosynthesis in SSc (Hutton et al., 1984, Sakamoto et al., 1999, Mikhailidis et al., 1986).

Measurement of metabolites of TxB\(_2\) in plasma compared to urine may tell us more about the degree of platelet activation that occurs within the peripheries. Sampling blood collected from the antecubital fossa will yield venous blood from the hands and digits where peripheral activation of platelets secondary to cold exposure and/or endothelial damage may have occurred. Measuring urinary metabolites however provides a more accurate assessment of total TxB\(_2\) formation. The present study was not designed to explore this hypothesis.

Previous work has identified higher levels of urinary 11-dehydro-TxB\(_2\) and F\(_2\)-isoprostanes in SSc compared with healthy controls but failed to identify a significant correlation between the 2 biomarkers (Cracowski et al., 2001). We did identify a strong correlation between urinary F\(_2\)-isoprostanes and 11-dehydro-TxB\(_2\). This provides evidence of the association between platelet function and oxidative stress that was discussed in section 6.1.2. The significant negative correlations we identified between urinary F\(_2\)-isoprostane levels and LTA to ADP is not be easily explained.

We identified a strong trend for higher urinary 2,3-dinor-6-keto-PGF\(_{1\alpha}\) in SSc compared with primary RP. A trend of this nature was previously noted in a study by Reilly et al. who reported higher urinary levels of 2,3-dinor-6-keto-PGF\(_{1\alpha}\) in SSc compared with healthy controls (Reilly et al., 1986). In their study, levels of 2,3-dinor-6-keto-PGF\(_{1\alpha}\) rose in response to total body cooling which the authors postulated was secondary to a platelet-vascular interaction. In contrast to urinary 11-dehydro-TxB\(_2\) and F\(_2\)-isoprostanes, levels of 2,3-dinor-6-keto-PGF\(_{1\alpha}\) were not influenced by seasonal variation.

In contrast to work by Cracowski et al., we did not identify higher levels of urinary F\(_2\)-isoprostanes in SSc compared with primary RP (Cracowski et al., 2002a). F\(_2\)-isoprostane levels were higher in the Autumn/Winter possibly due to increased oxidative stress secondary to ischaemia/reperfusion injury. This has not previously been demonstrated and provides interesting insight into the potential relationship that may exist between seasonal variation in temperature and oxidative stress.
Perhaps our most interesting findings were the relationship between eicosanoids and peripheral vascular function. This is the first study to demonstrate a negative correlation between urinary levels of both F$_2$-isoprostanes and 11-dehydro-TxB$_2$, (both powerful vasoconstrictors) with digital perfusion assessed using IRT. This needs to be further investigated in larger numbers of patients. In the next chapter, we shall explore whether anti-platelet therapy has the potential to augment the relationship between platelet function and peripheral vascular tone to promote vasodilation in patients with RP and SSc.

6.5.4 Plasma biomarkers

The choice of plasma biomarkers in this study was not restricted to molecules with a high specificity to platelets. Only soluble P-selectin has a significant degree of platelet specificity whereas sCD40 and TGF-β are produced by several cell types. Nonetheless, the major source of circulating levels of all 3 molecules is platelets and each has previously been used as a biomarker of platelet activation. The close correlation between sCD40L and TGF-β suggests common pathways for expression and release of these mediators, although the lack of correlation of sCD40L and TGF-β with circulating plasma levels of sP-selectin, platelet number and platelet morphology would suggest platelets may not be the primary source of these biomarkers in RP and SSc. Both sCD40L and TGF-β did, however, correlate with 11-dehydro-TxB$_2$ biosynthesis which may provide evidence of platelet activation contributing to the circulating levels of these biomarkers.

Several studies have identified higher circulating levels of the soluble form of P-selectin (sP-selectin) in SSc compared with healthy controls (Olewicz-Gawlik et al., 2010, Blann et al., 2003, Sfikakis et al., 1999, Iannone et al., 2008, Gruschwitz et al., 1995). We were unable to demonstrate a difference in circulating levels of sP-selectin between SSc and primary RP. Previous studies have not identified increased P-selectin expression in primary RP although platelet-monocyte and platelet-neutrophil interactions were enhanced in both primary RP and SSc compared with healthy controls ((Pamuk et al., 2007). We did identify associations between sP-selectin levels and both platelet number and structure in RP and SSc.

We were unable to demonstrate higher circulating TGF-β in patients with SSc compared with primary RP. There have been conflicting reports as to whether circulating levels of TGF-β are elevated in SSc compared with healthy controls (Dziadzio et al., 2005, Solanilla et al.,
Further work incorporating larger number of patients is required to evaluate circulating TGF-β levels and possible disease associations in SSc.

Increased circulating sCD40L levels have been identified in the plasma and serum of patients with SSc (Allanore et al., 2005a, Komura et al., 2004). Elevated levels of sCD40L have been associated with early disease course in lcSSc (Komura et al., 2004) and a more vascular phenotype (PAH and DU disease) (Allanore et al., 2005a). We were unable to demonstrate any association between sCD40L levels (or levels of TGF-β and sP-selectin) with objective or subjective assessments of peripheral vascular function in primary RP and SSc. This is the first study to specifically compare levels of sCD40L in patients with primary RP and SSc, and there was a strong trend to suggest levels of sCD40L were higher in SSc. We did not identify any disease associations in SSc with sCD40L levels, possibly owing to relatively small patient numbers.

6.5.5 Limitations

The chief limitation of this study is the relatively small cohort size. Approximately one quarter of patients recruited to the microvascular imaging study were already taking anti-platelet medication or NSAIDs, which precluded any useful assessment of platelet function.

6.6 Conclusions

In this chapter, I have reported the most comprehensive study of platelet function and eicosanoid biosynthesis in patients with primary RP and SSc. I have identified several novel areas of interest that may have important translational therapeutic implications and need to be explored further in larger studies of this kind. I shall further discuss the implications of our findings in the context of future work in Chapter 8.
Chapter 7 Evaluating the effects of asasantin retard on platelet function, oxidative stress and peripheral vascular function in primary Raynaud’s phenomenon and systemic sclerosis

“In its more aggravated forms diffuse scleroderma is one of the most terrible of all human ills. Like Tithonus, to “whither slowly”, and like him to be “beaten down and marred and wasted” until one is literally a mummy, encased in an ever-shrinking, slowly contracting skin of steel, is a fate not pictured in any tragedy, ancient or modern.”

William Osler, 1898
Journal of cutaneous genito-urinary diseases

7.1 Introduction

In previous chapters, I have outlined the potential contribution of platelets to peripheral vascular dysfunction in primary RP and SSc. I have also reviewed the evidence to support the contribution of platelets to dysregulated immune function and fibrosis in the context of SSc. A major contributory factor to the largely disappointing outcomes of previous therapeutic trials of anti-platelet agents in primary RP and SSc was the lack of sensitive validated endpoints for assessing digital vascular function. A large (multicentre) placebo-controlled RCT using a validated patient reported outcome measure of RP severity (such as the RCS diary) may be the preferred method for establishing the benefits of anti-platelet therapy on the frequency, duration and severity of RP attacks in primary RP, where the chief goal of therapy is arguably to improve subjective peripheral vascular function. This approach may not be the preferred method for evaluating the effects of anti-platelet agents in SSc for the following reasons. Firstly, in chapter 5 I demonstrated the lack of agreement that exists between the RCS diary parameters and objective peripheral microvascular assessment. This may lead to

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Tithonus was granted immortality by Zeus at the request of his lover Eos, but she failed to request eternal youth. Tithonus indeed lived forever but “when loathsome old age pressed full upon him...he could not move nor lift his limbs.....and no more has strength at all, such as once he had in his supple limbs” (Homeric Hymn to Aphrodite)
a type 2 error and failure to identify potentially disease-modifying improvements in vascular function (e.g. on frequency of DU) by subjective assessment of vascular function alone. This can be illustrated by the contrasting findings of previous studies of ERA therapy in SSc described in chapter 5. I have previously highlighted additional specific limitations of self-report assessment of RP severity in the context of SSc; most notably habituation and difficulty discerning discreet RP attacks from persistent vasculopathy secondary to irreversible changes in vascular morphology. Finally, restricting the evaluation of anti-platelet agents in SSc to peripheral vascular function alone fails to consider the potential beneficial effects of anti-platelet therapy on immune dysfunction and fibrosis in SSc. Consequently, the optimal study design for a therapeutic trial of anti-platelet agents in SSc would require a long-term (over several years), large (multicentre) RCT incorporating validated objective peripheral microvascular imaging endpoints and quantifiable objective clinical biomarkers of end-organ dysfunction (e.g. the forced vital capacity to assess lung function, frequency of DU, mean pulmonary artery pressure on right heart catheterization etc.). There is a fear amongst clinicians that such studies will never be undertaken in SSc due to the lack of industry support for trials exploring the pleiotropic effects of established, low-cost therapies. The challenges in demonstrating treatment efficacy using anti-platelet agents can be illustrated by considering the original RCTs that were undertaken to evaluate the efficacy of asasantin retard in the secondary prevention of stroke (ESPS-2 and PRoFESS studies) which necessitated recruitment of over 26,000 patients with established cerebrovascular disease.

Despite a lack of supportive evidence, recommendations are beginning to emerge to support the pragmatic use of low-dose aspirin in the management of SSc patients with severe digital ischaemia (Wigley, 2012). Whilst acknowledging the strong therapeutic rationale for using anti-platelet agents for vascular manifestations of SSc, this approach fails to consider the potential anti-inflammatory and anti-fibrotic benefits of anti-platelet agents. It also fails to consider the potential enhanced therapeutic potential of alternative anti-platelet agents beyond COX inhibition, or the combined use of low-dose aspirin and other anti-platelet agents, which have proved so effective in the management of atherosclerotic disease. These issues could be addressed by first undertaking proof-of-concept studies to establish the anti-platelet agent, or combination thereof that warrant further evaluation. There are 2 approaches by which this could be achieved. Experimental animal models of SSc (namely genetic or chemically induced forms of fibrosis) offer one potential avenue to explore the anti-fibrotic effects of anti-platelet therapy. This approach was successfully adopted in the study by Dees
et al. described in section 1.4.5 which identified a potential role for clopidogrel in reducing serotonin-induced dermal fibrosis in genetic and chemically-induced experimental models of fibrosis (Dees et al., 2011). The slowly progressive nature of skin and organ fibrosis in SSc, along with the challenges in quantifying fibrosis, would necessitate a large long-term study to demonstrate such an effect in human trials of SSc. The chief limitation of experimental models of fibrosis is the extent to which the findings of such work can be translated into humans. Murine models attempting to reproduce the vascular changes of SSc are under development and may play a more prominent role in biological proof-of-concept studies of vasoactive drugs in the future (Maurer et al., 2009). With the emergence of sensitive microvascular imaging techniques, there is greater enthusiasm to evaluate novel vasoactive medications by undertaking short-term proof-of-concept clinical trials in humans (e.g. (Herrick, 2012)). Small early-phase exploratory studies lack the power to draw conclusions on safety and efficacy but facilitate the incorporation of complex endpoints that would be unfeasible in large multicentre studies. This approach may reduce the number of novel compounds being evaluated in expensive phase 3 trials, which contributes so greatly to the high cost of novel therapies being licensed for use in humans. In this chapter I shall adopt such an approach to evaluate the effects of anti-platelet therapy in primary RP and SSc but before undertaking such work, I first had to consider the most appropriate anti-platelet agent, or combinations thereof, to investigate.

7.2 Anti-platelet agent(s) with the greatest therapeutic potential in primary RP and SSc

The ideal exploratory study of anti-platelet agents would use varying doses of several individual anti-platelet agents and combinations thereof. Such an approach would not be feasible in an orphan disease such as SSc and has forced us to consider the most attractive anti-platelet agent, or combination of agents, for further evaluation based on their biological action. Some agents were immediately excluded due to low future feasibility. For example, the non-peptide inhibitors of glycoprotein (GP) IIb/IIIa receptors (e.g. tirofiban and abciximab) are effective anti-platelet agents but require parenteral administration. This has limited their role to the acute management of coronary events and they could never be seriously considered for the management of chronic diseases such as SSc. Aspirin is the most established anti-platelet agent, irreversibly acetylating platelet COX, inhibiting the production of TxA₂ and preventing further platelet activation. In view the potent vasoconstrictive
properties of TxA$_2$ and previous work exploring endothelial dependent vasodilator effects of aspirin in primary RP (Easter and Marshall, 2005), we were keen to include aspirin therapy in this study. Using aspirin in combination with other agents enhances the effects of aspirin. For example, combining aspirin with the P2Y$_{12}$ receptor antagonist clopidogrel enhances the efficacy of aspirin in the early secondary prevention of acute coronary events. I have previously discussed a potential anti-fibrotic role for clopidogrel in SSc (Dees et al., 2011) and it has been suggested clopidogrel increases the bioavailability of NO which may have vasoprotective effects in SSc (Heitzer et al., 2006). The combination of aspirin and clopidogrel, however, increases the risk of gastrointestinal (GI) bleeding (Diener et al., 2004); a major consideration in SSc which itself can be associated with indolent GI bleeding. Furthermore, the principle mode of action of clopidogrel would not be expected to exert vasoactive effects that would necessarily improve vascular outcomes in primary RP and SSc. In contrast, the adenosine reuptake inhibitors (e.g. dipyridamole) directly promote vasodilation in addition to other pleiotropic effects, which may be beneficial in RP, particularly in the context of SSc (Kim and Liao, 2008). Dipyridamole inhibits the nucleoside transporter responsible for the uptake of adenosine. Adenosine is released by endothelial cells and platelets as a breakdown product of ATP and is a potent vasodilator and powerful inhibitor of platelet aggregation. Dipyridamole also selectively inhibits phosphodiesterase reducing platelet activation (by increasing intra-platelet levels of cAMP) and promoting vasodilation (both through potentiating the local action of NO and inhibiting phosphodiesterase-mediated degradation of cGMP which itself promotes relaxation of vascular smooth muscle cells). Other phosphodiesterase inhibitors (e.g. cilostazol) have been used specifically for their anti-platelet actions. Additional effects of dipyridamole include increased endothelial production of prostacyclin (a vasodilator), antioxidant properties and anti-inflammatory actions including inhibition of the monocyte-platelet interaction and reduced recruitment and activation of neutrophils and lymphocytes to sites of injury (Kim and Liao, 2008). Forearm vascular resistance studies suggest the vasodilatory properties of dipyridamole can be explained more by the potentiation of the adenosine mechanisms rather than via NO or other cGMP-mediated actions (Gamboa et al., 2005). Dipyridamole may also reduce fibrosis by inhibiting TGF-β-induced transdifferentiation of fibroblasts to myofibroblasts through NO-cGMP pathways (Dunkern et al., 2007). Dipyridamole has also been shown to reduce PDGF levels in the sera of patients with SSc (Takehara et al., 1987).

The co-administration of dipyridamole with aspirin (asasantin retard) increases the protective effect of aspirin in secondary prevention of stroke (Diener et al., 1996). Asasantin retard
contains a combination of aspirin 25mg and modified release dipyridamole 200mg. It is generally safe and well tolerated, and the bleeding rate is not significantly higher than that observed with aspirin as monotherapy, unlike other combinations of anti-platelet agents, e.g. clopidogrel plus aspirin (Diener et al., 2004, Diener et al., 1996).

As discussed in Chapter 1, the combination of aspirin and dipyridamole has been evaluated previously in primary RP and SSc with disappointing results (van der Meer et al., 1987, Beckett et al., 1984). Neither of these studies incorporated sensitive objective markers of peripheral vascular function, and may therefore have failed to identify improvements in vascular function with the potential to improve clinical outcomes in sufficiently powered long-term studies of RP and SSc. Consequently, I felt there was sufficient ground to reconsider the potential therapeutic benefits of this combination of anti-platelet agents in primary RP and SSc.

In this chapter, I shall report the findings of an investigator-led single-centre proof-of-concept study evaluating the short-term effects of asasantin retard therapy on peripheral vascular dysfunction, platelet function and eicosanoid biosynthesis in primary RP and SSc.

### 7.3 Methods

#### 7.3.1 Study design

The study was a short-term phase 2 exploratory open-label study and formed an extension to the initial 2 week assessment of peripheral vascular function and platelet function reported in previous chapters. This second phase to the study immediately following the initial run-in phase. The study design is summarised in Figure 7.1. Eligible participants were issued with untampered open-label asasrant retard (aspirin 25mg/modified release dipyridamole 200mg) at assessment 2 (week 2) to be taken as a single capsule orally twice daily. The study medication was commenced on the evening of their second assessment (week 2).
Figure 7.1. Summary of study design

The initial 2-week run-in phase formed the basis of the work described in chapters 5 and 6. Eligible patients were given the opportunity to enter the second interventional stage of the study, which involved open-label use of asasantin retard prior to a post-treatment assessment at the end of week 4.

Stage 1
No treatment

Stage 2
Asasantin retard

Baseline
assessment

2 weeks
Post run-in
assessment

2 weeks
Post-treatment
assessment

Patients were issued with a second 2-week RCS diary for completion during the treatment phase of the study. Participants attended the clinical measurement department of the RNHRD for a 3\textsuperscript{rd} assessment at week 4, taking the last of the asasantin retard capsules on the morning of their last assessment. The assessment at week 4 was identical to the assessment at week 2 (as described in section 6.3.2), incorporating a cold challenge assessment and collection of blood and urine for assessment of platelet function and eicosanoid biosynthesis.

7.3.2 Patient selection

This open-label study formed a sub-study of patients targeted and recruited using the eligibility criteria described in section 5.3.1. and 5.3.2. for the previously reported work evaluating peripheral vascular outcomes and platelet function in primary RP and SSc. Patients were excluded from entering the interventional second phase of the study if they fulfilled the following exclusion criteria:

1) current use of aspirin, dipyridamole or alternative anti-platelet agent
2) primary bleeding diathesis or platelet disorder
3) anticoagulation with warfarin
4) history of peptic or duodenal ulceration
5) history of intolerance or allergy to aspirin, dipyridamole or other non-steroidal anti-inflammatory medication.
6) Current use of oral corticosteroids and/or non-steroidal anti-inflammatory drugs

7.3.3 Trial endpoints
A summary of the trial-related procedures and outcomes is presented in Table 7.1.

Table 7.1. Assessments undertaken at each visit of study

IRT, infrared thermography; LSCI, laser speckle contrast imaging; LTA, light transmission aggregometry.
* participants with SSc  ** participants in stage 2 of the study

<table>
<thead>
<tr>
<th>Consent Form</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assess eligibility (for phase 1 and 2 of study)</td>
<td>X</td>
</tr>
<tr>
<td>Raynaud’s Condition Score (Black et al., 1998) (recorded by participant in 2 week period prior to assessment)</td>
<td>X X</td>
</tr>
<tr>
<td>Scleroderma Health Assessment Questionnaire * (Steen and Medsger, 1997)</td>
<td>X</td>
</tr>
<tr>
<td>Cold Stress Test (simultaneous IRT and LSCI assessments)</td>
<td>X X X</td>
</tr>
<tr>
<td>Urine sample (eicosanoid studies)</td>
<td>X X</td>
</tr>
<tr>
<td>Blood sample (LTA and plasma biomarkers)</td>
<td>X X</td>
</tr>
<tr>
<td>Document suspected adverse events/reactions</td>
<td>X</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Screening</th>
<th>Week 0</th>
<th>Week 2</th>
<th>Week 4 **</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening</td>
<td>Week 0</td>
<td>Week 2</td>
<td>Week 4 **</td>
</tr>
</tbody>
</table>

The following endpoints were chosen for analysis.

**Primary endpoints**

The primary endpoint for the study was the RCS diary during stages 1 and 2 to assess impact of asasantin retard on the mean daily frequency, mean daily duration and impact (RCS score) of RP attacks.

**Secondary endpoints**

The secondary endpoints were objective assessment of digital vascular function at baseline (assessment 2 at week 2) and following cold challenge using IRT and LSCI as described in section 5.3.3 and elsewhere.
Tertiary endpoints

Assessment of LTA, eicosanoid biosynthesis, circulating plasma biomarkers, platelet number, platelet morphology, PV, was undertaken as described in section 6.3.2 and elsewhere.

7.3.4 Tolerability, safety and compliance monitoring

Information on changes to medications, drug compliance and tolerability was collected at the final assessment. Patients were asked to report missed doses by documenting this on the relevant dates in the RCS diary. Participants were issued with a 60 capsules of asasantin retard and were asked to return unused medication at their final visit. The unused capsules were returned to pharmacy and counted for compliance monitoring. Participants were asked to report any changes to their medication usage or side effects at assessment 3. Medicine and Healthcare products Regulatory Agency (MHRA) definitions were used to classify adverse events (Table 7.2). Adverse events were considered adverse drug reactions (ADR) if they were reported as potential undesirable events in the summary of product characteristics (most recent version available on eMC dated 14.12.2011).

Table 7.2. Classification of adverse events

<table>
<thead>
<tr>
<th>Adverse event / experience (AE)</th>
<th>any untoward medical occurrence in a subject to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adverse drug reaction (ADR)</td>
<td>any untoward and unintended response in a subject to an investigational medicinal product which is related to any dose administered to that subject.</td>
</tr>
<tr>
<td>Unexpected adverse reaction</td>
<td>an adverse reaction the nature and severity of which is not consistent with the information about the medicinal product in question set out in the summary of product characteristics for that product.</td>
</tr>
<tr>
<td>Serious adverse event (SAE) or serious adverse drug reaction (SADR) or suspected unexpected serious adverse reaction</td>
<td>any adverse event, adverse reaction or unexpected adverse reaction, respectively, that: (a) results in death</td>
</tr>
</tbody>
</table>
Table 7.3.5 Statistical analysis

Assuming that the common standard deviation for the RCS is 1.8 (Black et al., 1998), it was estimated that a sample size of 19 patients in each group (primary RP and SSc) would have a 90% power to detect a difference in mean change in total RCS from baseline of -2.0 with a 0.050 two-sided significance level. Anticipating a potential 20% patient withdrawal during the study period, we hoped to recruit 25 patients with primary RP and 25 patients with SSc to the study.

All results are presented as median values (and interquartile range [IQR]), unless otherwise stated. Between group comparisons of unpaired data used the Mann Whitney U test. Comparison of paired data (e.g. comparing endpoints before and after treatment) used the Wilcoxon signed-rank test. The Fisher’s exact test was used to examine differences between categorical variables.

All data was analysed using SPSS version 18.0. All tests were two-tailed and a p value of <0.05 was considered statistically significant.
7.4 Results

7.4.1 Participants

Nineteen patients were recruited to the study. Of the 43 patients recruited for assessment in stage 1 of the study (described in section 5.4.1), only 22 were eligible for entry into the interventional phase of the study (stage 2) at screening. Five patients were taking anti-platelet agents at enrollment (n=4 aspirin, n=1 clopidogrel). Two patients were taking regular NSAID therapy. Five patients were taking corticosteroids (one of whom was also taking warfarin and another was taking aspirin). Six patients had a history of NSAID intolerance and 2 patients had a history of intolerance to aspirin. One patient had a history of peptic ulceration. One patient was excluded as a recent endoscopy had confirmed an erosive gastritis and it was felt inappropriate to treat with aspirin at the time of the study.

Of the 22 patients initially eligible for phase 2 of the study, 3 were excluded prior to treatment with asasatin retard; one patient who chose to withdraw from the interventional phase of the study as her RP symptoms were well controlled (enrolled during summer) and she did feel treatment was required and two patients who had inadvertently used aspirin in run-in phase of study (as reported in section 6.4.1).

Four patients withdrew from the study due to adverse events related to the study drug (see below for further details). The clinical features of the 15 participants who completed the study are presented in Table 7.3. There were no significant differences between patients with primary RP compared with SSc for age, gender, age at symptom onset, age at diagnosis, smoking history or vasodilator use (Table 7.3). All patients enrolled in the study had lcSSc (possibly reflecting exclusion of all patients receiving corticosteroids). Five patients carried ACA, 2 patients carried anti-Scl-70 and 2 patients carried anti-U3-RNP autoantibodies. The clinical features of the patients enrolled in the study with SSc are presented in Figure 7.2.
Figure 7.2 Clinical features of patients with SSc who completed the study (n=9).

Table 7.3 Baseline demographics of participants who completed the study

All data expressed as median (IQR) unless otherwise stated.
* Mann-Whitney U test for comparing distribution across unpaired groups and Fisher’s exact test to compare frequencies of categorical data

<table>
<thead>
<tr>
<th></th>
<th>Primary RP (n=6)</th>
<th>SSc (n=9)</th>
<th>p value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>7</td>
<td>1.00</td>
</tr>
<tr>
<td>Age in years (IQR)</td>
<td>45.6 (21.3)</td>
<td>53.6 (13.4)</td>
<td>0.07</td>
</tr>
<tr>
<td>Age at RP onset in years (IQR)</td>
<td>18 (23)</td>
<td>35 (10)</td>
<td>0.19</td>
</tr>
<tr>
<td>Age at diagnosis in years (IQR)</td>
<td>42 (17)</td>
<td>40 (13)</td>
<td>0.96</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>0 (0)</td>
<td>1 (11.1)</td>
<td>1.00</td>
</tr>
<tr>
<td>Ex</td>
<td>2 (33.3)</td>
<td>4 (44.4)</td>
<td>1.00</td>
</tr>
<tr>
<td>Never</td>
<td>4 (66.7)</td>
<td>4 (44.4)</td>
<td>0.61</td>
</tr>
<tr>
<td>Mean accrued pack year history (SD)</td>
<td>0 (6.88)</td>
<td>5 (38)</td>
<td>0.328</td>
</tr>
<tr>
<td>Vasodilator therapy (%)</td>
<td>1 (20)</td>
<td>6 (66.7)</td>
<td>0.08</td>
</tr>
</tbody>
</table>
7.4.2 Subjective assessment of RP severity

RCS diary
There was a significant reduction in both the mean daily RCS score (median 2.29 [2.27] vs. 1.11 [1.52], p=0.006) and the frequency of RP attacks (median 2.04 [2.67] vs. 1.32 [2.66] RP attacks per day, p=0.039) following treatment with asasantin retard (Figure 7.3). The duration of RP attacks was not affected by treatment with asasantin retard (median daily duration of RP attacks 20 [52.2] vs. 18.6 [40.6] minutes, p=0.19).

Figure 7.3 Impact of asasantin retard on Raynaud’s Condition Score
The bold lines indicate the median RCS score and boxplots indicate IQR. The whiskers reveal range of data points that sit within 1.5(IQR) of the 1st and 3rd quartiles respectively and equals the range in most instances. Individual outliers are numbered and depicted as circles if their value sits between 1.5(IQR) and 3(IQR) of the quartile. Outliers are depicted as stars if their value is 3(IQR) from the adjacent quartile.

7.4.3 Objective assessment of digital vascular function before and following cold challenge


**IRT assessments**
Descriptive data for thermographic assessment at each ROI and the DDD, at baseline and following cold challenge are presented in Table 7.4. There were no significant changes in skin temperature at any of the 3 regions of interest within the hands or for the DDD for the right 3\textsuperscript{rd} finger, at any time point before or following cold challenge, following treatment with asasantin retard therapy ($p>0.05$ for all comparisons, Table 7.4).

**LSCI assessments**
Descriptive data for LSCI assessment at each ROI, at baseline and following cold challenge, are presented in Table 7.4. There were no significant changes in perfusion flux values after treatment with asasantin retard therapy at any of the 3 regions of interest within the hands, before or following cold challenge ($p>0.05$ for all comparisons, Table 7.5).
**Table 7.4. Effect of 2 weeks asasantin retard therapy on thermographic assessment of digital vascular function in primary RP and SSc**

All values expressed as temperature in degrees Celsius (interquartile range). The distal dorsal difference (DDD) was calculated by subtracting the temperature overlying the dorsal aspect of the right middle MCP from the temperature from ROI 1. B, baseline assessment at 23°C; t 0, assessments immediately following 60s cold challenge at 15°C; t5, perfusion 5 minutes post cold challenge etc.

\[ p > 0.05 \] for all comparisons between perfusion before and after treatment.

\( n = 15 \) unless stated. \( *n = 14 \)

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>t0</th>
<th>t5</th>
<th>t10</th>
<th>t15</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ROI 1</strong></td>
<td><strong>Baseline</strong></td>
<td>25.3 (8.3)</td>
<td>21 (2.6)</td>
<td>24.3 (5.1)</td>
<td>25.5 (8.4)</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>27.9 (7.8)</td>
<td>21.4 (3.5)</td>
<td>23.3 (9.8)</td>
<td>25.8 (10.2)</td>
</tr>
<tr>
<td><strong>ROI 2</strong></td>
<td><strong>Baseline</strong></td>
<td>27.1 (8.6)</td>
<td>21.1 (3.3)</td>
<td>23.4 (4.9)</td>
<td>27.7 (7.8)</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>30.2 (7.0)</td>
<td>22.0 (3.3)</td>
<td>25.5 (7.9)</td>
<td>26.3 (9.8)</td>
</tr>
<tr>
<td><strong>ROI 3</strong></td>
<td><strong>Baseline</strong></td>
<td>28 (8.4)</td>
<td>21.1 (2.7)</td>
<td>23.4 (5.3)</td>
<td>26 (11.8)</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>26.5 (8.5)</td>
<td>21.7 (3.7)</td>
<td>23.4 (8.8)</td>
<td>25.5 (11.3)</td>
</tr>
<tr>
<td><strong>DDD</strong></td>
<td><strong>Baseline</strong></td>
<td>-0.64 (2.93)</td>
<td>-1.6 (2)*</td>
<td>-2.1 (4.5)</td>
<td>-1.6 (5.5)</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>-1.2 (2.8)</td>
<td>-1.3 (2.3)</td>
<td>-2.0 (6.4)</td>
<td>-1.5 (4.7)</td>
</tr>
</tbody>
</table>

**Table 7.5 Effect of 2 weeks asasantin retard therapy on LSCI assessment of digital vascular function in primary RP and SSc**

All values expressed as arbitrary flux units (interquartile range). ROI, region of interest; B, baseline assessment at 23°C; t 0, assessments immediately following 60s cold challenge at 15°C; t5, perfusion 5 minutes post cold challenge etc.

\[ p > 0.05 \] for all comparisons between perfusion before and after treatment.

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>t0</th>
<th>t5</th>
<th>t10</th>
<th>t15</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ROI 1</strong></td>
<td><strong>Baseline</strong></td>
<td>271 (261)</td>
<td>212 (172)</td>
<td>249 (182)</td>
<td>311 (311)</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>364 (289)</td>
<td>254 (172)</td>
<td>271 (370)</td>
<td>349 (413)</td>
</tr>
<tr>
<td><strong>ROI 2</strong></td>
<td><strong>Baseline</strong></td>
<td>147 (408)</td>
<td>89 (39)</td>
<td>122 (231)</td>
<td>161 (286)</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>264 (286)</td>
<td>103 (50)</td>
<td>89 (256)</td>
<td>176 (187)</td>
</tr>
<tr>
<td><strong>ROI 3</strong></td>
<td><strong>Baseline</strong></td>
<td>657 (920)</td>
<td>208 (315)</td>
<td>241 (696)</td>
<td>502 (823)</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>570 (760)</td>
<td>219 (392)</td>
<td>359 (674)</td>
<td>405 (791)</td>
</tr>
</tbody>
</table>
7.4.4 Eicosanoid biosynthesis

There was a reduction in urinary 11-dehydro-TxB$_2$ levels following treatment with asasantin retard (398 [198] vs. 157 [170] pg/mg, p=0.001, Figure 7.4). There was also a significant reduction in urinary 2,3-dinor-6-keto-PGF$_{1\alpha}$ following asasantin retard therapy (147 [93] vs. 135 [130] pg/mg, p=0.017). There was no significant change in urinary F$_2$-isoprostane levels following asasantin retard (median 1.00 vs. 0.938, p=0.39).

**Figure 7.4. Urinary 11-dehydro-TxB$_2$ levels before and following treatment with asasantin retard**

Values expressed as medians (interquartile range). The bold lines indicate the median value and boxplots indicate IQR. The whiskers reveal range of data points that sit within 1.5(IQR) of the 1st and 3rd quartiles respectively and equals the range in most instances. Individual outliers are numbered and depicted as circles if their value sits between 1.5(IQR) and 3(IQR) of the quartile. Outliers are depicted as stars if their value is 3(IQR) from the adjacent quartile.

7.4.5 Light Transmission aggregometry

The results of LTA analyses before and after asasantin retard therapy are presented in Table 7.6. There was a significant reduction in % aggregation (maximum % aggregation and at 5 minutes post-agonist) for all working concentrations of ADP and AA (Table 7.6, p<0.005 for all comparisons). There was a significant reduction in Gmax for aggregation with both working concentrations of AA but not with any working concentration of ADP (Table 7.6).
Table 7.6 Impact of asasantin retard on LTA assessments to varying concentrations of adenosine diphosphate (ADP) and arachidonic acid (AA) in primary RP and SSc

Max%, maximum % aggregation during 5 minutes post agonist; % 5 mins, % aggregation after 5 minutes post agonist; Gmax, maximum gradient of aggregation following addition of agonist. 1° RP, primary Raynaud’s phenomenon. All values expressed as median (IQ range).

§ p<0.005 vs. baseline

<table>
<thead>
<tr>
<th>Agonist and concentration</th>
<th>Adenosine diphosphate</th>
<th>Arachidonic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 μmol/L</td>
<td>10 μmol/L</td>
</tr>
<tr>
<td>Max %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>80 (11)</td>
<td>79 (7)</td>
</tr>
<tr>
<td>2 weeks</td>
<td>68 (7) §</td>
<td>62 (10) §</td>
</tr>
<tr>
<td>% 5 mins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>80 (11)</td>
<td>79 (7)</td>
</tr>
<tr>
<td>2 weeks</td>
<td>62 (10) §</td>
<td>55 (14) §</td>
</tr>
<tr>
<td>Gmax</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>44 (12)</td>
<td>42 (11)</td>
</tr>
<tr>
<td>2 weeks</td>
<td>47 (10)</td>
<td>42 (7)</td>
</tr>
</tbody>
</table>

7.4.6 Circulating plasma biomarkers

Treatment with asasantin retard did not lead to significant changes in levels of sP-selectin (18.1 (4.9) vs. 18.6 (5.7) pg/ml, p=0.21), sCD40L (62.0 (11.2) vs. 58.9 (34.6) pg/ml, p=0.53) or TGF-β (1290.9 (2159.8) vs. 2084.2 (3931.2) pg/ml, p=0.23).
7.4.7 Plasma viscosity and platelet number/structure

There was an apparent weak trend for the PV to fall following treatment with asasantin retard (1.68 vs. 1.61, p=0.13). The HCT, platelet count, PDW, PCT and MPV did not change following treatment with asasantin retard (p>0.05 for all comparisons).

7.4.8 Tolerability, safety and compliance

A list of all adverse events reported in patients receiving asasantin retard is presented in Table 7.7.

Table 7.7 Adverse events reported during trial

Data expressed as n (%) unless stated. Differences between primary RP and SSc evaluated using Fisher’s exact test.

<table>
<thead>
<tr>
<th>Adverse events</th>
<th>SSc (n=11)</th>
<th>Primary RP (n=8)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No adverse events</td>
<td>1 (9.1)</td>
<td>0 (0)</td>
<td>1.00</td>
</tr>
<tr>
<td>Fatigue</td>
<td>1 (9.1)</td>
<td>0 (0)</td>
<td>1.00</td>
</tr>
<tr>
<td>Sebaceous cyst</td>
<td>0 (0)</td>
<td>1 (12.5)</td>
<td>0.42</td>
</tr>
<tr>
<td>Cellulitis</td>
<td>1 (9.1)</td>
<td>0 (0)</td>
<td>1.00</td>
</tr>
<tr>
<td>Facial acne</td>
<td>1 (9.1)</td>
<td>0 (0)</td>
<td>1.00</td>
</tr>
</tbody>
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<table>
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<tr>
<th>Adverse Drug Reactions</th>
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<tr>
<td>Headache</td>
<td>9 (81.8)</td>
<td>7 (87.5)</td>
<td>1.00</td>
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<tr>
<td>Possible allergic reaction</td>
<td>0 (0)</td>
<td>1 (12.5)</td>
<td>0.42</td>
</tr>
<tr>
<td>Loose stool</td>
<td>1 (9.1)</td>
<td>0 (0)</td>
<td>1.00</td>
</tr>
<tr>
<td>Dyspepsia</td>
<td>1 (9.1)</td>
<td>0 (0)</td>
<td>1.00</td>
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<table>
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<tr>
<th>Serious Adverse Events</th>
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<tbody>
<tr>
<td>Oesophageal obstruction</td>
<td>1 (9.1)</td>
<td>0 (0)</td>
<td>1.00</td>
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<table>
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<tr>
<th>Withdrawn from study</th>
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<tbody>
<tr>
<td>Total number of withdrawals</td>
<td>2 (18.2)</td>
<td>2 (25)</td>
<td>1.00</td>
</tr>
<tr>
<td>Headache</td>
<td>2 (18.2)</td>
<td>1 (12.5)</td>
<td>1.00</td>
</tr>
<tr>
<td>Possible allergic reaction</td>
<td>0 (0)</td>
<td>1 (12.5)</td>
<td>0.42</td>
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There were no significant differences between AE event rates between primary RP and SSc. Only one participant completed the study without experiencing any new AEs. Headaches were the commonest reported ADR affecting 84.2% (16/19) of participants. The frequency of headaches was similar in primary RP and SSc (p=1.00). The mean headache severity was
5.3/10 when patients felt able to quantify (n=14 of 16 participants reporting headache). Three patients (15.8% of all participants) were withdrawn from the study due to headaches within the first 2 days of administration. In 7 of the participants reporting headache (43.8%), symptoms improved with repeat dosing and had resolved by the second week of therapy. Six patients experiencing headache (37.5%) reported symptoms persisting throughout the 2 week study period, although some reduction in frequency/severity of headaches was reported in 2 of these participants. One patient (5.3%) was withdrawn on day 2 after reporting symptoms consistent with hypersensitivity (globus sensation in throat). Hypersensitivity to asasantin retard is a commonly reported side effect affecting between 1 and 10% of recipients. Only one patient (5.3%) experienced dyspepsia after the first dose and was withdrawn after experiencing a severe headache after her second dose. One patient (5.3%) reported a single episode of diarrhoea. One patient with lcSSc and a longstanding history of upper gastrointestinal dysmotility experienced possible oesophageal obstruction and was admitted to hospital (constituting a serious adverse event). A therapeutic upper GI endoscopy was undertaken which appeared to resolve the blockage and excluded an associated upper GI bleed. There were no reported bleeding events and the Hb did not fall by greater than 1.0g/dL in any patient. Similarly, the median Hb level was similar before and following treatment with asasantin retard (13.0g/dL vs. 12.7g/dL, p=0.068). The serum creatinine did not rise above normal range for any patient following treatment and there was no significant difference between the median serum creatinine before and after asasantin therapy (n=14, 65 vs. 64.5, p=0.699).

7.4.9 Compliance monitoring

At the second assessment, participants were asked to disclose any missed doses on the RCS diary. Further enquiries were made about missed doses at the final assessment. In addition, the unused capsules were counted back by the pharmacists (n=32 for full compliance). Of the 15 patients who completed the study, 7 (46.7%) denied any missed doses and returned the correct number of capsules. Two patients admitted missing 1 and 3 capsules respectively, which was confirmed on recording number of capsules returned. One patient reported missing a single dose due to headache, but capsule counting confirmed a total of at least 8 missed doses. Another patient admitted 1 missed dose but returned 3 more capsules than expected. Three patients denied any missed doses but returned 1 more capsule than expected. One patient admitted omitting a single dose but returned fewer capsules than expected (n=29). She had inadvertently discarded some capsules in error and denied taking additional treatment to account for this discrepancy.
7.5 Discussion

The results of this study do not provide compelling evidence to support the use of asasantin retard in primary RP and SSc. We identified improvements in RP severity assessed using the RCS diary parameters but this may be secondary to the influence of the placebo effect and/or diary fatigue. The high prevalence of headaches suggests a vasodilatory effect but may be secondary to confirmation bias as patients were provided with verbal and written warning them of this potential side effect of treatment with asasantin retard. A double-blinded placebo-controlled cross-over study would have negated the influence of such bias and was originally considered for the study design. The over-encapsulation process required to manufacture identical placebo and active treatments would have created very large capsules and influenced tolerability. An alternative method we considered was to provide a separate combination of low-dose aspirin 25mg and modified release dipyridamole (and matching placebos). This approach would have increased the number of tablets and may also have affected tolerability and compliance. Each approach would have necessitated additional assessment visits which may have affected recruitment. I justified the open-label study design, despite the many inherent limitations, due to the objective nature of the majority of endpoints chosen for assessments. Another major limitation of the study was the study size. In chapter 1, I was critical of previous trials of anti-platelet agents due to the small study size and use of a heterogeneous mix of patients with primary RP and SSc. And yet, in this chapter, I have reported the findings of an equally small study and undertaken primary analysis using pooled data from patients with primary RP and SSc. As a feasibility study, we have demonstrated the challenges recruiting patients with primary RP and SSc to a single centre investigator-led study of anti-platelet therapy. We approached almost 200 potential patients for inclusion of the study and had expected interest to be higher. Enrollment was also influenced by strict eligibility criteria which prevented patients receiving NSAIDs, antiplatelet agents or steroids from entering the study. This accounted for a large proportion of our patients, particularly those with dcSSc and/or SSc-associated inflammatory arthropathy who were under-represented in this study. Other factors that may have contributed to low enrollment (some of which were expressed by potential participants) include low patient perception of likely treatment efficacy, need for invasive blood tests and an unnecessary concern regarding the use of “laser-derived” imaging modalities. Whilst I failed to achieve my original sample size calculation, the study was evidently adequately powered to identify improvements in the RCS diary parameters, although the relevance of the improvements we
identified is questionable as outlined above. Nonetheless, we would have preferred the study to be larger.

Despite these limitations, there were strengths to our study which has enhanced our understanding of the effects of anti-platelet therapy and the pathogenesis of peripheral vasculopathy in primary RP and SSc. I originally hypothesised that this combination of agents would promote basal digital vascular perfusion through several pathways including adenosine-mediated vasodilation, potentiation of local NO and reduced circulating vasoconstrictors such as thromboxane. There was no evidence to support this from the microvascular imaging data. I had also hypothesised that vasoconstriction to local cold challenge would be reduced following anti-platelet therapy due to reduced cold-induced platelet activation and local release of vasocontractors. Primary analysis of both thermographic and LSCI data failed to support this hypothesis which may represent a true lack of contribution of platelets in mediating cold-induced digital vasoconstriction. Irreversible morphological vascular changes in SSc may have blunted microvascular responses to intravascular factors in SSc. Previous studies have successfully used laser-derived methods to demonstrate the vasodilator effects of anti-platelet therapy in RP. For example, Easter et al. were able to demonstrate increased endothelium-dependent vasodilation in patients with primary RP (n=15) following aspirin therapy using laser doppler flowmetry (Easter and Marshall, 2005). In this study, a higher dose of aspirin was used (600mg) and the immediate vascular effects were assessed on the day of drug administration. I raised concerns regarding reproducibility of siting laser doppler probes in chapter 1, however this study benefited from assessing immediate changes in blood flow 30 minutes post-administration. We could have incorporated a similar assessment after administration of the first dose of asasantin retard although this would have been at the expense of the LTA studies which were undertaken by myself immediately following each assessment visit.

We demonstrated predictable effects of asasantin retard therapy on LTA, providing ex vivo confirmation of the influence of asasantin retard on platelet activation to ADP and AA. This confirmed that platelets were not resistant to the effects of treatment with anti-platelet therapy in primary RP and SSc. We also demonstrated an expected reduction in in vivo thromboxane biosynthesis. Nonetheless, the magnitude of the reduction in thromboxane synthesis was lower than I had anticipated. Previous studies have demonstrated reductions in thromboxane synthesis of 85-90% in stroke patients following low dose aspirin 40mg daily (Tohgi et al.,
In the present study, total thromboxane biosynthesis was reduced by approximately 60% of pre-treatment levels. This suggests higher than expected *in vivo* platelet activation despite anti-platelet therapy. Possible causes may include endothelial damage and high circulating levels of other platelet activators (such as the isoprostanes). We were also interested in the effects of asasantin retard therapy on prostacyclin production. Low-dose aspirin is generally thought to be insufficient to reduce COX-derived endothelial prostacyclin biosynthesis (Tohgi et al., 1992). Dipyridamole is thought to increase endothelial prostacyclin production (Kim and Liao, 2008). Furthermore, it has been noted that COX inhibition can shunt arachidonic acid down alternative pathways (Dufield-Llillico et al., 2009). Consequently, I hypothesised that we may identify an increase in urinary 2,3-dinor-6-keto-PGF$_{1\alpha}$ levels following asasantin retard therapy but I actually identified a significant reduction. This may be a consequence of endothelial dysfunction in RP. The combined effects of a suboptimal reduction in thromboxane biosynthesis, further offset by an unexpected reduction in endogenous prostacyclin production may have contributed to the lack of objective vasodilatory response to treatment with asasantin retard. Sakamoto et al. identified differing pulmonary vascular responses to cold challenge in patients with secondary RP (10 MCTD, 4 SSc) (Sakamoto et al., 1999). They identified an imbalance in thromboxane and prostacyclin biosynthesis favouring vasoconstriction in patients who exhibited a pulmonary vasopressor response to local cold challenge (Sakamoto et al., 1999). This suggests the contribution of platelets and eicosanoid biosynthesis may differ across patients with similar underlying conditions. I attempted a similar *post hoc* analysis in our patients but could not identify any trends to suggest a similar phenomenon in our cohort (data not presented).

Dipyridamole is thought to have anti-oxidant effects but we could not identify any change in urinary F$_2$-isoprostane levels following asasantin retard. Isoprostanes have been used successfully when assessing the efficacy of antioxidants such as vitamin E in clinical studies of cystic fibrosis, diabetes mellitus and homozygous homocysteinuria (Cracowski et al., 2002b). Similarly, urinary F$_2$-isoprostane levels have been used to demonstrate the anti-oxidant properties of established vasodilatory therapy such as iloprost in SSc (Erre and Passiu, 2009, Erre et al., 2008).

We were unable to demonstrate any reduction in circulating plasma levels of biomarkers of platelet function (sP-selectin, TGF-β and sCD40L). There was a trend for the PV to fall
following treatment but we could not present any convincing data to support a role for anti-platelet agents in potentially augmenting pro-inflammatory or pro-fibrotic pathways.

### 7.6 Conclusions

In summary, we have been unable to provide compelling evidence of improved digital vascular function following asasantin retard in primary RP and SSc. We have highlighted the challenges in undertaking single-centre proof-of-concept studies of this nature. We need a validated standardised approach to digital microvascular assessment that can be adopted across several specialist centres to support future studies of novel potential vasoactive mediators in RP. We have identified some interesting observations regarding the effects of anti-platelet therapy on eicosanoid biosynthesis in RP that would benefit from further work involving larger numbers of patients. Large cross-sectional studies incorporating both patients taking anti-platelet agents (for other indications) and those not receiving anti-platelet therapy may be the easiest way to undertake such work in the first instance. I shall address the important issue of whether the findings of this study should dissuade us from undertaking further work exploring the role of anti-platelet agents in primary RP and SSc in the next and final chapter.
Chapter 8 - Discussion

‘…and may the struggle continue until this most obstinate diseases is made to give up its secrets.’

Professor Gerald P Rodnan (1927-83)

8.1 Introduction

I have discussed the outcomes, limitations and the relevance of my findings in the context of previous research in the respective discussion sections of the previous chapters. I will not replicate such discussion here but shall attempt to bring together the broader aspects of my findings with a strong emphasis on the implications for future research.

8.2 Challenges surrounding patient-reported assessment of peripheral vascular function in RP and SSc

Assessment of peripheral vascular function, both clinically and objectively assessment using mirovascular imaging tools, is essential in the diagnosis of RP and SSc. The initial clinical diagnosis of RP typically relies upon a characteristic history, often in the absence of clinical signs. A history of increased sensitivity to cold in conjunction with biphasic digital colour changes has been proposed as the minimum requirement for a definite diagnosis of RP (Brennan et al., 1993). Indeed the presence of characteristic digital colour changes forms a central component of the classification criteria for early SSc (LeRoy and Medsger, 2001). In this thesis, I have highlighted specific limitations of patient self-report of RP severity using the RCS diary. Such limitations of self-report may extend to subjective assessment of digital vascular colour changes in possible RP. Vasoconstriction is an important component of healthy thermoregulation and we all experience a degree of visible digital colour change in response to cold exposure if the stimulus is sufficient. Such observations led an early 20th Century physician to dryly note ‘we are all subjects of Raynaud’s phenomena to a greater or lesser degree’ (Hutchinson, 1901). There are notable differences in psychosomatic testing in patients with primary and secondary RP that may result in over-reporting and under-reporting of symptoms in primary RP and in SSc respectively (Bayle et al., 1990). Such factors may account for the high reported prevalence of RP symptoms (~30%) in patients with fibromyalgia syndrome (FMS); a syndrome of sensitization of central pain pathways that
would not be expected to manifest with a peripheral vasculopathy. Indeed, the presence of true peripheral microvascular dysfunction in FMS would provide an interesting hypothesis for sympathetic medicated pain in this condition. The poor correlation between subjective and objective assessment of digital vascular function in patients with FMS would suggest "true" RP is not a feature of FMS (Dinerman et al., 1986, Bennett et al., 1991). It is my own conviction that such patients experience and report "cold intolerance" and objective assessment of digital microvascular function may help identify such patients. Similar issues may arise in primary RP and may explain the large variation we have identified in previous work looking at peripheral microvascular function in primary RP (mean DDD at 23°C = 1.79 with a standard deviation of 2.6) (Pauling et al., 2011b). In future, greater emphasis may be placed on objective assessment of microvascular dysfunction when diagnosing primary RP. This would have clear therapeutic implications, as "cold intolerance" in the absence of microvasculopathy would be expected to respond poorly to vasoactive medication whereas neuromodulatory agents may be highly effective at lessening the impact of such symptoms.

Surprisingly few studies have validated self-report of colour changes along side objective assessment of digital vascular function. We have commenced work evaluating self-reported colour changes in patients undergoing thermographic assessment of RP symptoms to examine the relationship between underlying diagnosis, self-report of colour changes and objective assessment of digital vascular function. This work may provide a platform for future studies designed to develop better methods for differentiating "true" RP from "cold intolerance" which would have obvious implications for the management of these potentially pathologically distinct conditions.

8.3 The need for improved peripheral vascular endpoints in therapeutic trials of RP and SSc

An additional important reason for assessing digital vascular function is to evaluate treatment efficacy, both in clinical practice and therapeutic trials of RP. Great strides have been made to refine clinical disease activity scores in other rheumatic disorders such as the successful application of the DAS-28 assessment in rheumatoid arthritis (RA). These tools have become invaluable in drug development and guiding clinicians in treatment decisions concerning the use of novel expensive therapies such as biological agents. In terms of outcome measures,
SSc is perhaps 20 years behind RA; in no small part due to the complexities associated with measuring disease activity in a slowly progressive clinically heterogeneous disease.

At present, only the RCS diary has been recommended for use in the assessment of peripheral vascular function in therapeutic trials of RP (Merkel et al., 2003, Khanna et al., 2008). Perhaps this is appropriate in the evaluation of treatments for primary RP, where the primary goal of treatment is to reduce symptom severity, and the vascular insult is generally insufficient to cause ischemic necrosis of the digits or vascular compromise in other organs. Even so, the magnitude of the placebo effect will necessitate large studies to confirm the superior efficacy of active treatment in placebo-controlled studies. The design of such studies would also need to consider the impact of seasonal variation on RCS diary outcomes, which may be a strong rationale for undertaking short-term rather than long-term studies.

In SSc, confining assessment of vasoactive treatments to RP symptoms has major limitations. We have noted specific issues arising from application of the RCS diary in SSc such as habituation, coping strategies and difficulty discerning discreet RP attacks from background vascular dysfunction secondary to irreversible morphological microvascular abnormalities. Such factors are likely to lead to under-reporting of RP symptoms in SSc. Figure 8.1 presents the visual appearance and corresponding objective digital microvascular assessments in a patient with long-standing ACA-positive lcSSc, complicated by recurrent DU and digital necrosis, who took part in my study. Despite compelling objective evidence of profound peripheral vascular dysfunction, the patient reported relatively mild symptoms (Figure 8.1). In this example, as across the group as a whole, there was no relationship between subjective and objective assessment of peripheral vascular function. I have previously used the findings of studies evaluating ERA therapy in SSc as an example of the disparity that can emerge between subjective and objective appraisal of the treatment efficacy (Nguyen et al., 2010, Rosato et al., 2010). The lower incidence of SSc-PAH in patients receiving dihydropyridine calcium antagonists provides a tantalizing glimpse into the potentially disease-modifying potential of modest vasodilator therapy on long-term outcomes in SSc (Steen and Medsger, 2003). It is possible only objective microvascular imaging is capable of identifying such modest improvements in peripheral vascular outcomes. The use of such tools may facilitate proof-of-concept studies examining the pleiotropic effects of widely available, cheap and safe treatments. The potential long-term impact of such drugs, even in the absence of notable beneficial effects of RP severity, should not be ignored.
Figure 8.1 Objective assessments of digital vascular function in a patient with lcSSc complicated by recurrent digital ulceration

The photograph demonstrates the visual appearance of the hands on arrival for assessment with evidence of digital shortening of index fingers due to previous necrotic episodes. The thermographic image was taken during the baseline of digital vascular function prior to cold challenge. The laser speckle contrast images taken at 13-second intervals during baseline and following cold challenge demonstrate poor perfusion of the digits at baseline and following cold challenge. Despite the convincing and consistent objective evidence of digital microvascular compromise, the same patient reported a SHAQ RP-VAS of 0.0/3.0, a mean daily RCS score 0.21/10, mean daily frequency of RP attacks of 2.36 and mean daily duration of RP attacks of 28.86 minutes which is consistent with relatively mild RP symptoms.
The work reported in this thesis has evaluated IRT and LSCI, but neither method has emerged as superior to the other. I have not considered other non-invasive methods for assessing cutaneous perfusion. A multicentre collaborative grant application has been submitted to Arthritis Research UK to evaluate IRT and LDI in SSc. This work will be an important step in validating such methods for future clinical trials. In Chapter 3, I have highlighted the need for a consensus statement from interested stakeholders to devise a standardised approach to thermographic protocol and endpoints in future clinical trials of RP. This will facilitate easier comparison of the efficacy of drugs across studies. The thermographic DDD (Clark et al., 1999, Anderson et al., 2007), which has been successfully applied to the classification of RP, may be similarly successful as an endpoint for use in therapeutic trials. There is great enthusiasm amongst members of the UK Scleroderma Study Group to undertake multicentre collaborative studies to work towards these objectives in the future.

8.4 Further validation of LSCI as a novel microvascular imaging tool in RP and SSc

The work I have undertaken thus far has concentrated on validating LSCI as a method for assessing digital microvascular reactivity (as a proxy for RP severity). I chose the cold challenge to facilitate dynamic assessment of microvascular function but other methods for testing autonomic function and vascular reactivity exist that I have not yet considered. Similarly, additional work is required to evaluate the clinical application of LSCI to assess other cutaneous microvascular abnormalities that occur in SSc including ischaemic cutaneous lesions (ranging from digital ulceration [DU] to gangrene) and telangiectasia. LSCI could be used to further evaluate the association between microvascular dysfunction and cutaneous fibrosis in SSc. In this section, I shall outline future research directions that will allow us to realise the full potential of LSCI in the assessment of RP, particularly in the context of SSc.

8.4.1 Dynamic assessment of digital vascular function
The cold challenge assessment

There are many methods for assessing peripheral vascular reactivity in humans. I have undertaken dynamic assessment of microvascular function using a local cold challenge. On face value, the cold challenge is attractive in its attempt to recreate the local conditions responsible for attacks of RP in vivo although it is not without its limitations. Most notably, few patients with RP actually experience what they would consider an attack of RP during the study, and the local cold challenge is more accurately an assessment of vascular reactivity to local cold exposure. In SSc, the cold challenge assessment assess the combined effect of reversible excessive vascular reactivity to cold and the irreversible obliterative miroangiopathy that occurs over time. A specific limitation of a thermographic cold challenge (upon which much of the existing literature exists) is the influence of conductive and convective heat transfer during the test itself (e.g. heat loss into water bath during cold challenge and heat gain from deep tissues during re-warming) on changes in skin temperature, which are generally attributed solely to changes in microvascular perfusion. The reproducibility of the cold challenge has been questioned (Herrick and Clark, 1998) and studies have identified better reproducibility of baseline assessments compared with those following cold challenge (Bartelink et al., 1993). The additional time considerations of a well-conducted cold challenge (between 30 and 60 minutes depending on protocol) is another limiting factor of the cold stress test.

Nonetheless, the cold challenge unquestionably provides useful information on vascular reactivity that can not be derived from static assessment alone, and further work should refine microvascular endpoints derived from the cold challenge and better define what these tell us about digital vascular structure and reactivity.

Other methods for the dynamic assessment of digital microvascular function

Other methods for dynamically assessing digital microvasculature function have been developed but not yet assessed using LSCI in RP and SSc. The warm challenge (e.g. 30°C waterbath) provides information on maximal vasodilatation, and has been incorporated as an initial step in subsequent cold challenges tests to assess vascular dysfunction in RP and SSc (Clark et al., 1999, Anderson et al., 2007). The iontophoresis of vasodilator chemicals has been used for LDI assessment of endothelial-dependent and endothelial-independent vasodilation (Anderson et al., 2003). LSCI could be similarly applied to these techniques and
may provide new insights into cutaneous microvascular abnormalities in RP and SSc. An initial observation of mine was the sensitivity of LSCI to stressors of autonomic nervous system function. For example, sneezing, deep breaths and emotion all affected the LSCI trace. LSCI may facilitate assessment of central and peripheral autonomic neural control of peripheral vascular function in primary RP and SSc. Moreover, LSCI may be more sensitive than existing methods for assessing autonomic dysfunction in humans such as heart rate variability and respiratory analysis.

There are other methods for assessing vasoreactivity and since we commenced our work, another group have published a study evaluating the discriminatory capacity of LSCI to differentiate between healthy controls, primary RP and SSc after dynamic tests of vascular function including the local cold challenge and post-occlusive reactive hyperaemia (PORH) (Della Rossa et al., 2012). This is an emerging field and other studies are underway evaluating LSCI in the dynamic assessment of digital vascular function in patients with RP and SSc (e.g. use of the PORH test in the ongoing SPECIES study [NCT01743612]).

8.4.2. Comparison of LSCI with other objective methods for assessing tissue perfusion

I have undertaken preliminary validation work of LSCI alongside the “silver standard” IRT. We are the first to undertake such work in RP and SSc. In section 1.5.4 I have outlined previous studies that have compared LSCI with other microvascular imaging modalities including NC, LDF and LDI. Only one study to date has compared LSCI with laser Doppler techniques in RP and SSc (Murray et al., 2009). This limited assessment with LSCI to the nailfold. Additional work is required to compare these techniques over broader regions of interest in view of the differing tissue penetration of LSCI over laser Doppler techniques. Without such work, it is difficult at this stage to comment as to which technique will emerge as superior. Both have advantages and limitations leading to my personal conviction that specific roles may be identified for each of these complementary techniques in the assessment of SSc.

8.4.3 Use of LSCI to assess natural history of DU progression and treatment response in SSc

To date, no studies have used LSCI to explore the natural history of DU progression and healing in SSc. LSCI has been successfully applied to the assessment of burns and may help to guide initiation of treatment and assessment of treatment efficacy of DU in SSc (Stewart et
al., 2005). A study of DU progression in SSc would facilitate development of LSCI (and IRT) parameters for the assessment of vasodilatory treatment efficacy as many of these patients go on to receive intravenous iloprost therapy. A study of this nature should include simultaneous assessment with other techniques (most notably IRT) and would be an important step in developing existing and novel endpoints for clinical trials and to justify ongoing use of costly therapies such as ERA therapy in SSc. A critical component of such a study would be to establish whether vasoactive therapies such as iloprost have any effects on peripheral vascular function in patients late in the course of SSc when the reversible microangiopathy has evolved into an obliterative vasculopathy with capillary loss and relative avascularity of affected tissues.

**8.4.4 Use of LSCI to assess telangiectasia morphology and function in SSc**

Telangiectases are benign cutaneous vascular malformations that are present in the skin of the majority of patients with SSc. They are an important cause of iron deficient anaemia when present within the gastrointestinal tract of patients with SSc. Cutaneous matt telangiectases are harmless but are associated with body image dissatisfaction in SSc (Ennis et al., 2013). Objective assessment of these dilated post-capillary venules may provide important clues on the pathogenesis of SSc. Novel treatments are under development to better manage telangiectases and LDI has been successfully used to assess treatment efficacy (Murray et al., 2012). No studies to date have used LSCI in the assessment of cutaneous telangiectases in SSc.

**8.4.5 Use of LSCI to explore the relationship between microvascular disease and cutaneous fibrosis in SSc**

The pathogenesis of cutaneous tissue remodelling and fibrosis in SSc is complex. LeRoy proposed a vascular hypothesis whereby microvascular dysfunction was an essential prerequisite to subsequent tissue fibrosis in SSc. In support of this paradigm, it had been noted that RP symptoms predate other clinical manifestations by several years in the majority of patients with SSc. Histological studies of skin biopsy material have helped delineate the temporal sequence of the key events that lead to tissue fibrosis. The initiating event is thought to be microvascular injury, perivascular inflammation involving cells of the innate and adaptive immune systems, secretion of extracellular factors that lead to remodelling of the
extracellular matrix, activation of fibroblasts/differentiation to myofibroblasts with subsequent increased collagen formation and tissue fibrosis. Against the “vascular hypothesis” is the paucity of evidence that vasoactive therapy can slow the progression of tissue fibrosis in SSc and the rapid progression of cutaneous fibrosis typical of patients carrying anti-RNA polymerase antibodies within weeks of the onset of RP symptoms (Cavazzana et al., 2009). Similarly, some patients with profound microvascular dysfunction never go on to develop skin fibrosis (Poormoghim et al., 2000) whereas other patients can develop significant fibrosis in the apparent absence of significant microcirculatory abnormalities. There are many other factors beyond microvascular dysfunction contributing to self-sustaining fibroblast activation in SSc and it is likely the exact contribution of pathological factors differs across the disease spectrum, with common pathways found within specific serological sub-groups of SSc (and their characteristic clinical phenotype).

I have previously discussed clinical assessment of cutaneous fibrosis in SSc using the validated modified Rodnan Skin Score [mRSS] (Clements et al., 1993, Clements et al., 1995). There are limitations to the mRSS. Firstly, it is a measure of disease severity and not activity. For example, it lacks the sensitivity to quantify specific components of cutaneous anatomy e.g. dermal thickness. Observer subjectivity on dermal thickness leads to unacceptably high levels of inter and intra-observer variability (25% and 12% respectively) for the purposes of small short interventional studies (Clements et al., 1995). The mRSS lacks the sensitivity to identify potentially important sub-clinical skin changes in unaffected skin and/or identify changes in short-term therapeutic studies. Finally, the mRSS loses discriminatory capacity when used in mixed cohorts of lcSSc and dcSSc (e.g. a score of 11/51 can describe a patient with mild dcSSc or severe lcSSc). Objective methods for assessing skin disease in SSc have been developed (e.g. plicometers, durometers, elastometers and cutometers) and used successfully in cross-sectional studies and clinical trials of SSc. These methods have been comprehensively described in a recent review (Moinzadeh et al., 2012) and whilst they overcome some of the limitations of clinical palpation, they also generally lack the sensitivity to identify sub-clinical changes in cutaneous re-modelling. For this reason, recent attention has focussed on methods that facilitate direct visualisation and quantification of cutaneous structure and function. High frequency ultrasound (HFUS) is a novel method for quantitative assessment of skin abnormalities in SSc. Initial studies applying HFUS to measure skin thickness easily distinguished between SSc and healthy controls (Serup, 1984, Akesson et al., 1986, Sedky et al., 2013). Measurements of the depth of dermis and subcutis can be
made alongside quantitative assessment of the echogenicity of the cutaneous tissues to provide information on the nature and extent of tissue remodelling that is present. Early pilot work reported good correlation between HFUS and assessment of skin thickness using palpation (Scheja and Akesson, 1997). In contrast, recent work evaluating HFUS in the digits of patients with SSc have identified poor correlation between dermal thickness and mRSS scores at the same sites (Di Geso et al., 2011). Correlation between skin thickness/echogenicity and fibroblast proteoglycan biosynthesis within biopsy specimens of assessed skin have been identified (Hesselstrand et al., 2002). Differences in HFUS appearances have been identified between lcSSc and dcSSc affected skin (Hesselstrand et al., 2008, Hesselstrand et al., 2002). HFUS assessment of skin thickness and echogenicity at the hand (between the second and third MCP joints of the right hand) and forearm (dorsal aspect, 3cm proximal to the wrist) correlates well with both local and global mRSS assessment, indicating assessment of a single site may provide an accurate reflection of total skin involvement (Hesselstrand et al., 2008). HFUS assessment of skin thickness can accurately discriminate between patients in the oedematous, fibrotic and atrophic phases of the disease (Kaloudi et al., 2010). Reliability of HFUS is better than the mRSS for assessing skin changes in SSc with excellent intra and inter-relater reliability (Scheja and Akesson, 1997, Kaloudi et al., 2010, Di Geso et al., 2011). An attempt have been made to use HFUS assessment of skin thickness and echogenicity in a therapeutic trial of SSc (Hashikabe et al., 2005). HFUS has also been used to assess tendon thickening in SSc (Tagliafico et al., 2011). HFUS can be adapted to evaluate tissue elasticity (Iagnocco et al., 2010, Di Geso et al., 2011). Ultrasound elastography (UE) has been used in oncology to help detect malignant lesions. Only one study has evaluated UE in patients with SSc, identifying dermal abnormalities in elasticity in clinically affected and unaffected skin (Iagnocco et al., 2010).

HFUS has also been used to assess microvascular changes at the nailfold in SSc and compared changes with those directly visualised using nailfold capillaroscopy (Keberle et al., 2000, Kim et al., 2008, Lee et al., 2006). Combining HFUS with power Doppler would provide a novel method for assessing cutaneous microcirculatory abnormalities such as telangietcasia. To date, no studies have used LSCI alongside HFUS to explore the relationship between microvascular abnormalities and fibrosis in affected and unaffected skin in SSc in vivo. Such work would provide fascinating insight into shared and divergent pathological pathways across the spectrum of SSc disease.
8.4.6 Use of LSCI to explore cutaneous microvascular abnormalities in animal models of SSc

Animal models have been developed to replicate the clinical phenotype of SSc to further understand the pathobiology of the disease and as a platform for pre-clinical proof-of-concept studies of novel therapeutics. I shall not provide a detailed description of the many available models that have emerged which have been reviewed elsewhere (Denton, 2012). In brief, early animal models such as the tight skin mouse 1 (Tsk1) focussed on cutaneous fibrosis. A second tight skin mouse (Tsk2) had an associated inflammatory phenotype with autoantibody formation that more closely mirrored human SSc. A well defined abnormality of TGF-β signalling was not a feature of either of these models which led to the development of a novel mouse model in which fibroblast-specific expression of a non-signalling TGF-β receptor (the TβRIIΔk-fib mouse) leads to features closely mirroring SSc including perivascular fibrosis and pulmonary vasculopathy (Denton et al., 2003, Derrett-Smith et al., 2010). The emergence of the Fra-2 transgenic mouse strain is an exciting new model which displays a clinical phenotype encompassing the pathological hallmarks of SSc; peripheral vasculopathy, inflammation and fibrosis (Maurer et al., 2009). In addition to genetically modified animal models of SSc, the clinical features of SSc can be induced experimentally e.g. hypoxia-induced PAH and bleomycin-induced fibrosis in susceptible mouse strains.

The use of imaging modalities provides an opportunity to evaluate the progression of fibrosis and vasculopathy in animal models of SSc non-invasively. Recent work has used HFUS to assess skin fibrosis in genetic and experimentally induced murine models of SSc (Elhai et al., 2013). The use of LSCI to assess peripheral vascular function in animal models of SSc has not yet been attempted but would has the potential to allow the dynamic assessment microvascular abnormalities in vivo. This could greatly improve the sensitivity of pre-clinical proof of concept studies of novel therapeutic agents in SSc.

8.5 The contribution of platelets to the pathogenesis of primary RP and SSc

The last few years has seen an re-emergence of interest in platelets and the pathogenesis of SSc. For example, Kahelah and colleagues have recently presented work at the American
College of Rheumatology meeting suggesting platelet-derived thrombospondin-1 may be an important contributor to endothelial cell apoptosis in the vasculopathy of early SSc (Kahaleh, 2012b). Improving our understanding of the contribution of platelets to the pathogenesis of RP and SSc was the overarching basis of this thesis. We have identified interesting differences in platelet function between primary RP and SSc. The work undertaken in this thesis has not allowed us establish whether excessive platelet activation is an important initiating event in the pathogenesis of these conditions or a secondary event of no pathobiological significance. There are many areas of platelet biology that are yet to be explored in RP and SSc which are beyond the remit of this discussion. In this section, I shall outline future research directions that may help us further establish the action of platelets (and endothelial cells) in RP and SSc, before offering final thoughts on the future use of anti-platelet agnets on RP and SSc.

8.5.1 Eicosanoid biosynthesis in RP and SSc

The vasoactive properties of eicosanoids, particularly the prostanoids is well established and they have an important role in the management of peripheral and pulmonary vascular complication of SSc (Kowal-Bielecka et al., 2009). In recent years attention has shifted to the role of eicosanoids in fibrosing conditions such as SSc and IPF. There are 2 major families of eicosanoids; the prostanoids produced following COX activity and leukotrienes/lipoxins formed by lipoxygenase (LOX) activity (Figure 8.2). The prostanoids PGE\textsubscript{2} and PGI\textsubscript{2} are antifibrogenic, but their local effects may be diminished at sites of fibrosis (e.g the lungs in IPF) by the competing more potent pro-fibrotic effects of PGF\textsubscript{2} (Castelino, 2012). Both PGE\textsubscript{2} and PGI\textsubscript{2} analogues have been shown to prevent experimentally-induced lung fibrosis in murine models (Castelino, 2012). Similarly, LOX activity leads to the biosynthesis of leukotrienes and lipoxins with opposing profibrotic antifibrogenic actions (Castelino, 2012, Chwiesko-Minarowska et al., 2012). Bronchoalveolar lavage (BAL) levels of profibrotic LTB\textsubscript{4} and LTE\textsubscript{4} are elevated in SSc-ILD compared with SSc without ILD and healthy controls (Castelino, 2012). Furthermore, LOX12/15 expression and activity differs between patients with SSc, RA and HC (Endo, 2012, Castelino, 2012). Genetic polymorphisms of LOX activating protein may enhance the susceptibility to ILD in SSc (Kowal-Bielecka, 2012).
My work has concentrated on prostacyclin, thromboxane and isoprostane biosynthesis in relatively small numbers of patients with primary RP and SSc. Nonetheless, we identified some interesting associations between urinary levels of these biomarkers with peripheral vascular function and seasonal variation. We also paradoxically identified significantly greater urinary 2,3-dinor-6-keto-PGF$_{1\alpha}$ levels in patients with SSc-ILD compared to patients with SSc without ILD. We would like to undertake additional work in larger numbers of patients, and with a larger variety of urinary eicosanoids (including products of LOX) to further evaluate the relationship between eicosanoid biosynthesis and vascular and fibrotic phenotypes in SSc.
8.5.2 Use of anti-platelet agents in primary RP and SSc

The original premise of this thesis was to evaluate the role of anti-thrombotic therapy in RP and SSc. My short-term open-label study of asasantin retard has not helped to establish a proof-of-concept for anti-platelet therapy in primary RP and SSc. In the presence of a good therapeutic rationale but in the absence of a good evidence base, the use of low-dose aspirin has been proposed for patients with SSc and severe digital ischaemia (Wigley, 2012). This pragmatic approach seems intuitive but does not consider the breadth of anti-platelet treatments available and the combination thereof that offers the greatest therapeutic potential. We chose the combination of asasantin retard and dipyridamole due to the vasodilatory potential of this combination.

This use of anti-platelet therapy in SSc may be greatly influenced by emerging evidence of increased macrovascular disease in SSc (Man, 2012). Greater recognition of macrovascular disease in SSc may increase the use of anti-platelet therapy in patients with established macrovascular abnormalities but is unlikely to extend to the use of anti-platelet agents early in the course of the disease where their impact could be greatest.

My attempts of developing a proof-of-concept for asasantin retard use within the context of a short-term interventional study illustrate the challenges facing researchers attempting work of this kind. The strict exclusion criteria and possible patient-perception on likely efficacy had a major impact on our ability to recruit to this study. The optimal study would be a long-term multicentre study including large numbers of patients with SSc. The costs of a study of this nature make it very unlikely that such work will ever be undertaken. We were unable to extend this study to additional participating centres because current microvascular imaging set-up across centres has yet to be standardised to a level whereby this would be feasible. I believe proof-of-concept studies of this nature should initially concentrate on the immediate vasoactive effects of a single-dose of therapy. Promising results should lead to longer term studies evaluating the short-term impact of therapy on a wide range of vascular and biological outcomes, before finally embarking on larger phase 3 studies to evaluate safety and efficacy of therapy. This approach to drug development, whilst laborious, may have the desired effect of excluding non-contenders early in the course of drug development, and actually expedite the emergence of new treatments at a lower cost.
8.5.3 Use of murine models of SSc to establish a proof-of-concept for the use of anti-thrombotic therapy in SSc

Only a single study has used murine models of SSc to develop a proof-of-concept for anti-platelet therapy in SSc (Dees et al., 2011). Despite the obvious limitations of animal models of disease, this approach does allow us to undertake dose-ranging studies of one or more anti-platelet agents and combinations thereof. As animal models of SSc are refined to more closely resemble human disease, such work will prove invaluable in developing therapeutic strategies in the pre-clinical setting. Such models may now provide the only feasible method for investigating pleiotropic action of existing low-cost drugs (e.g. statins) in SSc, helping to increase therapeutic options in this disease.

8.6 Conclusions

This thesis exemplifies the many challenges facing clinicians seeking new avenues for investigating and managing the peripheral microvascular abnormalities that occur in RP and SSc. I hope that the findings of this thesis have made a useful contribution to existing knowledge and bring us a little closer to understanding the complex relationship between RP and SSc, and better ways of assessing and managing these conditions. The work has certainly instilled in me a passion for the complexities of these disorders and I hope to contribute to the shared efforts until, to quote the late Gerald Rodnan, “this most obstinate of diseases is made to give up its secrets”.


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PARTICIPANT INFORMATION SHEET

Assessment of the cold stress test in healthy volunteers

Researchers: Dr J Pauling MRCP, Dr J Shipley PhD, Dr N Harris PhD, & Prof N J McHugh MD FRCP

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. Dr Pauling will go through the information sheet with you and answer any questions you have. We’d suggest this should take about 15 minutes. Talk to others about the study if you wish. Part 1 tells you the purpose of this study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study. Please ask if anything is not clear.

PART 1

What is the purpose of this study?
This is a study of healthy volunteers with 2 objectives. Firstly, it is designed to assess how reproducible measurements of finger blood flow (after submerging your hands in cool water) are when measured on 2 occasions, one week apart. This information will help inform the design of future trials that will assess finger blood flow in patients with Raynaud’s phenomenon and systemic sclerosis (patients who often experience problems with the circulation in their fingers). The second part of the study will assess how background room temperature affects finger blood flow response to submerging hands in cool water.

Why have I been invited?
We wish to undertake this study on 15 healthy volunteers.

Do I have to take part?
No. It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason.
What will happen to me if I take part?
We will ask you to attend the Clinical Measurement Department at the Royal National Hospital for Rheumatic Diseases, on 3 occasions over a 2 week period. Each assessment will last approximately 40 minutes. At each assessment we will measure the effects of submersing your hands in cool water on the blood flow to your fingers.

Expenses and payment
We are unable to pay volunteers for taking part but participants can be reimbursed any travel expenses they have incurred should they wish.

What will I have to do?
We will ask you to attend the Clinical Measurement Department at the Royal National Hospital for Rheumatic Diseases, on 3 occasions over a 2 week period. Each assessment will take place between 9 o’clock and 12 o’clock in the morning and last approximately 40 minutes. All participants will be asked to refrain from alcohol, nicotine and caffeine for 4 hours prior to the assessment. At each assessment you will be seated in a quiet room, wearing your light clothing (i.e. jumpers and overcoats removed) for a period of 20 minutes acclimatisation at either 23 degrees Celsius (for assessments 1 and 2) or 18 degrees Celsius (assessment 3). We will then measure the surface temperature of your skin (using a thermal imaging camera), and the blood perfusion (using a laser Doppler imager), of your fingers held at a comfortable position in front of you. We will place your hands in cellophane gloves and place them into a bowl of water cooled to 15 degrees Celsius for 1 minute. We will then remove the gloves and place your hands back in position on the platform in front of you. Repeat measurement of the surface temperature and blood perfusion of your fingers will be performed on removal of the gloves and at subsequent regular intervals, for a period of 15 minutes. This is the end of the experiment.

What are the possible disadvantages and risks of taking part?
We do not anticipate any risks or side-effects from undertaking the assessments. The only risk or burden relates to your attendance to the clinical measurement department on 3 separate occasions. The measurements do not involve any contact with your body and you will not feel the measurements being taken. Neither device involves any ionising radiation (e.g. X-rays). There are no needles or blood tests. The laser used in the perfusion imager is very weak in strength and when used appropriately is incapable of causing harm to humans e.g. burns to the skin or eyes.

What are the potential benefits of taking part?
The study will not help you but the information we get from this study will help improve the management of people with Raynaud’s phenomenon and systemic sclerosis.

What happens when the research study stops?
Once all participants have completed the study, the findings will be analysed. A summary of the findings will be sent to you and more detailed discussion can be arranged at your request. The results may be used in order to make a published report in a scientific journal as this may help other rheumatologists in their research.

What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?
Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

PART 2

What will happen if I don’t want to carry on with the study?
You are free to withdraw from the study at any time without giving a reason. Any data collected prior to your withdrawal may still be used in the final data analysis.

What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions [01225 465 941]. If you remain unhappy and wish to complain formally, you can do this by contacting the Patient Advice and Liaison Service (PALS). Details can be obtained from Laura Davies (telephone: 01225 473424).

In the event that something does go wrong and you are harmed during the research and this is due to someone’s negligence then you may have grounds for a legal action for compensation against the Royal National Hospital for Rheumatic Diseases but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (as outlined above).

Will my taking part in the study be kept confidential?
Yes. All participants will be issued with a unique identifier code which will be used to label all data collected as part of the study e.g. laser Doppler and thermal imaging results etc. All data will be stored in a locked filing cabinet or electronically on password protected computers. A cross-referencing list matching participants with their unique identifier code will be locked in a filing cabinet along with an electronic version stored on an NHS password protected computer. All identifying information will be removed if the data is written up for publication. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised. Monitors and auditors from NHS Research & Development (R&D) offices may require access to participants’ personal data to verify or cross check data.

Data will be retained for a period of 5 years to allow any necessary cross checking of data. The anonymised data collected may be used in future studies. Professor Neil McHugh will be responsible for the safe storage and disposal of the data collected from this study.

Who has reviewed the study?
All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by Bath Research Ethics Committee.

What will happen to the results of the research study?
The results from all the participants will be grouped together for analysis and used to write a scientific paper which will be published. Individual participants will not be identified as part of the publication. A summary will be sent to all participants and arrangements can be made to discuss the relevance of the results in person with Dr. Pauling should participants wish.

Who is organising and funding the research?
The research will be undertaken by Dr John Pauling and is being sponsored by the Royal National Hospital for Rheumatic Diseases. It is being funded by the Raynaud's and Scleroderma Association.

**Further information and contact details**
Thank you for taking the time to read this Participant Information Sheet. Dr Pauling is happy to answer any questions you may have. If you wish to receive further information please contact Dr John Pauling via telephone (01225 465 941) or via e mail (John.Pauling@rnhrd.nhs.uk).
Participant Number:

CONSENT FORM

Assessment of the cold stress test in healthy volunteers

Name of Principal Researcher:
Dr John D Pauling, Research Fellow, Royal National Hospital for Rheumatic Diseases

1. I confirm that I have read and understand the information sheet dated 11.11.09 (version 2.1) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I understand that data collected during the study, may be looked at by individuals from regulatory authorities or from the Royal National Hospital for Rheumatic Disease NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

4. I understand that anonymised data from this study may be used in future studies undertaken at the Royal National Hospital for Rheumatic Diseases.

5. I agree to take part in the above study

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PARTICIPANT INFORMATION SHEET

Asasantin retard therapy in the management of Raynaud's phenomenon

Researchers: Dr J Pauling MRCP, Dr J Shipley PhD, Dr N Harris PhD, & Prof N J McHugh MD FRCP

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. Dr Pauling will go through the information sheet with you and answer any questions you have. We’d suggest this should take about 15 minutes. It is important that you are entirely happy with the study so you may wish to discuss it with your family, friends and other professionals who treat you. Part 1 tells you the purpose of this study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study. Please ask if anything is not clear.

PART 1

What is the purpose of this study?
This study will investigate a new treatment for use in the management of Raynaud’s phenomenon. The treatment is called asasantin retard which has already been used for many years in the management of stroke disease. Asasantin retard is a combination of low dose aspirin and dipyridamole, and works by altering the function of platelet cells that are involved in clot formation following injury. It also acts as an antioxidant. It is generally a safe and well tolerated drug (see below for further details).

Previous studies have demonstrated that platelet cells contribute to the development of Raynaud’s phenomenon. We believe that asasantin retard, by altering platelet cell function and reducing oxidative stress, may improve the symptoms of patients with Raynaud’s phenomenon. Our short-term study (4 weeks) is designed to explore whether treatment with asasantin retard reduces frequency and severity of attacks of Raynaud’s phenomenon, improves blood flow to the fingers during cold stress test (see below), alters levels of blood stream markers of platelet function and reduces oxidative stress.

Why have I been invited?
You have been invited to take part because you have Raynaud’s phenomenon. Your Raynaud’s phenomenon may be part of another disease called systemic sclerosis.

Do I have to take part?
No. It is up to you to decide to join the study. You are free to withdraw at any time, without giving a reason.

**What will happen to me if I decide to take part?**
We will arrange an appointment to discuss the study in more detail. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form indicating you have read and understood the information you have been given.

Dr Pauling will have access to your medical records to assess your eligibility for inclusion in this study. It is also important for the researchers to record information about you and your conditions. Some of this information is general, such as age, ethnicity, and occupation. Other information is more specific, such as the length of time you have been ill and the seriousness of your condition. In addition we will gather information about all the medication that has been prescribed for you, currently and in the past. We will also ask questions about your smoking history. Other clinical information, such as your auto-antibody profile will be relevant to the study. One of the reasons for collecting this information is so that the researchers can exclude from the study any person for whom asasatin retard may potentially pose a risk.

The study itself lasts a total of 4 weeks and is divided into two parts, each lasting 2 weeks. Part 1 involves monitoring your condition over a 2 week period without additional treatment. Part 2 involves monitoring your condition whilst taking asasatin retard tablets. Throughout the study period you will be asked to keep a diary of the frequency and severity of your Raynaud’s attacks. People who suffer from Raynaud’s phenomenon adopt a variety of strategies to avoid precipitating attacks. It is important that you continue these strategies but your behaviour at home or outside should remain as consistent with the best ways you have found of managing your condition as possible.

We will ask you to attend the Clinical Measurement Department at the Royal National Hospital for Rheumatic Diseases, on 3 occasions during the 4 week period. These assessments will take place at the start of the study, at the end of the first 2 weeks and at the end of the 4 weeks period. Each assessment will last approximately 40 minutes. The following diagram may make the process more clear.

![Week 0](Assessment 1)

2 week “run-in” phase
(no study medication)

2 week treatment period
(asasatin retard)

Week 2
Assessment 2

Assessment 3

Week 4
Assessment

Any patients who cannot be prescribed asasatin retard, or any patients who would rather not take a study medication can still provide valuable information for the study by allowing us to measure their response to the cold stress test for Assessment 1, and then keeping a diary for two weeks and allowing us to repeat the cold stress test for Assessment 2. This will help to broaden our knowledge of the condition and the assessment techniques we use to monitor disease severity and response to treatment.

**Expenses and payment**
We are unable to pay volunteers for taking part but participants will be reimbursed any travel expenses they have incurred attending the assessment visits.

What will I have to do?
We will make an appointment for you to attend for a baseline assessment at the Clinical Measurement Department at the Royal National Hospital for Rheumatic Diseases. This will take place between 9 o’clock and 12 o’clock in the morning and last approximately 40 minutes. You will be asked to refrain from vigorous exercise, alcohol, nicotine and caffeine for 4 hours prior to the assessment. You will be seated in a quiet room, wearing your light clothing (i.e. jumpers and overcoats removed) for a period of 20 minutes acclimatisation at a comfortable 23 degrees Celsius. We will then measure the surface temperature of your hands (using a thermal imaging camera), and the blood perfusion of your fingers (using a laser Doppler imager), held at a comfortable position in front of you. We will place your hands in cellophane gloves and place them into a bowl of water cooled to 15 degrees Celsius for 1 minute. We will then remove the gloves and place your hands back in position on the platform in front of you. Repeat measurement of the surface temperature and blood perfusion of your fingers will be performed on removal of the gloves and at subsequent regular intervals, for a period of 15 minutes. In addition to the above, participants with systemic sclerosis will be asked to complete a health assessment questionnaire (the Scleroderma Health Assessment Questionnaire) at the first assessment visit only. You will be issued with a diary to allow you to record the frequency and severity of your Raynaud’s phenomenon on a daily basis for the following 2 weeks. A second assessment will take place after the first 2 weeks has elapsed. You will undergo a further cold challenge test as outlined above. We will also collect a urine and blood sample from you at your second visit. You will not take any study medication in the first 2 weeks.

Those taking part in the second part of the study, will be issued with a prescription for a 2 week supply of asasantin retard at the second assessment. The study medication will be dispensed from a local pharmacy without you incurring any dispensing charge. After 2 weeks treatment with asasantin retard, a third and final assessment will be undertaken with a further cold challenge, blood and urine tests. Enquiry will be made as to whether you have experienced any possible side effects of the treatment. The study ends following the third assessment.

Pregnant ladies will not be recruited to the study and all ladies of child bearing age will be required to practice a medically acceptable form of contraception throughout the duration of the study.

What alternative treatments are available for the management of Raynaud’s phenomenon?
There are other treatments available for the management of Raynaud’s phenomenon such as calcium channel antagonists (e.g. nifedipine) and selective serotonin reuptake inhibitors (e.g. fluoxetine). If you are already taking these treatments, you will be able to continue with them during this study. If you have not tried them previously, then we would not start them at the same time as this study as they might interfere with the results of the trial. However, you may wish to consider their use as an alternative to the trial or alternatively, after the study has been completed.

What are the possible disadvantages and risks of taking part?
The majority of patients who take asasantin retard don’t experience any side effects. The commonest side effect patients have reported is a headache. This is caused by vasodilation (opening up) of the blood vessels within the brain and is regarded as a harmless side effect. This vasodilation (opening up) is one of the reasons we wish to investigate asasantin retard use in Raynaud’s phenomenon and headaches are a reported side effect of all the effective treatments available for managing Raynaud’s phenomenon. Thankfully, headaches usually subside within a few days, even if treatment is continued, and would be expected to disappear on stopping treatment (which is short acting).
The second potential side effect is gastric irritation and bleeding. Aspirin can irritate the wall of the gastrointestinal tract and any subsequent bleeding could be more extensive due to the altered platelet function and disturbed clot formation. The risk of this side effect is extremely low. In a previous large study the risk was approximately 4 in 100 when treatment was continued for a period of 2 years. The maximum period of treatment for this study is 2 weeks which will reduce this risk of this potential side effect significantly. Pre-menopausal ladies may notice their period is slightly heavier if they have a period during the 2 week treatment period.

We do not anticipate any significant risks or side-effects from undertaking the assessments and the greatest inconvenience for yourself is the time you would commit to attending the hospital. Blood samples at each assessment will only be taken by a medically trained Research Fellow with considerable experience, but there is a possibility of some pain or a small bruise. The “cold stress test” involves placing your hands in a bowl of cool water (around 15°C) for 1 minute and then observing your hands for the following 15 minutes as they warm up. This test has been used in many trials of patients with Raynaud’s phenomenon and it would be considered unusual for this test to set off an episode of Raynaud’s phenomenon. The measurements we perform do not involve any contact with your body and you will not feel the measurements being taken. Neither device involves any ionising radiation (e.g. X-rays). The laser used in the laser Doppler imager is very weak in strength, and when used appropriately is incapable of causing harm to humans e.g. burns to the skin or eyes.

**What are the potential benefits of taking part?**

You may notice some improvement in the frequency and severity of your Raynaud’s attacks but this cannot be guaranteed, and the purpose of the study is to establish whether asasantin retard therapy could have a role in the treatment of Raynaud’s phenomenon. You may benefit from closer monitoring and supervision during the study period. Participants may also gain satisfaction from knowing that their contribution may help future patients with Raynaud’s phenomenon and systemic sclerosis.

**What happens when the research study stops?**

At the end of the study, the study medication will be stopped even if you have experienced benefit. Additional research would be required before we could consider long-term use of asasantin retard for the management of Raynaud’s phenomenon. You will have the opportunity to discuss alternative treatments for Raynaud’s phenomenon with Dr Pauling. Once all participants have completed the study, the findings will be analysed. A summary of the findings will be sent to you and more detailed discussion can be arranged at your request. The results will be used in order to make a published report in a scientific journal.

**What if there is a problem?**

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

**Will my taking part in the study be kept confidential?**

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.
PART 2

What will happen if I don’t want to carry on with the study?
You are free to withdraw from the study at any time without giving a reason. Any data collected prior to your withdrawal may still be used in the final data analysis. Existing samples stored can be removed and destroyed on withdrawal from the study at your request.

What if there is a problem?
If you have any acute medical problems during the study it is important that you let us know. We will provide you with contact details of who to contact should you develop any new problems.

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions [01225 448 444]. If you remain unhappy and wish to complain formally, you can do this by contacting the Patient Advice and Liaison Service (PALS). Details can be obtained from Laura Davies (telephone: 01225 473424).

In the event that something does go wrong and you are harmed during the research and this is due to someone’s negligence then you may have grounds for a legal action for compensation against the Royal National Hospital for Rheumatic Diseases but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (as outlined above).

Will my taking part in the study be kept confidential?
Yes. All participants will be issued with a unique identifier code which will be used to label all data collected as part of the study e.g. Raynaud’s diary, laser contrast speckle imaging and thermal imaging results etc. All data will be stored in a locked filing cabinet or electronically on password protected computers. A cross-referencing list matching participants with their unique identifier code will be locked in a filing cabinet along with an electronic version stored on an NHS password protected computer. All identifying information will be removed before data is written up for publication. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised. Monitors and auditors from NHS Research & Development (R&D) offices may require access to participants’ personal data to verify or cross check data.

Data will be retained for a period of 15 years to allow any necessary cross checking of data. Anonymised data collected may be used in future studies. Professor Neil McHugh will be responsible for the safe storage and disposal of the data collected from this study.

What if relevant new information becomes available?
Sometimes during the course of a research project, new information becomes available about the treatment which is being studied. If this happens, the lead researcher will tell you about it and discuss whether you wish to continue in the study. If you decide to continue, you will be asked to sign an updated consent form. It is also possible, that on receiving new information, the lead researcher may consider it to be in your best interests to withdraw from the study. They will explain the reasons for this. If the study is stopped for any other reason, you will be told why.

Involvement of the General Practitioner/Family Doctor
It is essential that we inform your GP if you choose to take part in the study to ensure your GP is aware that you are taking study medication should you encounter any problems. For this reason, we will not recruit you to the study if you are unwilling for us to inform your GP. We will provide your GP with a copy of this information sheet as well as a copy of a document called the ‘study protocol’
which gives the same information as this sheet but in more technical terms. If you wish to have a copy of this study protocol, please ask the research fellow.

**What will happen to any samples I give?**
Blood samples will be analysed in the laboratory of the Bath Institute for Rheumatic Diseases and within the laboratory of the Royal United Hospital in Bath. They will be stored in a locked freezer before and after analysis. The samples will be anonymised and labelled with your unique identifier code. They will be stored by the research team for a period of 15 years before being destroyed along with the cross referencing list. With your permission, if research within the 15 year storage time identifies any additional blood markers relevant to this field of research then the frozen samples may be used to measure these. Urine samples will be stored anonymously in a locked freezer within the Bath Institute for Rheumatic Diseases prior to analysis. They will then be destroyed. The samples will be used to assess platelet function and levels of oxidation stress.

**Will any genetic tests be done?**
No.

**Who has reviewed the study?**
All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by Bath Research Ethics Committee.

**What will happen to the results of the research study?**
The results from all the participants will be grouped together for analysis and used to write a scientific paper which will be published. Individual participants will not be identified as part of the publication. A summary will be sent to all participants and arrangements can be made to discuss the relevance of the results in person with Dr. Pauling should participants wish.

**Who is organising and funding the research?**
The research will be undertaken by Dr John Pauling and is being sponsored by the Royal National Hospital for Rheumatic Diseases. It is being funded by the Raynaud’s and Scleroderma Association.

**Further information and contact details**
Thank you for taking the time to read this Participant Information Sheet. Dr Pauling is happy to answer any questions you may have. If you wish to receive further information please contact Dr John Pauling via telephone (01225 448 444) or via e mail (John.Pauling@rnhrd.nhs.uk)
Appendix 4 Study Interest Form

Royal National Hospital for Rheumatic Diseases
NHS Foundation Trust
Upper Borough Walls
Bath. BA1 1RL
Telephone: 01225 465841
Facsimile: 01225 421202

Study Interest Form
Asasantin Retard Therapy in the management of Raynaud’s phenomenon

Please fill in your details below, read the following statements and tick as many boxes as appropriate.

Forename(s) ____________________________
Surname _______________________________
Address ______________________________________
____________________________________
____________________________________
Phone No _________________________________
Email ___________________________________

I AM INTERESTED IN TAKING PART IN THIS STUDY

I am interested in taking part in the study and would like you to contact me by telephone. □
I am interested in taking part in the study and would like you to contact me by Email. □
I am interested in taking part in the study and would like you to contact me by Post. □

I AM NOT INTERESTED IN TAKING PART IN THIS STUDY
I am not interested in taking part in this study. □
Appendix 5 Asasantin Retard Trial consent form

Participant Number:

CONSENT FORM

Asasantin retard therapy in the management of Raynaud's phenomenon

Name of Principal Researcher: Dr John D Pauling

1. I confirm that I have read and understand the information sheet dated 13.08.10 (version 1.2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from regulatory authorities or from the Royal National Hospital for Rheumatic Disease NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to access my records.

4. I understand that anonymised data from this study may be used in future studies undertaken at the Royal National Hospital for Rheumatic Diseases.

5. I am not currently pregnant or breastfeeding and agree to practice a medically acceptable method of birth control throughout the study period (or withdraw from the study in advance)

6. I agree to extra blood and urine samples being taken for this research.

7. I agree for my blood samples to be used for future research within the next fifteen years and that it may not be possible to inform me of the nature or results of this future research.

8. I agree to my GP being informed of my participation in the study.

9. I agree to take part in part 1 of the study

10. I agree to take part in part 2 of the study
<table>
<thead>
<tr>
<th>Name of participant</th>
<th>Date</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of person taking consent</td>
<td>Date</td>
<td>Signature</td>
</tr>
</tbody>
</table>

When completed: 1 for participant, 1 for medical records and 1 for researcher site file
Appendix 6 Scleroderma Health Assessment Questionnaire

Connective Tissue Diseases
Scleroderma Health Assessment Questionnaire

Forename
Surname
Date of Birth

Section 1 - Health Assessment questionnaire

We are interested in learning how your illness affects your ability to function in daily life. Please feel free to add any comments at the end of this form.

Please tick the one response which best describes your usual abilities over the past week.

1) Dressing and Grooming
   Are you able to:
   - Dress yourself including shoelaces and doing buttons?
   - Shampoo your hair?

2) Rising
   Are you able to:
   - Stand up from an armless straight chair?
   - Get in and out of bed?

3) Eating
   Are you able to:
   - Cut your meat?
   - Lift a full cup or glass to your mouth?
   - Open a new carton of milk (or soap powder)?

4) Walking
   Are you able to:
   - Walk outdoors on flat ground?
   - Climb up 5 steps?

Please tick any AIDS or DEVICES that you usually use for any of these activities:

- Walking stick
- Devices used for dressing (button hook, zipper pull, long handled shoe horn etc)
- Walking frame
- Built-up or special utensils
- Crutches
- Special or built-up chair
- Wheelchair
- Other (specify)

Please tick any categories for which you usually need ASSISTANCE FROM ANOTHER PERSON

- Dressing and Grooming
- Rising
- Eating
- Walking
Please tick the one response which best describes your usual abilities over the past week.

1) **Hygiene**
   Are you able to:
   - Wash and dry your entire body? [ ] Without ANY difficulty [ ] With SOME difficulty [ ] With MUCH difficulty [ ] Unable to do
   - Take a bath? [ ] Without ANY difficulty [ ] With SOME difficulty [ ] With MUCH difficulty [ ] Unable to do
   - Get on and off the toilet? [ ] Without ANY difficulty [ ] With SOME difficulty [ ] With MUCH difficulty [ ] Unable to do

2) **Reach**
   Are you able to:
   - Reach and get down a 5lb object (e.g., bag of potatoes) from just above your head? [ ] Without ANY difficulty [ ] With SOME difficulty [ ] With MUCH difficulty [ ] Unable to do
   - Bend down to pick clothing from the floor? [ ] Without ANY difficulty [ ] With SOME difficulty [ ] With MUCH difficulty [ ] Unable to do

3) **Grip**
   Are you able to:
   - Open car doors? [ ] Without ANY difficulty [ ] With SOME difficulty [ ] With MUCH difficulty [ ] Unable to do
   - Open jars which have previously opened? [ ] Without ANY difficulty [ ] With SOME difficulty [ ] With MUCH difficulty [ ] Unable to do
   - Turn taps on and off? [ ] Without ANY difficulty [ ] With SOME difficulty [ ] With MUCH difficulty [ ] Unable to do

4) **Activities**
   Are you able to:
   - Run errands and shop? [ ] Without ANY difficulty [ ] With SOME difficulty [ ] With MUCH difficulty [ ] Unable to do
   - Get in and out of a car? [ ] Without ANY difficulty [ ] With SOME difficulty [ ] With MUCH difficulty [ ] Unable to do
   - Do chores such as vacuuming, housework or light gardening? [ ] Without ANY difficulty [ ] With SOME difficulty [ ] With MUCH difficulty [ ] Unable to do

Please tick any AIDS or DEVICES that you usually use for any of these activities:

- [ ] Raised toilet seat
- [ ] Bath Rail
- [ ] Bath seat
- [ ] Long-handled appliances for reach
- [ ] Jar opener (for jars previously opened)
- [ ] Other (specify)

Please tick any categories for which you usually need HELP FROM ANOTHER PERSON:

- [ ] Hygiene
- [ ] Reach
- [ ] Gripping and opening things
- [ ] Activities

---

For office use only

- [ ] Dressing & grooming
- [ ] Rising
- [ ] Eating
- [ ] Walking
- [ ] Hygiene
- [ ] Reach
- [ ] Grip
- [ ] Activity
We are also interested in learning whether or not you are affected by pain because of your illness.

1. **How much Pain have you had because of your illness IN THE PAST WEEK?**
   PLACE A MARK ON THE LINE TO INDICATE THE SEVERITY OF THE PAIN
   
   NO PAIN  
   0  
   100  
   VERY SEVERE PAIN

2. **IN THE PAST WEEK, How much have your intestinal problems interfered with your daily activities?**
   PLACE A MARK ON THE LINE TO INDICATE THE LIMITATION OF ACTIVITY
   
   INTESTINAL PROBLEMS DO NOT LIMIT ACTIVITIES
   0  
   100  
   VERY SEVERE LIMITATION

3. **IN THE PAST WEEK, How much have your breathing problems interfered with your daily activities?**
   PLACE A MARK ON THE LINE TO INDICATE THE LIMITATION OF ACTIVITY
   
   BREATHING PROBLEMS DO NOT LIMIT ACTIVITIES
   0  
   100  

4. **IN THE PAST WEEK, How much has Raynaud’s interfered with your daily activities?**
   PLACE A MARK ON THE LINE TO INDICATE THE LIMITATION OF ACTIVITY
   
   RAYNAUD’S DOES NOT LIMIT ACTIVITIES
   0  
   100  
   VERY SEVERE LIMITATION

5. **IN THE PAST WEEK, How much have your finger ulcers interfered with your daily activities?**
   PLACE A MARK ON THE LINE TO INDICATE THE LIMITATION OF ACTIVITY
   
   FINGER ULCERS DO NOT LIMIT ACTIVITIES
   0  
   100  
   VERY SEVERE LIMITATION

6. **Overall, considering how much pain, discomfort, limitations in your life and other changes in your body and life, how severe would you rate your disease today?**
   PLACE A MARK ON THE LINE TO INDICATE THE SEVERITY OF YOUR DISEASE
   
   NO DISEASE
   0  
   100  
   VERY SEVERE DISEASE
Appendix 7 Example of Raynaud’s Condition Score Diary

Raynaud’s Condition Score Diary

<table>
<thead>
<tr>
<th>Attack</th>
<th>Duration (minutes)</th>
<th>Please tick box if no attacks of Raynaud’s occur today</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>10</td>
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<tr>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(if more than 12 attacks occur in one day then please use a second sheet)

Raynaud’s Condition Score

Please rate the difficulty you had today with your Raynaud’s condition by circling the appropriate number below.

Please consider the following in choosing your score:
The number of Raynaud’s attacks; the duration of the attacks; whether you had, for example, numbness, pain, burning and tingling, and the effect cold had on your ability to use your hands and to perform other activities.

<table>
<thead>
<tr>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
</table>

Raynaud’s Condition Score Diary

<table>
<thead>
<tr>
<th>Attack</th>
<th>Duration (minutes)</th>
<th>Please tick box if no attacks of Raynaud’s occur today</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>4</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(if more than 12 attacks occur in one day then please use a second sheet)

Raynaud’s Condition Score

Please rate the difficulty you had today with your Raynaud’s condition by circling the appropriate number below.

Please consider the following in choosing your score:
The number of Raynaud’s attacks; the duration of the attacks; whether you had, for example, numbness, pain, burning and tingling, and the effect cold had on your ability to use your hands and to perform other activities.

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
Appendix 8  Platelet aggregometry Quality Control work

Full title:
A short-term open-label single site pilot study evaluating the use of asasantin retard therapy in the management of Raynaud’s phenomenon.

Eudract Number: 2009-013468-37

Platelet Aggregometry Quality Control Assessments
Introduction

The PAP4 platelet aggregometer was purchased from alpha laboratories with the intention for its sole use as part of this study. The aggregometer was fully serviced and calibrated prior to delivery. In view of the infrequent use of the device (for approximately 30 minutes, up to 3 times per week) and the short duration of the study (18 months), it was agreed no additional formal servicing or calibration would be undertaken by alpha laboratories during the study itself. The aggregometer should be sent for a formal service should it be used in future studies. This summary shall describe in house QC assessments undertaken to evaluate the competency of the operator (Dr J Pauling), precision of the aggregometer and reproducibility.

Methods

All Quality Control (QC) assessments were undertaken by Dr J Pauling using plasma donated by Dr J Pauling. The QC assessments were derived from approved recommendations concerning quality control (H58-A Platelet Function Testing by aggregometry; Approved guideline).

Assessments of the standardisation of the instrument was undertaken setting 100% transmission with PPP and baseline with PRP. The PRP cuvette was removed and a second PPP cuvette inserted to ensure the chart deflected to 100%. A second PRP cuvette was added to ensure the chart deflected back to 0%. Finally, 0.1 ml or 0.225ml of PPP was added to the cuvette containing PRP diluting the sample by a factor of 33% and 20% respectively.

Aggregometry experiments were undertaken across the dose range of ADP (1.25 to 10 μmol/L) used in the study. These QC assessments were undertaken to evaluate the instrument and operator, and not the agonists used. For this reason, no studies were undertaken using alternative agonists such as arachidonic acid. QC assessments were undertaken over 3 consecutive days (6th-8th February 2012) using plasma donated by J Pauling. Six 0.109M 3.2% citrate vacutainer bottles were filled. Platelet rich plasma (PRP) and platelet poor plasma (PPP) was prepared as undertaken in the study and described in detail in the platelet aggregometry SOP. All assessments were undertaken using ADP (lot no 07000076, Exp 02.2013). The ADP had been reconstituted on the 26th January 2012 to a working concentration of 10 μmol/L. Working concentrations of 5, 2.5 and 1.25 μmol/L were created with repeated 1:1 dilution of 125 μL of agonist and purified water.

On assessments 1 and 2, the assessments were undertaken with the agonists as per the study protocol (10, 5, 2.5 and 1.25 μmol/L) on 2 occasions (allowing duplicate assessments). Surplus PRP at assessment 2 allowed additional assessments with the 10 μmol/L concentration. This was repeated at the 3rd assessment, although the order of the assessments was changed to facilitate analysis of duplicate samples across separate channels. Additional PRP allowed additional assessments with the 1.25μmol/L concentration. In total, 13 duplicate assessments were undertaken exceeding the recommended minimum of 10 assessments.

Finally, assessment of the temperature within the cuvettes was undertaken using post-aggregation samples on the 1st March 2012. The temperature was assessed using a calibrated digital thermometer (Model 2751-K Digitron Instrumentation Ltd).
Statistical Analysis

A descriptive analysis of the tests for standardisation and linearity are reported. The coefficient of variation (CV) for each aggregation endpoint (maximum aggregation, aggregation at 5 minutes and initial slope of aggregation) is calculated using the following formulae:

\[
SD = \sqrt{\frac{\sum (x_1 - x_2)^2}{2n}}
\]

\[
Mean = \frac{\sum (x_1 + x_2)}{2n}
\]

\[
CV(\%) = 100 \times \frac{SD}{Mean}
\]

\(n\) being the number of data pairs and \(x_1\) and \(x_2\) duplicate measurements.


Results

All data can be verified using enclosed hard copy printed reports generated by the PAP-4 aggregometer on the day of the assessments.

Instrument set-up

The instrument is designed to only allow aggregation experiments to be undertaken when the incubation chambers have reached a temperature of 37°C. The instrument is switched on 30 minutes before operation and indicates when the channels are ready for analysis.

The stir bar speed for each channel can be checked by pressing the stir bar speed buttons at any stage. These are routinely checked prior to aggregation experiments to ensure they remain set at 920rpm.

Instrument functionality and linearity

Assessments of instrument standardisation confirmed accurate standardisation with repeated values of 100% for PPP and 0% for PRP. These assessments were undertaken primarily using channel 1 of the aggregometer. At assessment 2, all channels were checked individually. No transmissions of >103% were obtained for PPP providing reassurance of the quality of the instrument and PPP preparation (i.e. platelet free). Tests for linearity gave readings slightly below expected (up to 8%) values. The mean deflection for the 33% dilution was 27.4%. The 20% dilution undertaken on 08.02.2012 was slightly below at 19%.

Table 1. Assessments of instrument functionality and linearity.
Platelet aggregation curves. For each aggregation experiment, the endpoints chosen were (as in study) %aggregation at 5 minutes, maximum percent aggregation and initial slope of aggregation. The results are presented in Table 2.

**Table 2. Aggregation results performed for each agonist concentration in duplicate.**

<table>
<thead>
<tr>
<th>Date</th>
<th>Agonist conc (µmol/L)</th>
<th>Channel</th>
<th>5 minutes</th>
<th>Maximum aggregation</th>
<th>Initial slope of aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>06.02.2012</td>
<td>ADP 10</td>
<td>1</td>
<td>79</td>
<td>79</td>
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<tr>
<td></td>
<td>ADP 5</td>
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<td>80</td>
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<tr>
<td></td>
<td>ADP 2.5</td>
<td>3</td>
<td>78</td>
<td>78</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>ADP 1.25</td>
<td>4</td>
<td>20</td>
<td>23</td>
<td>25</td>
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<tr>
<td></td>
<td>ADP 10</td>
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<tr>
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<td>ADP 5</td>
<td>2</td>
<td>79</td>
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<td>4</td>
<td>33</td>
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<tr>
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<tr>
<td></td>
<td>ADP 5</td>
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<td>77</td>
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<td>4</td>
<td>8</td>
<td>18</td>
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<tr>
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<td>ADP 1.25</td>
<td>4</td>
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<td></td>
<td>ADP 10</td>
<td>2</td>
<td>80</td>
<td>80</td>
<td>42</td>
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<td>ADP 1.25</td>
<td>3</td>
<td>33</td>
<td>33</td>
<td>19</td>
</tr>
</tbody>
</table>

The CVs for the combined analysis are presented in Table 3. The CVs for all endpoints were approximately 15%. This is likely to reflect the fact that reproducibility of aggregation
curved is dependent upon biological variation of responses to agonist, operator competency and instrument accuracy. There was greater variability to low agonist concentrations. Table 4 presents the CVs for samples stimulated with higher dose agonists (10, 5, 2.5 μmol/L concentrations only). The CVs for %aggregation improved significantly to 7.2%. There was no change in the CV for initial slope of aggregation (~14%). The CVs for %aggregation improve further to 1.28% if analyses are restricted to 10 and 5 μmol/L alone (despite the use of fewer analyses which would be expected to increase variability). High agonist concentrations tend to result in complete aggregation (80% deflection on aggregation curves) whereas low agonist concentrations result in a more finely balanced ex vivo equilibrium between aggregation and disaggregation. This leads to repeatable primary wave aggregation, followed by subsequent disaggregation. At that stage, platelets can continue to disaggregate, stabilise or the release of platelet activators from platelet granules can result in a secondary wave of aggregation. It is likely subtle differences in the biological responses of samples to low agonist concentrations account for the increased variation in these samples. Consistent findings across different channels of the aggregometer with higher doses suggest this variation is not the result of instrument failure or poor operator competency.

Table 3. CVs generated for each aggregation endpoint for all studies (n=13 in duplicate).

<table>
<thead>
<tr>
<th></th>
<th>% aggregation at 5 minutes</th>
<th>Maximum aggregation</th>
<th>% Initial slope of aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>62.42307692</td>
<td>62.92307692</td>
<td>34.61538462</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>9.790105</td>
<td>8.600089</td>
<td>4.988448</td>
</tr>
<tr>
<td>Coefficient of Variation</td>
<td>15.68347</td>
<td>13.66762</td>
<td>14.41107</td>
</tr>
</tbody>
</table>

Table 4. CVs for each aggregation endpoint for higher concentrations of agonists (10, 5 and 2.5 μmol/L only). n=9 duplicate pairs.

<table>
<thead>
<tr>
<th></th>
<th>% aggregation at 5 minutes</th>
<th>Maximum aggregation</th>
<th>% Initial slope of aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>76.94444444</td>
<td>76.94444444</td>
<td>40.44444444</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>5.542763</td>
<td>5.542763</td>
<td>5.925463</td>
</tr>
<tr>
<td>Coefficient of Variation</td>
<td>7.203591</td>
<td>7.203591</td>
<td>14.65087</td>
</tr>
</tbody>
</table>

Cuvette temperature

The PAP-4 aggregometer will only allow assessments to be undertaken when the cuvette holding bay is heated to 37°C. The temperature in each channel was assessed using a calibrated digital thermometer (Model 2751-K Digitron Instrumentation Ltd). The results are reported in Table 5.

<table>
<thead>
<tr>
<th></th>
<th>Channel 1</th>
<th>Channel 2</th>
<th>Channel 3</th>
<th>Channel 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>36.5 ºC</td>
<td>36.8 ºC</td>
<td>36.7 ºC</td>
<td>36.7 ºC</td>
</tr>
</tbody>
</table>
All temperature measurements were within 0.5°C of the target temperature which was considered acceptable performance.

Conclusions

These experiments have confirmed acceptable instrument functionality and operator competency. We have highlighted increased variability at lower agonist concentrations which will need to be considered when analysing the results of assessments using lower agonist concentrations in the study. None of the QC checks have raised concerns regarding the instruments functionality or accuracy.

Enclosed

Print outs from aggregometry experiments 6th – 8th February 2012-02-08