PERIOCULAR MALIGNANCY AND EYELID RECONSTRUCTION

A thesis submitted for the degree of Doctor of Philosophy

Dr Michelle Tian Sun MBBS

Discipline of Ophthalmology and Visual Sciences

South Australian Institute of Ophthalmology

The University of Adelaide and Royal Adelaide Hospital

August 2016

DEDICATION

To my parents, Kim and Eileen, and my husband Chris.

TABLE OF CONTENTS

DEDICATION 2

TABLE OF CONTENTS 3

ABSTRACT 10

DECLARATION 12

ACKNOWLEDGEMENTS 13

PUBLICATIONS AND PRESENTATIONS 15

CHAPTER ONE: LITERATURE REVIEW 18

1.1 Overview 18

1.2 Periocular Skin Cancer 20

1.2.1 Introduction to Periocular Skin Cancer 20

1.2.2 Basal Cell Carcinoma 23

1.2.2.1 Introduction 23

1.2.2.2 Basal Cell Carcinoma Histological Subtypes 23

1.2.2.3 Basal Cell Carcinoma with Orbital Invasion 26

1.2.2.4 Clinical Presentation and Investigation of Basal Cell Carcinoma with Orbital Invasion 28

1.2.2.5 Management of Basal Cell Carcinoma with Orbital Invasion 30

1.2.3 Squamous Cell Carcinoma 36

1.2.3.1 Introduction 36

1.2.3.2 Histological Features and Subtypes 36

1.2.3.3 Prognostic Factors 38

1.2.3.4 Surgical Management of Squamous Cell Carcinoma 40

1.2.3.5 Non-Surgical Management 42

1.3 The AJCC TNM Staging System 44
1.3.1 The TNM Staging System for Eyelid Carcinoma 44
1.3.2 Implementing the TNM Staging 46
1.3.3 TNM as a Predictor of Outcomes 47
Table 1: Definitions of TNM for Eyelid Carcinoma, AJCC Cancer Staging Manual, Seventh Edition 51
Table 2: Stage Grouping for Carcinoma of the Eyelid 53
1.4 Eyelid Reconstruction and Eyelid Tarsal Substitutes 54
1.4.1 Indications for Eyelid Reconstruction 54
1.4.2 Anatomic Considerations 54
1.4.3 Established Tarsal Substitutes in Eyelid Reconstruction 55
Figure 1: The Anatomy of the Eyelid 59
1.5 Principles of Bioengineering 60
1.5.2 Introduction 60
1.5.2 The Importance of Biomechanics 61
1.5.2.1 *In Vivo* Stress and/or Strain 62
1.5.2.2 Functional Demands: Sub-Failure and Failure Conditions 62
1.5.2.3 Prioritisation of Mechanical Properties as Design Parameters 62
1.5.2.4 Regulation and Interaction of Cells with an Extracellular Matrix *In Vivo* 63
1.5.2.5 Mechanical and Physical Factors Impacting Tissue Repair 63
1.5.2.6 Outcome Based Success Criteria and Methods to Model Tissue Growth 64

1.5.2.7 Biomechanical Studies within Ophthalmology 64
1.5.2.8 Specific Considerations for Eyelid Tarsus 66
1.5.3 The Role of the Scaffold 69
1.5.4 Tissue Engineering in Ophthalmology 72
1.5.4.1 Cornea 72
1.5.4.2 Glaucoma 74
1.5.4.3 Conjunctiva 75
1.5.4.4 Dry Eye 76
1.5.4.5 Orbital Fractures 77
1.5.5 Bioengineering Tarsus 80
1.5.6 Role of Cell Culture in Bioengineering 81
1.5.6.1 Fibroblast Culture 81
CHAPTER 2: BASAL CELL CARCINOMA 84
2.1 Introduction 84
2.2 Methods 86
2.3 Results 88
2.3.1 General Characteristics 88
2.3.2 Correlation between Biopsy and Excision 88
2.3.4 Biopsy and Excision Dimensions 89
2.4 Discussion 91
2.4.1 Summary of Findings 91
2.4.2 Characteristics of Basal Cell Carcinoma 91
2.4.2 Diagnostic Accuracy of Biopsy 92
2.4.3 Reporting of Margins 94
2.5 Study Limitations 97

2.6 Conclusion 98
Table 3: Basal Cell Carcinoma Subtypes at Initial Biopsy and Excision 99
Table 4: Biopsy Compared to Excision for Periocular Basal Cell Carcinoma of Mixed Histology 100
CHAPTER 3: SQUAMOUS CELL CARCINOMA 101
3.1 Introduction 101
3.2 Methods 103
3.2.1 Data Collection 103
3.2.2 Statistical Analyses 104
3.3 Results 105
3.3.1 Squamous Cell Carcinoma in Situ (Bowen Disease) 105
3.3.2 Invasive Squamous Cell Carcinoma 106
3.3.3 Metastatic Disease 107
3.3.4 Perineural Invasion and Histological Data 107
3.3.5 Management 108
3.3.6 Follow-Up 109
3.4 Discussion 111
3.4.1 Summary of Findings 111
3.4.2 Previous Reports 111
3.4.3 Factors Associated with Recurrence and Metastases 112
3.4.4 Squamous Cell Carcinoma in Situ (Bowen Disease) 114
3.5 Study Limitations 116
3.6 Conclusion 117
Table 5: TNM Stages for Patients with Periocular Squamous Cell Carcinoma 118

Table 6: Recurrent Cases of Periocular Squamous Cell Carcinoma 119
CHAPTER 4: BIOMECHANICAL STUDIES OF THE EYELID TARSUS 123
4.1 Introduction 123
4.2 Methods 125
4.2.1 Sample Selection 125
4.2.2 CellScale BioTester 125
4.2.3 Biomechanical Parameters 126
4.3 Results 129
4.4 Discussion 130
4.4.1 Summary and Relevance 130
4.4.2 Alternate Tarsus Substitutes and Their Biomechanical Properties 130
4.3.3 Application in Tissue Engineering 133
4.5 Study Limitations 134
4.6 Conclusion 135
Table 7: Biomechanical Properties of Tarsus Tissue 136
Figure 2: The CellScale BioTester 137
Figure 3: Tarsus Biomechanical Testing 139
Figure 4: Stress-Strain Curve for Tarsus Tissue 140
CHAPTER 5: SCAFFOLD DESIGN FOR BIOENGINEERED TARSUS 141
5.1 Introduction 141
5.2 Methods 143
5.2.1 Scaffold Material 143
5.2.2 Scaffold Fabrication 144
5.2.3 Scaffold Characterisation and Preliminary Assessment 145

5.3 Results 146
5.3.1 Scaffold Characteristics and Biomechanics 146
5.3.2 Characterisation of Scaffolds 146
5.4 Discussion 147
5.4.1 Summary of Findings 147
5.4.2 Chitosan as Scaffold Material 147
5.4.3 Previous Studies of Bioengineered Tarsus 148
5.5 Study Limitations 150
5.6 Conclusion 151
Figure 5: Formation of Scaffolds by Cryogelation 152
Figure 6: Effect of Chitosan Concentration of Pore Size of Chitosan Scaffolds 153
Figure 7: Tensile Elastic Modulus Scaffolds and Native Tarsus Tissue ... 154
Figure 8: Pore Architecture of Chitosan Scaffolds 155
Figure 9: Chitosan Scaffolds Support Fibroblast Culture 156
CHAPTER 6: FIBROBLAST CULTURE FOR BIOENGINEERED TARSUS 157
6.1 Introduction 157
6.2 Methods 158
6.2.1 Orbital Skin Fibroblast Culture 158
6.2.2 Characterising Differentiation 159
6.2.3 Culture of Orbital Fibroblasts onto Bioengineered Scaffolds .. 159
6.3 Results 161
6.3.1 Orbital Fibroblast Culture 161
6.3.2 Culture of Fibroblasts Over Scaffolds 161
6.4 Discussion 162

6.4.1 Summary of Findings 162
6.4.2 Previous Studies 162
6.4.3 Applicability in Clinical Practice 163
6.5 Limitations 165
6.6 Conclusions 166
Figure 10: Immunological and Gene Expression Analysis of Orbital Skin Fibroblasts 167
Figure 11: Scanning Electron Microscopy of Fibroblast Culture of Scaffolds 168
CHAPTER 7: FINAL DISCUSSION 169
CHAPTER 8: FUTURE DIRECTIONS 172
CHAPTER 9: REFERENCES 174

ABSTRACT

Non-melanoma skin cancer is the most common cancer in Australia. Basal cell carcinoma and squamous cell carcinoma are the two most frequently encountered types of non-melanoma skin cancer, and together they make up over 90% of all skin cancers. The periocular region is involved in 10% of cases and is associated with significantly more disease-related morbidity due to the local effect of both the disease and the surgical treatment on ocular adnexa. Therefore, it is imperative that high-risk tumours are correctly identified to ensure appropriate management and surveillance. Surgical excision remains the gold standard treatment but functional reconstruction of the eyelid represents an ongoing challenge. Despite the wide range of autologous and artificial eyelid substitutes, there is yet to be an ideal replacement for the specialised eyelid tissue called the tarsus. The tarsus is responsible for both structural support and physical form, making its adequate substitution fundamental to functional outcomes. Numerable uncertainties remain regarding the staging and management of periocular non-melanoma skin cancer which, combined with our lack of ideal eyelid tarsus substitutes, represents the basis for work undertaken as part of this thesis.

Previous studies contributing to our knowledge of periocular basal cell carcinoma histological subtypes and treatment of invasive disease are first reviewed in Chapter 2. Chapter 3 subsequently summarises our understanding of periocular squamous cell carcinoma with a particular focus on the utilisation and prognostic role of the most up-to-date American Joint Committee on Cancer (AJCC) staging system for the eyelid carcinoma.

In order to determine the required properties for the ideal tarsus tissue substitute, Chapter 4 analyses the normal biomechanical properties of the eyelid tarsus tissue. This study, the first of its kind for human tarsus tissue, provides a benchmark for bioengineering studies described in the following chapter. In Chapter 5, we describe the development of a novel bioengineered three-dimensional scaffold which is tailor-made to behave biomechanically like natural tarsus. In order to improve *in vivo* compatibility, we also successfully cultured fibroblasts from eyelid skin samples which were then seeded onto our bioengineered scaffolds, the results of which are described in Chapter 6.

Finally, insights into the presentation, staging and management of periocular basal cell carcinoma and squamous cell carcinoma, along with our novel bioengineered eyelid tarsus substitute are placed in the context of the previous literature in Chapter 7, before possible directions for future studies are discussed in Chapter 8.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Michelle T. Sun

August 2016

ACKNOWLEDGEMENTS

This doctoral thesis would not have been possible without the help, support and assistance of numerous people.

It has been my great privilege to have been taught by Professor Dinesh Selva. He has provided unwavering support and incredible mentorship throughout the past seven years. I continue to be inspired by his pursuit of excellence and attitude towards life and medicine. He has been instrumental in guiding me through my research development and I hope that this represents only the beginning of a long and fruitful collaboration. Similarly, I am extremely thankful to Professor Robert Casson, my co-supervisor. In addition to his academic talents and leadership, I will always admire Professor Casson's enthusiasm and commitment towards research and ophthalmology. I also look forward to continuing to work and collaborate with him in the years to come.

There are a number of other researchers and clinicians who I have learnt so much from and who I am also grateful to. Dr WengOnn Chan has been an immense support during the last few years and has been incredibly generous with his time, offering insightful advice on research, ophthalmology and life. I have also learned much from Dr John Wood, who has been an invaluable source of knowledge on laboratory-based research. Furthermore, Associate Professor Andrea O'Connor has helped me significantly in the area of bioengineering. Too many to mention are my many other colleagues and friends, whom I am thankful to for all their support and encouragement over the years.

Finally, a special thanks to my family. To my parents, Kim and Eileen – I can never be grateful enough for the sacrifices you have made to ensure I have had every opportunity in life. To my brother David – I am so glad to have your humour and companionship. Most importantly, I thank my husband Chris. Sometimes you meet someone truly exceptional, someone who believes and inspires you, encourages and pushes you to be better than you could imagine for yourself. I am lucky enough to have married that someone.

PUBLICATIONS AND PRESENTATIONS

Chapter One

1. Review: Sun MT, O'Connor AJ, Wood J, Casson R, Selva D. Tissue Engineering in Ophthalmology: Implications for Eyelid Reconstruction. Ophthalmic Plastic and Reconstructive Surgery 2016 [epub ahead of print]
2. Review: Sun MT, Wu A, Figueira E, Huilgol SC, Selva D. Management of periorbital basal cell carcinoma with orbital invasion. Future Oncology 2015;11:3003-10.
3. Manuscript: Wu A, Sun MT, Huilgol SC, Madge S, Selva D. Histological subtypes of periocular basal cell carcinoma. Clinical and Experimental Ophthalmology 2014;42:603-7.
4. Manuscript: Herbert HM, Sun MT, Selva D, Fernando B, Saleh G, Beaconsfield M, Collin R, Uddin J, Meligonis G, Leatherbarrow B, Atuallah S, Irion L, McLean C, Huilgol S, Davis G, Sullivan T. Merkel Cell Carcinoma of the Eyelid: Management and Prognosis. JAMA Ophthalmology 2014 Feb;132(2):197-204.
5. Manuscript: Watanabe A, Sun MT, Pirbhai A, Ueda K, Katori N, Selva D. Sebaceous Carcinoma in Japanese Patients: Clinical Presentation, Staging and Outcomes. British Journal of Ophthalmology 2013 Nov;97(11):1459-63
6. Presentation: Sun MT, Herbert HM, Selva D, Fernando B, Saleh G, Beaconsfield M, Collin R, Uddin J, Meligonis G, Leatherbarrow B, Atuallah S, Irion L, McLean C, Huilgol S, Davis G, Sullivan T. Merkel Cell Carcinoma of the Eyelid: Management and Prognosis. Annual

Royal Australian and New Zealand College of Ophthalmologists Annual Scientific Congress 2013

7. Presentation: Watanabe A, Sun MT, Pirbhai A, Ueda K, Katori N, Selva D. Sebaceous Carcinoma In Japanese Patients: Clinical Presentation, Staging and Outcomes. Annual Royal Australian and New Zealand College of Ophthalmologists Annual Scientific Congress, 2013

Chapter Two

8. Manuscript: Sun MT, Wu A, Huilgol SC, Selva D. Accuracy of Biopsy in Subtyping Periocular Basal Cell Carcinoma. Ophthalmic Plastic and Reconstructive Surgery 2015;31:449-51.
9. Research Letter: Sun MT, Figueira E, Huilgol SC, Selva D. Minimum Histological Safety Margins in Periocular Basal Cell Carcinoma. British Journal of Ophthalmology 2014;98:706.
10. Presentation: Sun MT, Wu A, Huilgol SC, Selva D. Accuracy of Biopsy in Subtyping Periocular Basal Cell Carcinoma. Annual Royal Australian and New Zealand College of Ophthalmologists Annual Scientific Congress 2014

Chapter Three

11. Manuscript: Sun MT, Andrew NH, O'Donnell B, McNab A, Huilgol S, Selva D. Periocular Squamous Cell Carcinoma: TNM Staging and Recurrence. Ophthalmology 2015;122:1512-6.
12. Presentation: Sun MT, Andrew N, O'Donnell B, McNab A, Huilgol S, Selva D. Periocular Squamous Cell Carcinoma: TNM Staging,

Management and Prognosis. European Society of Ophthalmology Annual Congress, 2015

Chapter Four

13. Manuscript: Sun MT, Pham DT, O'Connor AJ, Wood J, Casson R, Selva D, Costi J. The Biomechanics of eyelid tarsus tissue. Journal of Biomechanics 2015;48:3455-9.
14. Presentation: Sun MT, Pham D, O'Connor A, Wood J, Casson R, Selva D, Costi J. The Biomechanics of Eyelid Tarsus Tissue. British Oculoplastic Surgical Society Annual Congress, 2015

Chapter Five and Six

15. Presentation: Sun MT, O'Connor A, Wood J, Casson R, Milne I, Biswa D, Selva D. Bioengineering Eyelids. Annual Royal Australian and New Zealand College of Ophthalmologists Annual Scientific Congress 2016

CHAPTER ONE: LITERATURE REVIEW

1.1 Overview

Australia experiences some of the most intense ultraviolet radiation levels worldwide with resultant rates of skin cancer amongst the highest around the globe.[1, 2] Basal cell carcinoma is the most commonly encountered skin cancer, accounting for 68% of all cases, and squamous cell carcinoma is the second most common skin cancer, making up 28% of all cases.[3] Although these cancers are not reportable and thus generally not recorded in cancer registries, they are by far the most frequently diagnosed malignancies nation-wide.[1] The eyelid and periocular region is highly susceptible and represents one of the most common sites for non-melanoma skin cancers. This region is involved in up to 10% of all cases, of which 95% are either basal cell carcinoma or squamous cell carcinoma.[4] Although metastasis is uncommon, local invasion results in notable associated morbidity, especially when involving vital ocular structures. It is therefore imperative that these periocular tumours are recognised early, staged appropriately, and high-risk features identified promptly to ensure adequate treatment and surveillance. Despite how frequently these cancers are encountered and treated, significant deficiencies remain in our understanding of the diagnosis, staging and outcomes of disease occurring in the periocular region.

Surgical excision remains the gold-standard treatment for skin cancer and is the most common indication for eyelid reconstruction. Whilst excision with a margin of 3-4mm for uncomplicated tumours and 4-5mm for more aggressive cases remains relatively straightforward in most anatomic locations,[5] the

eyelids represent a significant challenge given the delicate tissue and vital surrounding ocular structures. Poorly reconstructed eyelids can result in marked local morbidity and visual impairment either from malposition, corneal exposure and keratopathy and/or tear drainage dysfunction. In cases of large tumours which involve the full thickness of the eyelid, adequate substitution of the tarsus tissue is fundamental to the overall outcomes of reconstruction. The tarsus tissue is a specialised fibrous connective tissue found in both the upper and lower eyelid which provides both physical form and structural support.[6] It is vital for normal eyelid function, movement and cosmetic appearance but there remains a lack of knowledge regarding the normal biomechanical properties of this highly unique tissue. Thus, finding an appropriate replacement for tarsus tissue remains a challenge, and despite numerous autologous and artificial substitutes, there is yet to be an entirely satisfactory solution. Increasing interest in tissue engineering over the past decade has opened up new and exciting opportunities to create artificial tissue replacements tailor made to mimic native tissue. This, however, remains relatively unexplored in the field of oculoplastic ophthalmology but has the potential to refine and advance the way we approach reconstructive surgery.

1.2 Periocular Skin Cancer

1.2.1 Introduction to Periocular Skin Cancer

Rates of skin cancer in Australia are amongst the highest in the world, and the periocular region is highly susceptible. Skin cancers account for around 80% of all newly diagnosed malignancies and the incidence of skin cancer in Australia has continued to rise in the past few decades, with two in three Australians being diagnosed with skin cancer before the age of 70.[7]

Basal cell carcinoma, squamous cell carcinoma and melanoma represent the three main types of skin cancer. Non-melanoma skin cancers (basal cell carcinoma and squamous cell carcinoma) are the most commonly diagnosed cancers in Australia and involve the eyelid in up to 10% of all cases. Each year in Australia, over 434,000 people are treated for one or more non-melanoma skin cancers, with a 14% increase in general practitioner consultations between 1998 and 2007.[8] In 2001, it was estimated that 2% of the entire Australian population were treated for non-melanoma skin cancer, costing a total of $264 million and placing significant constraints on our health care system. This figure is only set to rise with the ageing population of Australia.

Non-melanoma skin cancers develop due to a combination of factors which include environmental, genetic and phenotypical. However, exposure to ultraviolet light represents the most important risk factor, and is associated with 90% of non-melanoma skin cancers.[9] The timing and pattern of exposure, along with certain complexions, are also known to affect the risk of

skin cancer development, with fair-skinned individuals suffering repeated episodes of sunburn during childhood most at risk.[10-12] A previous study has found that each 8-10^{o} of latitude closer to the equator is associated with a doubling in squamous cell carcinoma incidence.[13] DNA damage caused by exposure to ultra-violet light results in the activation of various oncogenes and inactivation of tumour suppression genes which ultimately give rise to abnormal cell proliferation. The two main genes that contribute to the pathogenesis of malignancy when mutated include oncogenes and tumour suppressor genes.[14] In 1996, mutation of the *patched 1* gene (*PTCH1)* on chromosome 9q22.3 was found to be associated with basal cell naevus syndrome (Gorlin Syndrome), and a gene mutation of *PTCH1* has since been found in up to 70% of sporadic basal cell carcinomas.[15, 16] *PTCH1* normally acts as a tumour suppressor, blocking the sonic hedgehog signalling pathway by antagonising Smoothened receptor activity. While basal cell carcinoma has no precursor lesion, squamous cell carcinoma is known to develop from actinic keratosis, which tend to occur on chronically sun-exposed areas. Squamous cell carcinoma development is thought to involve cell proliferative and anti-apoptotic pathways including mutations of the oncogene *RAS*, inactivation of the tumour suppressive gene *TP53* and *p16/CDKN24*, and overexpression of epidermal growth factor receptor.[17, 18] In an attempt to target the above genetic pathways which are known to cause cancer, a number of therapeutic agents have been studied and are discussed in more detail in the relevant sections below.

Other established risk factors for the development of both basal cell carcinoma and squamous cell carcinoma include ionising radiation, immunosuppression, chemical exposure, psoralen and long-wave ultraviolet radiation.[19-22] The risk of developing both basal cell carcinoma and squamous cell carcinoma following solid organ transplant patients is significantly increased, with rates of squamous cell carcinoma several hundred times higher than the general population.[23, 24] Furthermore, human papillomavirus infection, specifically HPV-16, has been implicated as a significant risk factor for squamous cell carcinoma of the head and neck.[25]

1.2.2 Basal Cell Carcinoma

1.2.2.1 Introduction

Basal cell carcinoma represents the most common form of skin cancer in Australia, Europe and the United States, accounting for 90% of all eyelid malignancies.[26] While basal cell carcinomas rarely metastasise, they can be locally destructive and lead to significant morbidity, especially when located in the periocular area.[27] Histologically aggressive basal cell carcinoma (infiltrative, micronodular and basosquamous subtypes) are associated with higher rates of recurrence, as well as increased risk of perineural and perivascular invasion.[28] The various histological subtypes and challenges associated with the management of more aggressive tumours in the periocular area are discussed in further detail below. Furthermore, in an invited review of basal cell carcinoma with orbital invasion, we summarised the available literature and provided recommendations for the management of this challenging condition.[29]

1.2.2.2 Basal Cell Carcinoma Histological Subtypes

The classifications of histological subtypes advocated by the World Health Organisation and the Royal College of Pathologists have been widely adopted in the recent literature.[30, 31] The main growth patterns recognised include superficial, nodular, infiltrative and micronodular, with the latter two subtypes associated with significantly increased risk of local recurrence and overall morbidity.[31] Nodular basal cell carcinoma has been known to occur more frequently on head and neck, whilst infiltrative basal cell carcinoma predominates over superficial basal cell carcinoma on the face.[32-36] This is of

particular significance in the periocular region, where local invasion can result in significant morbidity and thus higher rates of aggressive basal cell carcinomas may require changes to standard treatment.

To better define the histological subtypes of basal cell carcinoma occurring in the periocular region, we retrospectively reviewed the pathological reports of consecutive basal cell carcinomas treated at the at the Institute of Medical and Veterinary Science (IMVS) Main Laboratory, Adelaide, in the 7-year period from 2006 to 2013.[37] Of the 1915 periocular basal cell carcinomas reviewed, the most common histological subtype was nodular (1393, 65.1%) followed by infiltrative (377, 17.6%), superficial (277, 12.9%) and micronodular (93, 4.3%). Tumours occurred on the lower lid most frequently (822, 51.8%), followed by the medial canthus (466, 29.4%, the lateral canthus (178, 11.2%) and upper lid (120, 7.6%). Infiltrative basal cell carcinoma accounted for 25.6% of all lateral canthal tumours, compared to 19.1% of lower lid, 16.2% of medial canthal and 10% of lower lid lesions. Squamous differentiation was identified in 4.8% of cases and perineural invasion was present in 0.9% of cases. A quarter of specimens contained more than one subtype, with the most common subtype combinations being nodular with infiltrative (49.6%), and nodular with superficial (25.8%).

When comparing our results to other reports in the literature, we found higher rates of infiltrative basal cell carcinoma compared to the superficial subtype, consistent with previous reports from Townsville, Brisbane and the United Kingdom of facial and periocular basal cell carcinoma.[33, 38, 39] Our findings

may be attributable to the theory which suggests that superficial basal cell carcinoma results from acute, intense sun exposure to skin that is less exposed to sunlight and thus less tanned.[40] As the face and periocular region are chronically exposed to sunlight and more tanned, fewer superficial basal cell carcinoma may develop at these sites.[32-34] Another theory suggests that basal cell carcinoma subtype progresses from superficial to nodular to infiltrative and therefore if chronic sun exposure triggers progression to infiltrative basal cell carcinoma, more infiltrative basal cell carcinoma may develop in the periocular region.[41]

We found that there was mixed histology in 24.9% of our specimens while a previous study by Ho and colleagues identified mixed histology in 17.9% of their periocular basal cell carcinomas.[39] Our most common subtype combinations of nodular with infiltrative and nodular with superficial were consistent with previous studies.[32] Mixed histology has been reported to occur in up to 38.5% of all basal cell carcinoma and can result in an initial biopsy which misses one or more additional aggressive subtypes.[42-49] If an aggressive subtype is missed, initial treatment may be inadequate, thereby predisposing to recurrent disease. As the proportion of mixed basal cell carcinoma is high and aggressive subtypes require modified treatment, surgical excision with margin control may be favoured over nonsurgical destructive modalities for periocular basal cell carcinoma.[28, 50, 51] Further studies examining the accuracy of biopsy for periocular basal cell carcinoma are required to better guide our interpretation of these initial histology reports.

1.2.2.3 Basal Cell Carcinoma with Orbital Invasion

The reported incidence of orbital invasion by periocular basal cell carcinoma ranges from 0.8% to 5.5%.[27, 38, 52-57] Risk factors of orbital invasion include male gender, multiple recurrences, large lesion size, aggressive histologic subtype, perineural invasion, medial canthal location, and advanced patient age.[27, 55] Although rare, local invasion of periocular basal cell carcinoma into orbital tissues can lead to intracranial involvement and death.[52, 58]

A higher incidence of orbital invasion by periocular basal cell carcinoma in male patients has been reported, with up to 77% of patients being male.[27, 52, 53, 55, 59-61] This has been attributed to more aggressive disease,[27, 59] with delayed tumour diagnosis also potentially playing a role.[62] Although basal cell carcinoma is most commonly diagnosed between the ages of 40 and 80 years, the mean age of patients presenting with orbital invasion tends to be higher at around 68 to 70 years.[27, 53, 55]

The most common initial site for basal cell carcinomas demonstrating orbital invasion is the medial canthus (53.6% to 56.2%), followed by the lower eyelid (20.3% to 35.7%), the upper eyelid (4.7% to 7.1%), and the lateral canthus (3.6% to 18.7%).[27, 55] Previous studies have suggested that sheets of tumour can spread deeply along the orbital wall periosteum, which may predispose the lateral and medial canthal locations given the close proximity of the skin to the periosteum in these regions.[27, 63] This theory has been supported by previous reports in the literature which have found a higher risk of orbital invasion and mortality with medial canthal basal cell carcinomas.[6,15-17]

The most commonly encountered basal cell carcinoma histological subtypes are the less aggressive nodular and superficial types, with the more aggressive subtypes such as infiltrative, morpheaform/sclerosing and basosquamous making up only 5-7% of all cutaneous basal cell carcinoma. However, in cases of basal cell carcinoma with orbital invasion, the more aggressive subtypes account for more than 80% of cases, with infiltrative basal cell carcinoma being the most commonly reported subtype encountered, making up 51.6%-78.6% of these cases.[27, 52, 53]

Perineural invasion occurs in less than 1% of basal cell carcinoma, and tumours with perineural invasion are regarded as more aggressive and associated with higher rates of recurrent disease.[64] Basal cell carcinoma with perineural invasion can be challenging to manage, as tumours can present long after primary tumour removal, and can be associated with skip areas which complicate margin control.[27, 28, 64] It has been suggested that low resistance cleavage plans of the perineural sheath can facilitate rapid and broad tumour extension.[27, 28] In a review of periocular basal cell carcinoma with orbital invasion, Leibovitch and colleagues found that 19.3% of patients had evidence of perineural invasion, and of these, 91.7% were recurrent tumours and all were of an aggressive histologic subtype.[27]

Long-standing or neglected tumours, as well as recurrent or incompletely excised tumours, are contributing factors to more aggressive behaviour of basal cell carcinoma.[27, 28] Previous reports have revealed that 71.4% to

84.4% of patients with orbital invasion by basal cell carcinoma have either a recurrent or previously incompletely excised tumours, while the remainder present with orbital invasion at the first visit.[27, 52, 53, 55] The presence of scar tissue after previous excision may obscure monitoring and delay clinical detection of recurrence.[27, 28, 52, 65] In addition, fibrosis may entrap malignant cells and favour orbital extension by preventing superficial migration of cells.[27, 52]

The mean duration from the first tumour excision to the diagnosis of orbital invasion in recurrent tumours have been reported to range from approximately 7.8 to 9.8 years.[27, 53, 66] The mean duration from first symptoms or signs of the tumour to the diagnosis of orbital invasion in primary cases may be 3.5 years,[27] which may reflect delayed diagnosis or neglect.[54]

1.2.2.4 Clinical Presentation and Investigation of Basal Cell Carcinoma with Orbital Invasion

Almost all patients with orbital invasion present with a visible or palpable mass,[27, 53, 55] which is fixed to bone in 35.7% to 40.6% of cases.[27, 55] Other common signs previously reported include limitation of ocular motility in 30.4% to 40.0% of cases, and globe displacement in 17.6% to 18.7% of cases.[27, 55, 67] Additional clinical signs can include immobile lids and ptosis.[27, 52, 54, 58] Epiphora, secondary to either canalicular or nasolacrimal sac involvement, may be common (60%) in medial canthal basal cell carcinomas.[52] Although rare, perineural spread of basal cell carcinoma may potentially lead to sensory loss in the periocular region and facial nerve involvement.[54] In two major

series, 31.3% and 35.7% of patients had only a visible or palpable mass with no clear signs of orbital invasion.[27, 55] Thus, orbital invasion may exist without the presence of orbital signs, in which case the diagnosis requires a high index of suspicion.[27, 68] The presence of risk factors including visible or palpable mass fixed to bone, limited ocular motility, globe displacement, ptosis, epiphora and sensory disturbance should raise the possibility of subclinical orbital invasion and further investigation with imaging should be considered.[27, 55]

Imaging of patients with suspected orbital invasion can demonstrate bone and soft tissue involvement. Computed tomography with bone windows is preferred for visualising bony destruction, whereas T1-contrast enhanced, fat suppressed magnetic resonance imaging is best for demonstrating soft tissue changes and perineural invasion.[27, 53-55, 62, 69] In 20.6% to 30.8% of patients, there is evidence of orbital bone destruction on computed tomography.[27, 53, 55] Soft tissue involvement can usually be visualised as a homogenous, mildly enhancing mass, with irregular borders. Other imaging findings can include: rectus muscle infiltration, lacrimal sac involvement, or extension into the ethmoid sinus, cribriform plate, or invasion through the superior orbital fissure to involve the dura, cavernous sinus, and cerebral tissue.[27] Magnetic resonance imaging can provide useful information regarding the extent of perineural invasion but is unable to exclude this definitively as many patients with symptomatic perineural invasion have no radiologic evidence of disease.[52, 64, 70]

1.2.2.5 Management of Basal Cell Carcinoma with Orbital Invasion

The management of periocular basal cell carcinoma with orbital invasion is challenging and often requires a multidisciplinary approach incorporating ophthalmology and oculoplastics, radiation oncology, craniofacial surgery, otolaryngology, dermatology, and, with the advent of targeted therapy, opinions from medical oncology may also prove beneficial.[62] Treatment should be individualised for each patient, taking into account factors such as the extent of orbital involvement, visual function, as well as general medical health.

Exenteration is the treatment of choice for patients with bulbar or extensive orbital invasion.[27, 53, 55, 56, 60-62, 66, 69] Total orbital exenteration involves complete removal of the globe and all orbital contents including periorbital while subtotal orbital exenteration involves removal of the globe with partial preservation of orbital tissues.[62, 71] More aggressive tumours may extend beyond the orbit into the ethmoid and frontal sinuses or involve the bony orbital walls. These tumours may require extended exenteration with removal of bone and extraorbital tissue often with a combined craniofacial approach.[62, 72]

The aim of orbital exenteration, as with any radical oncological operation, should be a surgical cure. This usually involves removal of bone where there is fixation of the tumour mass to the bone or bony erosion with the extent of removal determined by clinical and imaging findings. Monitoring of surgical margins with intraoperative frozen section, or delayed paraffin sections can be

performed and repair of the surgical defect delayed until clear margins are obtained. Reconstruction of the exenterated orbit usually involves the use of split-thickness skin grafts, temporalis muscle flaps or free flaps. If the orbital walls are intact, then healing by secondary intention is also an option. Patients who undergo exenteration can be fitted with a prosthesis to improve the aesthetic outcome.[62]

Complications are known to occur in just under a quarter (23.5%) of patients, and can include fistula formation into the nose, a sinus, or nasolacrimal duct, tissue necrosis with eschar formation, chronic drainage and/or infection, chronically exposed bone, cerebrospinal fluid leak, and pain.[71, 73] Management of large fistulae and exposed bone involves the use of a temporalis muscle flap or other local flaps.[73-75] Less commonly, a cerebrospinal fluid leak may result from cautery or other trauma to the orbital roof.[62, 71, 73, 76, 77]

In selected cases, globe-sparing local excision with or without radiotherapy and close follow-up with regular imaging (preferably magnetic resonance imaging) is an alternative option.[27] These cases include patients with anterior orbital involvement only, in patients with a single eye or where a patient declines exenteration.[27, 54] Margin control should be strongly considered for cases treated with local excision. Mohs micrographic surgery is usually not appropriate in the setting of deep orbital invasion because of the difficulty of obtaining correctly orientated specimens in orbital soft tissues.[54] Additionally, there is a significant risk of false-negative results with standard frozen section

techniques.[52, 58, 78] Therefore the use of paraffin section remains the margin control of choice for basal cell carcinoma with orbital invasion, as it provides high-quality tissue morphology, including cases involving orbital fat.[52]

Adjuvant radiotherapy or exenteration may be considered if margins are involved and further resection with globe preservation is not possible. Complications of globe-sparing surgery are usually treatable and include restriction of ocular motility, epiphora secondary to loss of the nasolacrimal apparatus and abnormal lid position.[52] Follow-up imaging should be performed in all patients, and magnetic resonance imaging is preferable as it has the ability to detect early posterior recurrences, which may sometimes occur in the absence of superficial recurrence due to the presence of scar tissue.[27, 52, 65] Annual postoperative magnetic resonance imaging for at least 5 years has been recommended for patients treated with globe-sparing surgery for basal cell carcinoma with orbital invasion.[52]

The decision to exenterate or to pursue a conservative surgical course is one to be made after a detailed discussion with the patient. In the setting of primary medial canthal basal cell carcinoma with limited anterior orbital invasion, a globe-sparing approach may be considered. There should be a high expectation of postoperative complications and possible revision procedures. In the presence of features associated with a higher risk of recurrence, such as recurrent disease, perineural invasion, or restriction of extraocular movements preoperatively, exenteration may be the preferred treatment choice.[52]

Exenteration may be combined with adjunctive radiotherapy in cases in which margins are not clear, in high-risk aggressive tumours with perineural invasion, or in residual inoperable tumours.[27, 55, 79, 80] The role of radiotherapy as an adjunct to surgery requires further controlled studies, and thus far, studies have demonstrated no differences in recurrence rates of those treated with exenteration alone and exenteration followed by radiotherapy.[55] The value of radiotherapy as adjunctive therapy to excision with clear margins should be weighed against the possible ocular side effects if the globe is to be retained.[27, 54] Possible side effects of periorbital radiation therapy include cataract formation, ectropion, stenosis of the lacrimal duct, radiation retinopathy, neovascular glaucoma, radiation optic neuropathy, and blindness.[27, 62, 81, 82] Ocular side effects from radiotherapy may occur in up to 20% of patients treated with either local excision and radiotherapy or radiotherapy alone.[27] Due to the propensity for tumour development in irradiated skin, this should be considered carefully in younger patients who are at risk of developing secondary skin cancers related to radiation therapy.[62, 81] Basal cell carcinomas recurring after radiotherapy are more difficult to diagnose and manage due to the tissue changes induced by radiation which are similar to those induced by the tumour.[83, 84]

Although exenteration remains the best treatment for basal cell carcinoma with orbital invasion, patients who are poor surgical candidates and those who decline surgery may undergo radiation therapy alone. Around 25% of patients who undergo radiation therapy alone for orbital basal cell carcinoma with

invasion develop recurrent disease.[27, 62] Cisplatin-based chemotherapy, given alone or in combination with doxorubicin, bleomycin, 5-flurouracil, or methotrexate are the most commonly used agents for locally advanced basal cell carcinoma.[62, 85] Overall response rates from small studies range from 68-77%, with complete response rates reported at 28-44%.[62, 85, 86] Chemotherapy may also be used to reduce tumour size prior to surgery or as an adjuvant therapy in patients who have failed surgery and/or radiation therapy.[62, 87] Previous studies have also demonstrated success with systemic cisplatin and doxorubicin in treating recurrent invasive basal cell carcinoma of the medial canthus and orbit.[88, 89]

More recently, oral Vismodegib has been trialled in patients with locally advanced periocular basal cell carcinoma with response rates in about half of all cases.[90] Vismeodegib is an oral inhibitor of the Hedgehog pathway which counteracts Smoothened receptor activity and was approved by the FDA in 2012 for treatment of locally advanced and metastatic basal cell carcinoma.[91] It is important to note however, that serious adverse effects were reported in 25% of patients in an initial study on the safety and efficacy of Vismodegib.[91] Further studies are currently underway and are required prior to more mainstream use.

Regular, long-term follow up should be conducted in all patients. The recurrence rate may be lower for exenteration than local excision or radiotherapy alone, with recurrence rates reported to range from 2.8% to 28.5% after mean follow-up periods of approximately 3 years.[27, 55] The

recurrence rate for medial canthal basal cell carcinoma with orbital invasion treated with globe-sparing surgery is reported to be 5% at a mean follow-up of 3.2 years.[52] However this study included less advanced tumours with fewer patients presenting with limited ocular movements (5%) and perineural invasion (5%) compared to other series of mainly exenterated patients.[27, 52] It is likely that the recurrence rate will be significantly higher with longer follow up and further studies are required.

1.2.3 Squamous Cell Carcinoma

1.2.3.1 Introduction

Squamous cell carcinoma is the second most common cutaneous malignancy and accounts for 5-10% of periocular cutaneous tumours.[92, 93] Established risk factors as described above include ultraviolet light exposure, toxin exposure, radiation, immunosuppression, pre-existing chronic skin lesions, albinism and several genetic skin disorders.[94, 95] Although less common than basal cell carcinoma, squamous cell carcinoma is associated with significantly higher morbidity as these tumours tend to behave more aggressively and are known to metastasise. Locally recurrent disease can be particularly difficult to manage, as these tumours are generally more locally destructive, complicating reconstruction following excision. Numerous factors have been associated with increased risk of local recurrence including increased tumour size, thickness, or depth, poor histologic differentiation, perineural invasion, locally recurrent tumours and immunosuppression.[96-98] The histological subtypes of squamous cell carcinoma and management of aggressive tumours in the periocular region is discussed in more detail below.

1.2.3.2 Histological Features and Subtypes

There are numerous variants of squamous cell carcinoma with a wide range of clinical behaviours impacting upon prognosis.[99, 100] Widely accepted histological subtypes included classic, adenoid, verrucous and spindle cell squamous cell carcinoma, with additional subtypes including clear cell, signet ring cell, pigmented, inflammatory, basaloid, desmoplastic and infiltrative squamous cell carcinoma.[101]

Most recently, Cassarino and colleagues performed a comprehensive review of the available literature on squamous cell carcinoma variants and using established behaviours of various subtypes, devised a classification for squamous cell carcinoma based upon risk of metastasis.[99, 100] Low risk squamous cell carcinomas (≤ 2% recurrence risk) included squamous cell carcinoma arising from actinic keratosis, squamous cell carcinoma associated with human papilloma virus, spindle-cell squamous cell carcinoma and tricholemmal carcinoma. Intermediate risk squamous cell carcinoma (3-10% recurrence risk) included adenoid/acantholytic squamous cell carcinoma, lymphoepithelioma-like carcinoma of the skin and intraepidermal epithelioma (Jadassohn type) with invasion. High risk squamous cell carcinomas (>10% recurrence risk) included invasive Bowen disease, de novo squamous cell carcinoma, squamous cell carcinoma secondary to predisposing factors including immunosuppression, radiation and burns, adenosquamous carcinoma and squamous cell carcinoma arising in a proliferating pilar tumour or cyst. There were also a number of rare subtypes of indeterminate malignant potential and these include clear cell squamous cell carcinoma, signet cell squamous cell carcinoma, papillary squamous cell carcinoma, pigmented squamous cell carcinoma, follicular squamous cell carcinoma, squamous eccrine ductal carcinoma and squamous cell carcinoma arising from adnexal cysts.

1.2.3.3 Prognostic Factors

Regional or distant metastatic disease in cutaneous squamous cell carcinoma is associated with a significantly worse prognosis with 5-year mortality rates ranging from 55 to 75%.[22, 96, 102] In addition to the high-risk histological subtypes, there are a number of other factors which predispose to higher rates to metastasis and recurrence.[22] In 1932, Broders first proposed a grading system based upon cell differentiation, with grade I being tumours with more than 75% of cells differentiated, grade II with 50-75% differentiation, grade III with 25-50% differentiation and grade IV with <25% cell differentiation.[103] This grading was never widely adopted though, with the majority of pathologists tending to refer to lesions as well differentiated (low proportion of anaplastic cells with architecture closely resembling normal epidermis), moderately differentiated (at least half the cells are anaplastic) and poorly differentiated (all cells are anaplastic) instead.[22, 92] Rowe and colleagues found that poorly differentiated squamous cell carcinomas were associated with a metastatic rate of 32.8%, compared to 9.2% of well differentiated tumours and the risk of local recurrence was twice that of well differentiated squamous cell carcinomas (28.6% vs 13.6%).[96] Squamous cell carcinomas that are moderately and poorly differentiated are also associated with higher rates of subclinical extension.[104, 105]

Location plays an important role. The periocular region, along with the auricular and nasal regions are considered high-risk areas.[106] Recurrent squamous cell carcinomas are themselves associated with a five-fold higher rate of metastasis and three-fold higher rate of further recurrence compared

with primary tumours.[96, 107] Squamous cell carcinomas measuring greater than 2cm have been found to be twice as likely to recur and be associated with three-fold increased metastatic rate.[96, 97] Previous studies have also shown that increasing thickness is associated with higher metastatic potential,[22, 108] with depth greater than 4mm being found to have 45% rate of metastasis compared with just 6.7% for superficial tumours.[96, 97] In keeping with this, tumours invading into deep structures such as muscle, bone or cartilage have also been found to confer a higher risk of metastasis and recurrence.[96, 108]

Perineural invasion is known to be associated with higher rates of metastasis, recurrence and morbidity and is widely recognised as a poor prognostic factor.[109-112] Perineural invasion can be both intratumoural, with invasion noted within the tumour mass, and extratumoural, with perineural invasion outside of the main tumour. Intratumoural perineural invasion is not known to affect clinical prognosis and is relatively common, and thus perineural invasion associated with more aggressive tumours always refers to extratumoural invasion.[22] Rates of recurrence and metastases have been reported to be as high as 47% and 35% respectively for head and neck squamous cell carcinoma with perineural invasion treated with excision only.[111]

Underlying predisposing factors including immunosuppression,[104] especially after solid organ transplantation,[96, 113] and chronically injured skin from previous radiotherapy, chronic ulceration and thermal injuries are known to be

associated with more aggressive tumours and higher rates of both recurrence and metastases.[96, 97, 100] Younger patients and male patients have been both previously been found to have higher rates of recurrence compared to patients over 60 years of age and female patients respectively.[114, 115] Leibovitch and colleagues also previously noted an associated between male gender and poorly differentiated squamous cell carcinoma.[105]

1.2.3.4 Surgical Management of Squamous Cell Carcinoma

Surgical excision with histological margin control or with Mohs micrographic surgery remains the treatment of choice with the lowest associated recurrence rates.[4, 88] For low-risk squamous cell carcinoma, 4-mm margins have been found to achieve a 95% clearance rates in a study of 141 cutaneous squamous cell carcinomas by Brodland and Zitelli.[116] For tumours measuring 20mm or larger, more aggressive histological grade, high-risk location or subcutaneous tissue invasion, margins of 6mm with excision to include subcutaneous fat were recommended.[116] However, when these guidelines are applied to the delicate periocular region, significant morbidity can result from challenging functional reconstruction and thus immediate histological margin monitoring with frozen section may be preferred to preserve maximal tissue whilst still ensuring complete tumour excision.[22] In a review of 51 cases of periocular squamous cell carcinoma treated with frozen-section controlled excision, Donaldson et al found a recurrence rate of 2.0% during a mean follow-up of 31.1 months.[94] Without margin control, reported 5-year recurrence rates range from 5% to 18.7% for primary cutaneous squamous

cell carcinomas in all locations and is likely to reflect subclinical extension.[117, 118]

Standard frozen section histological analysis with bread-loafing sections is known to examine less than 1% of the margins, is subject to skip areas, and up to 5% of tumours reported to have clear margins have been later found to be associated with incomplete excision.[119, 120] As such, several studies advocate the use of Mohs micrographic surgery or en-face frozen section, which examines the entire deep and peripheral margins. Mohs first reported a 5-year cure rate of 98.1% for eyelid squamous cell carcinoma excised using Mohs micrographic surgery,[121] and more recently Malhotra and colleagues confirmed comparably low recurrence rate of 3.6% during a median follow-up of 5 years.[104] Nemet and colleagues utilised a 5mm safety margin for periocular squamous cell carcinoma with margin control using immediate frozen section or Mohs micrographic surgery. The authors reported a combined recurrence rate of 5.9%, but found that for cases with incomplete initial excision, there were no reports of further recurrence following re-excision with Mohs micrographic surgery compared to 4.7% of tumours which recurred following re-excision with frozen-section control.[122] However, widespread use Mohs micrographic surgery and en-face frozen section control are sometimes limited by time and expense, and thus adequate assessment of high-risk features combined with functional reconstruction remain important considerations when managing periocular squamous cell carcinoma.

1.2.3.5 Non-Surgical Management

For patients who are poor surgical candidates or who decline surgical intervention, non-surgical options include cryotherapy, radiotherapy, chemotherapy and newer targeted oral therapies.[22]

Cryotherapy is generally reserved for small, low-risk squamous cell carcinomas with well-delineated borders when other treatment options are unavailable and should be used with caution near the lacrimal system due to potential for local damage.[123, 124]

Radiotherapy alone has been found to be associated with a 5-year recurrence ate of 12.5% for periocular squamous cell carcinoma which increases to 50% for recurrent or advanced lesions.[125, 126] Adjuvant radiotherapy combined with surgical excision has been recommended for high-risk cases, in particular perineural invasion, and can improve outcomes and reduce the risk of morbidity and mortality associated with recurrent and/or metastatic disease.[94, 127]

Chemotherapy agents previously studied for squamous cell carcinoma include cisplatin, doxorubicin, bleomycin, peplomycin, 5-fluorouracil and methotrexate with the majority of cases responsive.[128] Targeted therapies have also emerged in recent years but have generally been reserved for advanced cases and those with distant metastatic disease. Gefitinib is a tyrosine kinase inhibitor that selectively inhibits EGFR-stimulated tumour cell proliferation and has been trialled for recurrent or aggressive squamous cell carcinoma.[129]

Erlotinib is also a tyrosine kinase inhibitor with similar mechanism of action but clinical trials have yet to be completed for squamous cell carcinoma, however early case reports have been promising for advanced cases.[130] Cetuximab is a monoclonal antibody to EGFR and is currently in phase II trials for metastatic and unresectable squamous cell carcinoma.[131]

1.3 The AJCC TNM Staging System

1.3.1 The TNM Staging System for Eyelid Carcinoma

The American Joint Committee on Cancer (AJCC) and International Union for Cancer Control TNM staging system for eyelid carcinoma was established to provide a standardised approach to staging in this unique anatomical location (Table 1).[132] Several changes to the 6th edition of the AJCC staging were made to allow for finer discrimination between tumour size and various invasion depths in the most updated 7th edition. The T2 category was subdivided into T2a and T2b to differentiate between tumours which exceeded 10mm but were less than 20mm, and/or those which involved the tarsal plate or eyelid margin. Similarly, T3 was subdivided into T3a and T3b. Stage T3a included all tumours which invaded adjacent ocular or orbital structures or any evidence of perineural invasion and T3b referred to tumours which required enucleation, exenteration or bony resection to fully excise.[133] The subdivision of T3 and changes to T4 were implemented with the goal of improving surgical correlation. The previous T4 stage in the 6th edition of AJCC staging including any tumour which invaded into the bulbar conjunctiva, sclera, globe and deeper structure, which meant that tumours with bulbar conjunctival invasion suitable for globe-sparing surgery were staged the same as deep orbital invasion requiring exenteration.

Additional differences in the updated staging system include subdivision of the nodal component into cN0 and pN0 depending on if the nodal metastasis is determined on the basis of clinical or pathological lymph node biopsy. Stages from 0-IV were also added, and are described in Table 2.[132] Stage I refers to

disease localised to the eyelid. Stage II refers to an eyelid lesion involving varying adjacent tissue which remains surgically resectable. Stage III includes any tumour with associated nodal disease, or an advanced tumour requiring enucleation, exenteration or a tumour that is non-operable. Any sign of distant metastatic disease then becomes stage IV.

Several limitations have previously been raised regarding TNM staging for eyelid carcinoma and general cutaneous skin cancers. Some authors have noted that the 7th edition eyelid carcinoma T4 designation does result in some subjective differences between various institutions, with an example being that of a large eyelid carcinoma invading the orbit and paranasal sinuses which may be considered unresectable in certain hospitals but resectable in a tertiary care specialist centre. Additionally, in the introduction to the 7th edition of the eyelid carcinoma staging, Ainbinder et al. raised the potential limitation of medial canthal tumours being without their own biomarker status.[132] Tumours involving the medial canthal region are known to be associated with a higher risk of locoregional recurrence and orbital invasion and thus are often managed in the same fashion as tumours with high-risk features.[132] Furthermore, Buethe and colleagues previously raised concern regarding the grouping of non-melanoma and non-Merkel cell skin cancers together despite significant clinical variation between subtypes in the AJCC cutaneous squamous cell carcinoma and other skin cancers section. The authors noted that certain tumours such as keratocanthoma can be inappropriately upstaged despite their benign behaviour requiring only very conservative management. As such, the authors suggested that clarification is required for very benign

tumours including basal cell carcinoma and other adnexal tumours which may not require full pathological staging evaluation.[134] Although the eyelid is regarded a high-risk location, basal cell carcinomas are known to still behave in benign manner in the majority of eyelid cases, and thus staging may also be more prudent for squamous cell carcinoma and other more aggressive skin cancers.

Since establishment, however, there have been few studies on periocular malignancy utilising this updated staging system and these are discussed in detail below. As such, the role of the AJCC TNM staging system in predicting outcomes and guiding management remains unclear and further studies are required to better evaluate its application and prognostic ability.

1.3.2 Implementing the TNM Staging

As mentioned, since the establishment of the AJCC staging system for eyelid carcinoma, there have been few studies which studied its application, although these have increased in the last few years as the staging system becomes more widely implemented.

A number of studies have examined the feasibility of the AJCC staging system. Shinder et al.[133] retrospectively staged 27 patients with eyelid carcinoma which included 17 cases of basal cell carcinoma, 3 cases of squamous cell carcinoma, 6 patients with sebaceous gland carcinoma and a single case of Merkel cell carcinoma. The authors compared the 6th and 7th editions of the AJCC staging and noted significant differences, with the

number of T2 and T3 tumours increasing (7 to 11 and 4 to 9 respectively) and the number of T4 tumours decreasing (from 10 to 1). Similarly, Crawford and colleagues retrospectively staged 50 cases of eyelid carcinoma using the AJCC 7th edition, with nodular basal cell carcinoma being the most common histology (85%), followed by morpheaform basal cell carcinoma (9%), squamous cell carcinoma (4%) and sebaceous gland carcinoma (2%).[135] The authors found that the majority of tumours (72%) were staged at IA, 22% at IIA, 4% at IIB and 2% at stage III. High-risk features such as perineural spread, pagetoid spread, tumour necrosis and associated carcinoma syndromes were associated with a stage of IIB or higher in 80%, and all tumours stage II and higher. It is unclear however, which types of tumours were graded at which stage, and correlation of outcomes with initial AJCC stage were not evaluated.

1.3.3 TNM as a Predictor of Outcomes

There has been only one previous study of periocular squamous cell carcinomas utilising the AJCC 7th edition of the TNM staging system to correlate initial stage with outcomes. Nasser and colleagues studied 51 primary and 14 recurrent periocular squamous cell carcinomas in order to investigate whether T-stage correlated with risk of regional nodal metastases.[136] The authors retrospectively staged patients based upon clinical, pathological and radiological data and found that all but two patients presented with disease localised to the eyelid with T3aN0M0 being the most commonly encountered TNM stage (22/51 cases, 43%). The other TNM stages were as follows: T1N0M0 (6 patients), T2aN0M0 (11 patients),

T2bN0M0 (17 patients), T2bN1M0 (2 patients), T3bN0M0 (2 patients), T3bN1M0 (1 patient). During a median follow-up time of 67.0 years, there were seven local recurrences occurring patients with the following TNM stages: T2aN0M0 (2 cases), T2bN0M0 (1 case) and T3aN0M0 (5 cases). The authors also found that regional nodal metastases occurred in 6% of cases and was only observed in patients presenting with T2b tumours or higher and tumours measuring 18mm or greater. These included four patients with nodal metastasis at presentation involving tumours with T-stages of T2b (two patients), T3b (one patient) and T4 (one patient) and two patients with T3aN0M0 and T4N0M0 tumours who developed nodal disease following initial treatment. The authors concluded that tumour size and AJCC T-stage correlated to metastases, and suggested consideration of sentinel lymph node biopsy or stricter nodal surveillance in patients with stage T2b or greater or tumours measuring 18mm or more.[136] However, it is unclear how many cases of nodal metastasis involved recurrent tumours, and there was a clear need for further studies utilising the updated AJCC TNM staging system to better characterise its role in periocular squamous cell carcinoma.

Sebaceous carcinoma is a rare but potentially fatal malignant neoplasm usually arising from the sebaceous glands of the eyelid. These tumours are notorious for masquerading as benign conditions, and are classically associated with significant morbidity and mortality due to delayed diagnosis.[137] In the first study correlating TNM staging with outcomes for sebaceous carcinoma of the eyelid, Esmaeli and colleagues reviewed fifty consecutive patients and found that disease-specific survival was poorer

among patients with T stage of T3a or worse.[138] The authors found that no tumours 9mm or less were associated with lymph node metastases and that T category at presentation was significantly associated with nodal metastasis but not local recurrence. Similarly, Choi et al. retrospectively reviewed forty patients treated for sebaceous gland carcinoma, correlating clinical tumour size, AJCC clinical stage and pathological AJCC T-stage with regional nodal or distant metastatic disease. The authors found that the clinical and pathological T-stage of T2b or higher were both significantly associated with metastatic disease as compared to T1 or T2a tumours but tumour size alone was not predictive.[139] In the largest review utilising the 7th edition of the TNM staging, Watanabe et al. studied 63 cases of eyelid sebaceous gland carcinoma and found that T3a tumours and greater were significantly associated with local recurrence but not regional nodal metastasis. Furthermore, in contrast to the findings by Esmaili and colleagues, the authors found that two patients with primary T2aN0M0 tumours measuring less than 9mm (5mm and 7mm) developed nodal metastasis during follow-up and thus recommended consideration of sentinel nodal biopsy or strict nodal surveillance in all patients regardless of initial TNM stage.[140]

Merkel cell carcinoma is an extremely rare but aggressive skin tumour which involves in the eyelids in 10% of cases and is associated with high rates of metastasis and estimated mortality of 25 to 35%.[141] In the largest study of patients with eyelid Merkel cell carcinoma, Herbert and colleagues reviewed 21 patients from five oculoplastic units within the United Kingdom and Australia.[142] All cases were staged with the AJCC 7th edition criteria for eyelid

carcinoma and Merkel cell carcinoma, and the study found that majority of eyelid Merkel cell carcinoma presented with localised eyelid disease of T-stage T2 (using the eyelid staging) or T1 (using the Merkel staging). The eyelid staging was found to enable finer discrimination of tumour size and invasion extent compared to the Merkel carcinoma staging, with tumours being staged into 6 different eyelid TNM stages and 4 different Merkel cell TNM stages. The authors found a local and regional nodal recurrence rate of 10%, and distant metastatic recurrence rate of 19%. There were two disease related mortalities (10%) both associated with eyelid stage T3a tumours. Furthermore, this study found that the lowest-grade tumour associated with both regional, and distant metastatic disease was T2aN0M0, suggesting that there is a role for sentinel lymph node biopsy or radiological nodal assessment, in conjunction with strict surveillance in all patients with primary eyelid Merkel cell carcinoma. Sniegowski et al.[143] also studied the correlation of the AJCC staging with outcomes in periocular Merkel cell carcinoma, reviewing 18 patients retrospectively. The authors found that a T-stage of T2b or worse was associated with significantly reduced disease free survival at 3 years, and that lymph node metastasis at presentation was predictive of both increased risk of metastatic disease and shorter disease free survival.

Table 1: Definitions of TNM for Eyelid Carcinoma, AJCC Cancer Staging Manual, Seventh Edition

Primary Tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour ≤ 5mm in greatest dimension; not invading tarsal plate or eyelid margin
T2a	Tumour >5mm, but not >10mm, in greatest dimension; or, any tumour that invades the tarsal plate or eyelid margin
T2b	Tumour >10mm, but not >20mm, in greatest dimension; or, involves full thickness eyelid
T3a	Tumour >20mm in greatest dimension; or, any tumour that invades adjacent ocular or orbital structures; any T with perineural invasion
T3b	Complete tumour resection requires enucleation, exenteration, or bone resection
T4	Tumour is not resectable because of extensive invasion of ocular, orbital or craniofacial structures, or brain
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed

cN0*	No regional lymph node metastasis, based on clinical evaluation or imaging
pN0†	No regional lymph node metastasis, based on lymph node biopsy
N1	Regional lymph node metastasis
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

Abbreviations: AJCC = American Joint Committee on Cancer, * Clinical N0, † Pathological N0

Table 2: Stage Grouping for Carcinoma of the Eyelid

Stage	T-stage	N-stage	M-stage
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2a	N0	M0
Stage IC	T2b	N0	M0
Stage II	T3a	N0	M0
Stage IIIA	T3b	N0	M0
Stage IIIB	Any T	N1	M0
Stage IIIC	T4	Any N	M0
Stage IV	Any T	Any N	M1

1.4 Eyelid Reconstruction and Eyelid Tarsal Substitutes

1.4.1 Indications for Eyelid Reconstruction

Eyelid reconstruction represents one of the most challenging areas of reconstructive plastic surgery for orbitofacial surgeons. This is due to a combination of anatomic complexity, functional considerations and aesthetic concerns. The most common indication for eyelid reconstruction is for defects due to surgical resection of cutaneous malignancies. As discussed above, the most common skin cancers occurring the periocular region include basal cell carcinoma and squamous cell carcinoma. Other less common causes for eyelid defects include trauma and occasionally congenital abnormalities.

1.4.2 Anatomic Considerations

A detailed appreciation of eyelid anatomy is necessary for adequate assessment and management of patients requiring reconstruction. Given this body of work concerns the repair of large defects requiring tarsus tissue substitutes, this will be the focus of the discussion below.

The eyelid is a bi-lamellar structure, composed of the anterior lamella consisting of skin and the orbicularis oculi muscle, and posterior lamella, which comprises of the tarsal plate and palpebral conjunctiva (Figure 1).[144, 145] The medial and lateral canthi also have important structural and functional roles and involve numerous structures including the lacrimal apparatus, tarsal plate, orbicularis muscle, eyelid retractors, orbital septum and cheek ligaments.[144] Large, full thickness eyelid defects requiring reconstruction of both the anterior and posterior lamella are among the most difficult to repair

due to the lack of suitable tarsal substitutes. The tarsus is a fibrocartilagenous structure which provides both support and structural form, making it an essential component of the eyelid's function and physical appearance. It is tightly adherent to the eyelid skin, measuring approximately 25mm in length and 1mm thick and 7-10mm vertically in the upper eyelid and 3.8mm in the lower eyelid.[146] The tarsal plate migrates laterally from its centralised position of the mid-pupillary line from childhood.[144] Natural tarsus is a specialised tissue, histologically comprised of dense fibrous connective tissue and typical cartilage. Structurally, tarsus consists of fibroblastic cells surrounded by an extra-cellular matrix with types I and III collagen, as well as aggrecan.[6]

1.4.3 Established Tarsal Substitutes in Eyelid Reconstruction

The tarsus represents an important structure which requires adequate substitution during reconstruction in order to maintain proper eyelid function and appearance. In extensive eyelid defects where local or contralateral flaps are not suitable, tarsal substitutes are required to complete reconstruction. Tarsal substitutes described previously include hard palate mucoperiosteum, nasal septal chondromucosa, auricular cartilage and skin, preserved sclera, irradiated homologous tarsus, aorta and artificial tarsal plates.[144, 147-150] Issues encountered with each of these substitutes are explored below and include any or a combination of: difficulty with harvest, inadequate strength to support the reconstructed eyelid, thickness and rigidity of the material, deformity or shrinkage over time, difficult donor-site healing and local inflammation. Presently no substitutes are completely satisfactory and new alternatives are required to achieve the desired outcomes for patients.[147, 148, 151]

Ito and colleagues previously described the use of hard palate mucoperiosteum in the reconstruction of full thickness upper eyelid defects following tumour ablation.[147, 151] Although reported to be histologically similar,[151] ocular irritation found in 20% of patient is thought to be secondary to keratin patches in the stratified squamous epithelium. Additional complications in the recipient site include ocular irritation (20%), transient keratopathy (13%), partial graft dehiscence (13.4%), upper eyelid retraction (13%) and skin flap necrosis (7%).[145] Mucous production from presumed minor salivary glands within the graft has also been reported in a small number of cases.[152]

The nasal septal chondromucosa has also been used in the past for both upper and lower eyelid reconstruction, selected for its rigidity and non-keratinised epithelium which improves comfort.[153, 154] Donor tissue is usually harvested endoscopically, although an external per-alar approach has also been reported.[155] Chonchal chondro-perichondral grafts have also been described using a modified Matsuo's technique.[156] Cartilage usually requires thinning prior to use in upper eyelid reconstruction due to the differing thickness.[154] Reported complications include flap retraction and nasal valve distortion at the donor site caused by upper lateral cartilage harvest or abnormal intranasal scarring.[153]

Although less commonly used due to the lack of mucosal lining and risk of corneal damage, auricular cartilage has been described in full-thickness

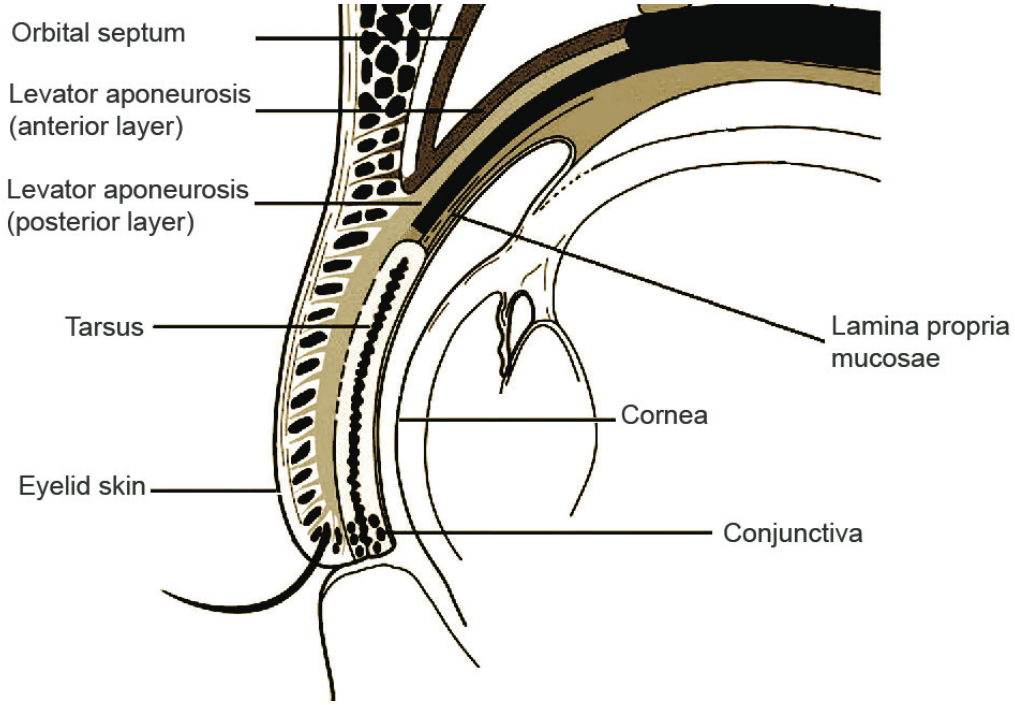
eyelid repair when combined with various flaps.[157, 158] Techniques for auricular cartilage harvest include both anterior[159, 160] and posterior approaches,[161] although donor site complications reported include wound dehiscence and infection.[157]

In a bid to eliminate the need for a donor site and associated complications, a number of alternate tarsal substitutes have been described with varying degrees of success. Preserved sclera has also been used in various reconstructive techniques since first being described as a tarsal substitute in 1971.[162] Limitations include the lack of pliability, issues with availability and degradation post implantation.[148, 163] Irradiated homologous tarsus has been described previously in a series of six patients by Jordan and colleagues. The authors found that the irradiated tarsus was able to provide sufficient support for the reconstructed lid but disadvantages included reabsorption at the exposed margin and the need for a myocutaneous flap for blood supply.[150, 164] Furthermore, this method relies upon donor tarsus, which can be difficult to source and there remains a theoretical risk of disease transmission with inadequate tissue sterilisation.

TarSys™ (IOP Ophthalmics, Costa Mesa, California, USA) is biomaterial derived from decellularised porcine small intestine submucosa and is designed to function as an artificial tarsus scaffold upon which native cells integrate and remodel. The eight-layer membrane is constructed based upon the tarsal plate and contains types I, III and IV collagen with glycosaminoglycan.[165] Designed to be used in the lower lid, reports in the

literature have been limited to use in lower lid retraction and lagophthalmos repair.[166, 167] Furthermore, disadvantages noted include the need to hydrate the implant for 20 minutes prior to use, gradual melting of the implant and retraction over time.[166] Furthermore, Munday and colleagues described two cases of foreign body giant cell reaction to TarSys™ which required graft removal,[166] while Kim et al. reported a case of bilateral lower lid cyst-like foreign body reaction to the implant which also required excision.[168]

Figure 1: The Anatomy of the Eyelid



1.5 Principles of Bioengineering

1.5.2 Introduction

Tissue engineering refers to the synthesis of living tissues using bioreactors, cells, scaffolds and/or growth factors. Tissue engineering aims to produce a functional tissue replacement to repair defects, and the use of engineered three-dimensional biomaterial constructs to reconstruct or repair living tissue has been widely investigated over the last two decades. [169, 170, 171] Ideally, tissue engineering would restore key functions of missing or defective tissues, and would degrade at a rate which best complements the natural rate of cellular differentiation and proliferation, ultimately integrating well with surrounding native tissue both in the immediate and long-term period. The basic principle of tissue engineering involves the combination of a polymer scaffold with a stem cell or precursor cell population.

Key components required for successful tissue engineering include a viable scaffold, cells, stimulating factors to encourage desired cell behaviour and a blood supply. Given the current challenges associated with sourcing tarsus tissue substitutes for use during large eyelid defect repair, tarsus represents an obvious target tissue for bioengineering. Furthermore, tarsus tissue has excellent potential to be engineered as it is thin, thereby easily allowing oxygen and nutrients to the cells within the developing engineered tarsus construction. The key principles of bioengineering are discussed in further detail below.

1.5.2 The Importance of Biomechanics

It has been shown that the behaviour of cells, including their adhesion, migration, proliferation, differentiation and gene expression, is affected by their local physicochemical microenvironment.[172, 173] Furthermore, studies have demonstrated that the interaction between cells and a scaffold can change depending upon biomechanical properties.[174] The majority of tissues have important biomechanical functions including force generation and/or transmission, load bearing or fluid transport. Various changes in the form of ageing, disease or injury results in significant dysfunction of the tissue. Given that biomechanical factors are critical in regulating cell behaviour in normal tissue, accurate assessment of target tissue biomechanical properties *in vivo* is critical for successful tissue engineering. In order to address the issues associated with the role of biomechanics in tissue engineering, the United States National Committee on Biomechanics proposed a set of guidelines for bioengineering of load bearing tissues.[175] The guidelines aimed to increase awareness about the biomechanical function of tissues being bioengineered, identify mechanical and structural requirements required for tissue engineering and encourage researchers to incorporate these criteria into future work. Since its development, functional tissue engineering has expanded to include several additional factors which have since been incorporated into the guidelines for researchers.[176] A number of key factors identified are summarised below.

1.5.2.1 *In Vivo* Stress and/or Strain

It is vitally important to understand the mechanical thresholds for normal tissues in a variety of activities such that tissue engineered replacements can meet functional demand. There have been numerous studies of *in vivo* stress and strain performed on ligaments and tendons, which have demonstrated the significant differences in failure force between the two tissues, reflecting the differences in their function.[177-179] Various tools including buckle and E-type gages, modified pressure transducers and implantable transducers have been used in numerous studies.[175, 180-182] Other tissues which have been invested for stress/strain histories include articular cartilage, bone and heart valves.[183-186]

1.5.2.2 Functional Demands: Sub-Failure and Failure Conditions

It is essential for tissue engineered replacements to meet failure and sub-failure properties of the native tissue in order to successfully restore function. Viscoelastic functions such as static or cyclic creep, or stress and relaxation testing can be used to determine the natural failure and sub-failure conditions of the target tissue.[175] Biological tissues have been shown to exhibit one or more of the following behaviours including viscoelastic, anisotropic, non-linear, multi-phasic, inhomogenous and transport.[175, 176]

1.5.2.3 Prioritisation of Mechanical Properties as Design Parameters

Despite our growing knowledge regarding the various mechanical properties and structure of our target native tissue, the relative importance of each property in the eventual success of a bioengineered replacement remains

relatively unknown.[175] Presently, it remains unrealistic to match each and every known property, and priority is often placed on a particular parameter such as tensile strength or compressive modulus, depending on the core function of the target tissue *in vivo*.[176] Given the tarsus tissue primarily provides structural support while also facilitating the blink movement, important parameters to understand and replicate would include the elastic modulus, tensile strength and maximum strain.

1.5.2.4 Regulation and Interaction of Cells with an Extracellular Matrix *In Vivo*

It has been well-established that the biomechanical and biological interaction between cells and artificial scaffolds have a significant effect upon cell behaviour, and hence are critical to eventual success of bioengineered tissue.[187] As such, numerous techniques have been developed in order to fabricate scaffolds with controlled mechanical properties and tunable architecture.[176] These are discussed in more detail below in the scaffold section.

1.5.2.5 Mechanical and Physical Factors Impacting Tissue Repair

Numerous studies have demonstrated the ability to enhance tissue regeneration *in vitro* by utilising 'bioreactors' to provide physical stimulation for bioengineered constructs.[188-190] These studies have yielded techniques which can be used to control cell growth and differentiation during the *in vitro* stage. Furthermore, various mechanical and chemical loading methods have been developed to enhance this process.[191, 192]

Once successfully implanted, cells within the tissue engineered construct will be subjected to the complex biological environment, which is known to influence cellular growth, differentiation and ultimately long-term outcomes. It is therefore important to develop methods allowing for monitoring of both *in vivo* mechanical factors and cell responses.[176]

1.5.2.6 Outcome Based Success Criteria and Methods to Model Tissue Growth

An ability to objectively assess the success of bioengineered tissue is required, and any criteria need to be tissue and organ specific. Minimally invasive methods to measure tissue function both *in vitro* during development and *in vivo* following implantation are continuously being developed.[176] In the last decade, significant advances have also been made in the area of computational models to allow simulation of various parameters involved in tissue repair and regeneration in order to help predict outcomes.[193, 194]

1.5.2.7 Biomechanical Studies within Ophthalmology

Within ophthalmology, the most extensive *in vivo* biomechanical studies have been performed on the cornea. Reichert developed an instrument called the Ocular Response Analyser (ORA; Reichert Corporation; Depew, USA) which measures the corneal response to indentation by using a rapid air pulse.[195] The Ocular Response Analyser utilises the principles of non-contact tonometry, whereby the intraocular pressure is determined by the air pressure required to applanate the central area of the cornea. The instrument records

two measurements of corneal response to an air pulse, the first being the force required to flatten the cornea as air pressure rises, and the second being the force at which the cornea flattens as the air pressure drops. The difference between the two readings reflects a direct measure of the cornea's biomechanical properties and is termed the corneal hysteresis.[195, 196] Additional parameters arising from the Ocular Response Analyser include the corneal-compensated intraocular pressure and corneal resistance factor, the latter reflecting the overall resistance of the cornea using an equations taking into account the stiffness of the cornea using Young's modulus of the collagen fibres.[197] It is important to note, however, that these studies have thus far been aimed at investigating the various biomechanical states of the cornea in relation to pathological conditions such as keratoconus, oedema and glaucoma rather than for the purposes of tissue engineering.

There are numerous previous *ex vivo* studies on the biomechanics of various structures in and around the eye. In early studies, Firberg and Lace[198] investigated the elastic properties of the human choroid and sclera using eye-bank eyes, and found an average stress at failure of 3.3 +/- $1.2x10^5 N/m^2$ (correlating to approximately 0.0000033MPa). Bisplinghoff and colleagues later investigated the dynamic pressure required to rupture human sclera, which was found to be both viscoelastic and anisotropic. The authors found an average maximum stress of 14.89 +/- 4.81 MPa in both directions and average maximum strain of 0.058 +/- 0.018 MPa along the meridional plane and 0.041 +/- 0.014MPa along the equator.[199] Most recently, Chen et al.[200] examined the elastic properties of the posterior retina, choroid and sclera in

twenty-four human donor eyes from the eye bank. Having already established that the retina exhibited both anisotropic and inhomogeneous characteristics,[201] the group found that the posterior layers behaved non-linearly, with significant differences in vertical and horizontal meridian of the retina which were not found in the choroid and sclera. While the above results are thus far been mainly used in mechanical models aimed at preventing injury and disease progression, they could certainly be applied to tissue engineering in the future.

In 2011, Chen and Weiland[202] studied the mechanical properties of orbital fat and encapsulating connective tissue in order to provide a benchmark for bioengineered orbital implant design. The authors utilised five pairs of human eyes obtained within 48 hours of death, and 33 porcine eyes within 6 hours of slaughter. Similar to their previous studies with the retina, choroid and sclera, the samples were studied at body temperature using a tensile-testing system. The authors found that the initial stress-strain relationship was linear, but later became non-linear and noted that the Mooney-Rivlin hyperplastic material model was particularly adaptive to the human model.

1.5.2.8 Specific Considerations for Eyelid Tarsus

As briefly mentioned previously, important parameters to consider when evaluating biomechanics of structures such as the tarsal plate include the following: the elastic modulus, tensile strength and maximum strain. The elastic modulus refers to the measured strain in response to being deformed elastically, and is defined as the slope of its stress-strain curve. The tensile

strength is defined as the maximum stress or strain a material can withstand before failing. The maximum strain refers to the total strain just prior to failure during tensile strength testing.

The viscoelasticity of human tarsus is determined by structural content and architecture, which is known to include collagen types I, III and VI, aggrecan, versican, tenascin, cartilage oligomeric matrix protein and a variety of glycosaminoglycans.[6] The collagen content of tarsus provides elasticity,[203] while the stiffness of tarsus has been attributed to the aggrecan content.[6] Aggrecan has been previously shown to have an estimated nanomechanical compressive stiffness of approximately 1 Pa at strains of <20% (toe region), which increases linearly from 0.1 to 1.5 MPa at strains >40% (corresponding to 40-80 mg/ml aggrecan).[204]

As discussed above, commonly utilised tarsal substitutes include hard palate mucoperiosteum, nasal septal chondromucosa and auricular cartilage.[151] To the best of our knowledge, there are no previous biomechanical studies investigating hard palate or nasal septa. The composition of auricular cartilage and tarsus are similar, however, as both contain glycosaminoglycan and collagen.[205] Auricular cartilage is known to also contain elastin, which differentiates it from other subtypes of cartilage and significantly alters its biomechanical properties.[205, 206] Collagen and elastin have been found to display very different biomechanical properties, with the tensile modulus of collagen being approximately 1000 times greater than elastin, and its extensibility being only 13% compared to 150% for elastin.[203] Preserved

sclera has also been utilised as a tarsus substitute,[148] and previous studies have demonstrated the stiffness of sclera ranges from 2.8-3.3MPa[207] and elastic modulus averages 2.9 +/- 1.4MPa with a maximum strain of approximately 20%.[198]

To the best of our knowledge, there are no previous studies of the normal biomechanical properties of human eyelid tarsus tissue which is critical to the success of any future bioengineered substitute.

1.5.3 The Role of the Scaffold

The use of a porous scaffold to provide support and facilitate synthesis of three-dimensional tissue represents one of the principal methods of tissue engineering. The role of the scaffold includes supporting and guiding cell attachment and tissue growth, providing mechanical support and maintaining the space for new tissue to develop. Key scaffold characteristics therefore includes: three-dimensional structure with adequate porosity, biocompatibility, biomechanical likeness and biodegradability.[208] Both the chemical and physical properties of scaffolds are thus important in determining their efficacy. Significant scaffold design criteria include material selection, biocompatibility, biodegradability and degradation profile (rate, by-products and strength characteristics), porosity (pore sizes, interconnections and volume fraction), surface chemistry, topography and cell-surface interactions.[209] As tissue and cell properties vary significantly around the body, the design of tissue engineering strategies including suitable scaffolds needs to be specific to the tissue type being targeted. Success in tissue engineering using polymeric scaffolds has been demonstrated for certain tissues, including skin and cartilage, although challenges remain in scaling up to large three-dimensional tissues.[169, 171]

Both synthetic and natural biopolymers may be used for tissue engineering of soft tissues. The most commonly used polymers include polylactide acid, polyglycolide acid, and a combination of the two, poly(lactic-co-glycolic) acid. Poly(lactic-co-glycolic) acid is a biodegradable synthetic polyester which is approved by the US Food and Drug Administration for human clinical use.

Poly(lactic-co-glycolic) has been extensively investigated for use in tissue engineering due to the versatility in fabrication and range of achievable chemical and mechanical properties.[210-212] Recently, strategies have been developed to modify the surfaces of polymers like poly(lactic-co-glycolic) to improve their cell and tissue interactions and moderate the inflammatory reactions which occur when biomaterials are placed in the body.[213-215]

Natural polymers may also be used to create tissue engineering scaffolds and hydrogels. Commonly used natural polymers include chitosan, collagen, gelatin, silk fibrin, elastin and glycosaminoglycans.[160, 216-218] Such materials can be biocompatible, provide favourable cell binding sites and are often degraded through natural metabolic pathways in the body. Naturally-derived polymers such as chitosan, possibly in combination with synthetic polymers for improved strength, have potential as tissue engineering scaffolds for soft tissues due to their biomimetic properties. Although known for their ease in forming macro-porous structures, natural polymers such as chitosan can be limited in their mechanical stability. Cross-linking chitosan structures has been shown to improve stability of resultant scaffolds.[209]

Porosity also represents a significant factor in promoting cellular infiltration and vascularisation. Appropriate pore size, shape, interconnectivity and alignment have been shown to be crucial in regulating morphogenesis of seeded cells.[210, 219, 220] Thermally-induced phase separation is one method of scaffold fabrication which has been investigated extensively, and various processes during the production phases have been shown to alter the final

morphological features of the scaffold. Previous studies have demonstrated success in tailoring pore size and internal architecture by controlling the mould geometry and freezing rates in poly(lactic-co-glycolic) scaffolds produced via thermally-induced phase separation.[210, 221, 222] The mechanical properties of resultant scaffolds vary significantly depending upon architecture,[223] and therefore it is crucial to understand the biomechanics of the native tissue prior to scaffold fabrication.

1.5.4 Tissue Engineering in Ophthalmology

Thus far, there have been promising studies investigating the role of tissue engineering in corneal disease, glaucoma, conjunctival reconstruction, dry eye disease and orbital fracture repair. These are discussed in more detail below.

1.5.4.1 Cornea

Tissue engineering has long been investigated as an alternative to human corneal transplantation to treat potentially blinding corneal disease. There have been numerous studies of acellular polymer matrices aimed at promoting re-epithelialisation *in vivo*. A number of groups have used Type I collagen scaffolds as artificial corneal extra-cellular matrices.[208, 216] Griffith and colleagues have conducted a number of studies using fibrillar recombinant human collagen type I and III (RHCI or RHCIII) as corneal stromal matrices.[224] RHCIII was found to be optically superior, and the group were later successful in implanting 10 cell-free corneal substitutes into human patients made with RHCIII and cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). After 6-7 months, the substitutes were well integrated with regeneration of corneal epithelium, stroma and nerves, although long-term outcomes are unknown.[217, 225] More recently, Zhang et al.[161] studied a novel collagen scaffold synthesised with rat tail Collagen I for use as a potential corneal tissue substitute for use in corneal transplantation. The scaffold was found to have comparable transmittance and thickness when compared with human cornea. Furthermore, the scaffold was successful in supporting re-

epithelialisation and keratocyte cellularisation *ex vivo* using porcine corneal epithelial cells.

Insler et al. first reported the concept of corneal endothelial cell transplant expanded *ex vivo* onto collagen-coated dextran in 1990,[219] and since then there have been many studies of corneal endothelial cell culture. Liang and colleagues developed a novel chitosan-based scaffold onto which they were successful in establishing corneal endothelial cell culture derived from rabbits *ex vivo*.[220] Following implantation into Wistar rabbits, the blended membranes demonstrated good histocompatibility and degraded steadily with less associated inflammation compared to control. Similarly, Ozelik and colleagues fabricated an ultrathin chitosan-poly(ethylene glycol) hydrogel film which was found to be >95% optically transparent and able to support sheep corneal endothelial cell culture.[226] Combinations of chitosan including keratin-chitosan and polycaprolactone-chitosan membranes have also been studied *in vitro* and been shown to support cell culture.[227, 228]

Lai et al. studied the use of corneal endothelial cell sheets fabricated with hydrogel carriers resembling the native corneal endothelium, which aimed to minimise some of the issues encountered with existing corneal endothelial substrates including optical interference, foreign body reaction and disturbance of physiological function.[218, 229, 230] Initially working with gelatin hydrogels, the authors recently investigated hydrogels made using hyaluronic acid, a biopolymer which is naturally found in the aqueous and vitreous. They found that cell sheet transplantation using these hydrogels resulted in superior

biological stability with minimal adverse effects in rabbit studies.[160] In an attempt to further improve the properties of collagen hydrogels, Takezawa et al. developed a collagen vitrigel with the key step of vitrification allowing water to evaporate in a controlled manner with resultant cross-linking and rearrangement of collagen fibrils.[231, 232] The group later studied the ability of collagen vitrigel to support the three main corneal cell layers, limbal explants, keratocytes and endothelial cells, with promising results during *in vitro* experiments.[233]

Graphene has also been studied as a potential biomaterial for use in the cornea, with Tan and colleagues culturing human corneal stromal fibroblasts onto graphene films for use as a synthetic keratoprosthesis skirt material.[234]

1.5.4.2 Glaucoma

Acellular matrices have also been investigated in glaucoma procedures. First studied in rabbit eyes, Chen and colleagues implanted biodegradable, porous collagen matrices made of 1% collage/C-6-S co-polymer into 17 eyes and found that post-operative intra-ocular pressure reduction was approximately equal compared to trabeculectomy controls.[235] Since then, there have been numerous studies of the Ologen implant, a bioengineered porcine collagen matrix composed of 90% lypophilised porcine collagen and <10% lypophilised glycosaminoglycan with pore size of 10-300mm aimed at replacing mitomycin C for trabeculectomy.[236] In 2014 Cochrane review found that intra-ocular pressure lowering with the Ologen implant was comparable with Mitomycin C for trabeculectomy but evidence and longer-term follow up still remains

limited.[237] The most recent study conducted by Cillino and colleagues reported extended follow-up of 5 years in a group of 40 patients assigned to either trabeculectomy with Mitomycin C or Ologen and found similar success rates and safety profile.[238]

1.5.4.3 Conjunctiva

There have been a few studies investigating the use of tissue-engineered implants in conjunctival reconstruction. Hsu et al. grafted porous collagen-glycosaminoglycan co-polymer matrices into the bulbar conjunctiva of rabbits with artificial full-thickness conjunctival wounds.[239] The authors found that by 28 days, the rabbits with matrix grafts had less wound contraction (6.8% +/- 3.2% fornix shortening) compared with controls who were ungrafted (26.4% +/-5% fornix shortening). Lee and colleagues later studied the use of modified poly(lactic-co-glycolic) acid 50/50 scaffolds modified with either hyaluronic acid and/or amniotic membrane in conjunctival reconstruction.[240] The authors used human stromal fibroblasts obtained from human corneal tissues and were successful in seeding scaffolds prior to implantation in albino rabbits. At 4 weeks post-operative, grafted wounds were found to contract 6% compared to 25% of ungrafted conjunctival wounds. In addition to their use in the cornea, collagen vitrigels have also been studied in conjunctival reconstruction.[241] Zhou et al. demonstrated that optimized vitrified collagen was able to successfully promote conjunctival epithelial cell growth and goblet cell repopulation during *in vitro* rabbit studies.[242]

1.5.4.4 Dry Eye

There have also been a number of studies aimed at creating a tissue engineered tear secretory device to treat patients with keratoconjunctivitis sicca who remain symptomatic despite conventional treatment. Many previous groups have reported successful animal and human cell culture onto basic extracellular matrices with collagen I and Matrigel ®,[243-245] a preparation derived from basement membranes of the Engelberth-Holm-Swarm mouse sarcoma line containing laminin, collagen IV, heparin-sulfate proteoglycans, entactin and nidogen.[163] Selvam and colleagues have also since shown culture of purified rabbit lacrimal acinar cells onto numerous matrix protein-coated polymers including co-polymers of poly(lactic-co-glycolic) acid (85:15 and 50:50) and poly-L-lactic acid with retention of secretory properties.[246] These co-polymers, as discussed previously, have the advantages of having adjustable biomechanical properties and the ability to be tailored to specific target tissues.

In a review of bioengineering for conjunctiva and dry eye, Lu and colleagues discussed the potential use of 'organ-on-a-chip' technology for the ocular surface.[241] Organ-on-a-chip refers to a bioengineered microdevice with cultured cells in an attempt to mimic target organ function, and some success has been reported with lung, liver, intestine, spleen and bone marrow studies.[247-251] Any successful tear secretory unit would require lacrimal gland cells, conjunctival epithelium and micro-fluid channels, and preliminary *in vitro* studies of conjunctival epithelium and artificial lacrimal glands provide a basis for further development.[252, 253]

1.5.4.5 Orbital Fractures

The orbital floor is the wall of the orbit most commonly affected by trauma, and post-traumatic changes can manifest in enophthalmos and diplopia. Restoration of orbital volume is therefore vital in preventing complications and maintaining normal globe function. There are numerous implant options for use during orbital wall repair, of which autologous bone graft remains the gold standard, although with the obvious limitation of donor site morbidity and harvesting challenges. As such, various biomaterials have been developed, and include non-resorbable alloplastic, resorbable alloplastic, and more recently, bioengineered bone. Non-resorbable biomaterials studied in the past include titanium mesh, porous polyethylene (Medpor) and bioactive glass.[254] Notable risks of non-resorbable biomaterials include foreign body reaction, risk for migration and infection.[255] In order to address some of these issues, biodegradable polymers have been studied as alternative options. Poly(lactic acid), PLGA, combinations of and derivatives of the two have been studied extensively in the past, and there have also been studies of polyglactin-910 mesh and a newer periosteum-polymer composite material.[254, 256] Kontio and colleagues compared polydioxanone (PDS) and poly(l/d)lactide implants in rat studies and found that PDS was mechanically unsuitable, losing form within 2 months but the poly(l/d)lactide polymers showed promising results at 7 months follow-up.[257] The group then progressed to human studies using poly(l/d)lactide 70/30, and found that the bioresorbable implants resulted in good clinical outcomes for patients with $2cm^2$ or larger defects with 36 weeks of follow-up.[255] The authors then compared outcomes with fractures repaired using autologous bone graft, and found no statistically significant differences

in complications.[258] However, long-term outcomes of resorbable implants are not well described, and suitability of use may depend on fracture size.[254]

There have been a number of recent studies investigating the potential for orbital bone tissue engineering after previous established studies for bone regeneration of the mandible, cranium and limbs.[259-261] Mesenchymal stem cells have been the most widely investigated cell line for craniofacial tissue engineering, and have been shown to proliferate well *in vitro* from small samples.[262] Additionally, there is a growing body of work surrounding bone morphogenetic proteins (BMP), which secrete signalling molecules stimulating differentiation of osteoprogenitor cells and thereby bone formation.[263] Currently BMP type 2 and 7 have been developed for clinical applications.[264, 265] Recent advances have used biodegradable 5-Ethyl-5(hydroxymethyl)- b,b-dimethyl-1,3-dioxane-2- ethanol (EH)-poly(ethylene glycol) (EH-PEG) hydrogels with integration of mesenchymal stems cells in order to deliver bone morphogenetic protein-2 (BMP-2) to injured tissue.[266] This is particularly of interest in orbital fracture repair, as the periosteium, which contains the osteoprogenitor and chondroprogenitor cells, are frequently injured in facial trauma, further delaying healing post fracture.[267] Betz and colleagues loaded EH-PEG with BMP-2 and implanted them into 8mm orbital floor defects in rabbits. The authors found that there was significant bone growth at 28 days, establishing the viability of this concept for future studies.[266] Rohner et al.[268] studied the use of polycaprolactone coated with bone marrow in pig orbital defects, which was shown to result in significantly more bone regeneration compared to polycaprolactone alone at 3 months post repair (14.1% vs 4.5%).

Medical grade polycaprolactone along with its composites created via fused deposition modelling have also been studied in orbital floor reconstruction with promising results in human patients.[269, 270] Other studies have focused more on craniofacial applications and include PLGA seeded with periosteal cells,[268] polycaprolactone with cultured calvarial osteoblasts and mesenchymal progenitor cells,[271, 272] and poly(propylene fumarate) scaffolds treated with growth factor and infused with bone marrow.[273] Such tissue engineered bone constructs therefore have the potential to not only provide immediate support and restoration of orbital volume, but long-term benefits due to early stimulation of bone regeneration.

1.5.5 Bioengineering Tarsus

To the best of our knowledge, there is only one previous study investigating the use of engineered polymeric scaffolds for tarsal repair.[274] This study used a type of polyhydroxyalkonaoates as synthetic tarsal substitutes in a rat study and found that they were successful in supporting eyelid reconstruction, fibroblast growth and fibrous encapsulation. Polyhydroxyalkonaoates are biodegradable and thermoprocessable polyesters produced by microorganisms.[244] Polypoly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) consisting of 12% mol% 3HHx were used to produce scaffolds with an average thickness of 0.7mm and resultant micropores of 5μm diameter. These scaffolds were cut into 1.0x1.0mm pieces and were implanted into the upper eyelids of three-month-old Sprague-Dawley rats, with acelluar dermal matrices of same size and thickness used as controls. Post-operative histological studies demonstrated high density of inflammatory cell infiltrate around the PHBHHx scaffold in the first 2 weeks post-operatively compared with few inflammatory cells in the control group. At 8 weeks, the reaction had shifted to one of chronic inflammation, with ongoing macrophage and lymphocyte infiltration with the percentage of fibroblasts measuring 32.13 ± 1.47% compared to 100% in the acellular dermal matrice and unoperated rat groups.[274] The significant inflammatory response demonstrated in this animal study suggests that further refinement is required to improve tolerability once implanted.

1.5.6 Role of Cell Culture in Bioengineering

In addition to numerous studied strategies to improve the biocompatibility of engineered scaffolds,[213-215] culturing native cells pre-implantation onto the scaffolds also aims to reduce such inflammatory responses. Given the importance of fibroblasts within the histological structure of tarsus, seeding of these cells onto a bioengineered scaffold aims to both improve biocompatibility and also enhance the biomechanical properties.

1.5.6.1 Fibroblast Culture

Fibroblasts represent the principal cell of the stroma and both produces and modulates the extracellular matrix.[275, 276] Fibroblasts are the most common cell type in animals and are known to be responsible for growth factor secretion and mediation of tissue fibrosis.[277, 278] They have also been used extensively in cell culture experiments and are easily maintained *in vivo*. Since Georgy Fey first reported HeLa cell culture in 1951,[279] numerous fibroblast lines have been developed for scientific experiments including NIH3T3 fibroblasts from Swiss mouse embryos,[280] and other lines of murine embryonic fibroblasts.[281, 282]

In humans, fibroblasts have been cultured from varying anatomical sites ranging from cutaneous to deep tissue including lung. Most recently, Fernandes and colleagues investigated the culture of fibroblasts from varying cutaneous locations including the eyelid, post-auricular area and abdominal scar. Samples of 0.3cm^2 (post-auricular and abdominal) and 0.2cm^2 (eyelid) were placed in culture dishes containing Dulbecco's modified Eagle's medium

(Life Technologies, Inc., Grand Island, NY) 10 % of Fetal Bovine Serum (FBS, Invitrogen, Carlsbad, CA, USA), 2 mML–glutamine (Invitrogen), 19 MEM NEAA (Invitrogen), 29 antibiotic antimycotic solution (penicillin/streptomycin/amphotericin—final concentration of 200 U/mL, 0.2 and 0.5 l g/mL, respectively—Sigma) and maintained at 37 degrees with 5% carbon dioxide. The authors found that skin samples from all three sites were successful in supporting fibroblast culture with viability being highest from the scar at 81%, compared to 67% for the eyelid and 51.5% for post-auricular skin in cryopreservation studies.[283] The culture of lung fibroblasts is well established in the literature and utilised smaller samples of tissue.[284-287] Lung fibroblasts have been previously derived from lung tissue obtained from scleroderma patients at autopsy. The tissue was dissected into 0.5 x 0.5mm pieces before being cultured using Dulbecco's modified Eagle's medium with various supplements at 37 degrees in 10% carbon dioxide. The medium was changed every three days to facilitate removal of non-attached and dead cells until fibroblasts were confluent. Crystal violet staining, along with immunofluorescent staining using a monoclonal antibody specific for human fibroblasts was used to confirm fibroblast culture.[288] Regardless of their origin, fibroblasts can be identified via their spindle-shaped morphology and stain uniformly for the mesenchymal marker vimentin but are negative for markers of epithelial, smooth muscle, endothelial, perineural and histocytic cells.[275]

Previous studies have demonstrated that fibroblasts from differing origin and anatomical location exhibit varying phenotypes and characteristics.[276, 289] In a study comparing fibroblasts cultured from pre-tarsal eyelid skin and presternal

skin, Li and colleagues found that the dermal fibroblasts isolated from the two locations differed in their mechanical properties and response to inflammatory cytokines and growth factors. Furthermore, although the presternal fibroblasts demonstrated greater contractility *in vitro* under stimulation from pre-inflammatory cytokines, when tested under physiological conditions, pretarsal fibroblasts were found to generate greater contractile forces with greater overall stiffness.[290] This study reflects the fact that eyelid skin generally scars minimally post-operatively[291] compared to the propensity of presternal skin to scar heavily into keloid scars.[292] When applied to bioengineering, this study also highlights the need to harvest fibroblasts from the target tissue area to ensure that the resultant tissue engineered construct is as biomechanically similar as possible. Given the relative ease of taking eyelid skin samples and standard practice of taking a biopsy prior to excision, orbital skin fibroblast culture could be easily be established for tissue engineering purposes, although this is yet to be described in the literature.

CHAPTER 2: BASAL CELL CARCINOMA

2.1 Introduction

Basal cell carcinoma is the most common cancer worldwide, with 80% of all basal cell carcinomas occurring in the head and neck region.[32] The histologic subtype of basal cell carcinomas influences the biologic behaviour of the tumour and recurrent basal cell carcinomas are often associated with a primary tumour of an aggressive subtype.[293-295] Although more recently dermoscopy and confocal microscopy represent a promising new technique to identify basal cell carcinoma subtypes,[296, 297] biopsy still remains the most commonly utilised technique to examine histology. Surgical excision is the gold standard for treatment with high efficacy and low recurrence rates.[26] Accurate assessment of surgical margins is essential for determining the adequacy of surgical excision of basal cell carcinomas. Confusion regarding the meaning of surgical margins results in inconsistent histological reporting and subsequent difficulty interpreting pathological reports.[120] Terms such as 'close' and 'narrow' in relation to surgical margins are relative, and their correct interpretation relies upon mutual understanding between the surgeon and pathologist, which may not be consistent. Previous studies have demonstrated varying definitions of 'close' surgical margins including tumour involvement within a few cells, to 0.1 to 5mm adjacent to the tumour edge.[120]

Non-surgical treatment options for periocular basal cell carcinoma include radiotherapy and photodynamic therapy and topical treatments (imiquimod or 5-fluorouracil).[298] However, non-surgical treatment options are primarily suitable for basal cell carcinomas of non-aggressive histology, and therefore

patient selection relies upon the accuracy of the initial biopsy. This is particularly significant in the periocular region, where recurrent basal cell carcinomas are associated with significant morbidity, and increased risk of orbital invasion.[27] We therefore reviewed the accuracy of initial biopsy, with a particular focus in the aggressive histological basal cell carcinoma subtypes.[299] We also discuss the implications of 'close' and 'narrow' surgical margins in the context of periocular basal cell carcinoma and review the available literature.[300]

2.2 Methods

Histological data was analysed from patients diagnosed with primary basal cell carcinoma of periocular region (upper lid, lower lid, medial canthus or lateral canthus) by a single pathology laboratory servicing South Australia from 2006-2013. Patients who underwent a biopsy prior to treatment and tumour excision were included. Data was retrieved from the Institute of Medical and Veterinary Science (IMVS) Main Laboratory database and the following characteristics were analysed: gender, age, tumour location, histological subtype on biopsy, biopsy type (punch, incisional, shave), biopsy and excisional dimensions and depth, surgical excision technique and histological subtype on excision. All specimens were processed with paraffin sections and subsequently diagnosed by an IMVS pathologist. Histological diagnosis of basal cell carcinoma subtype(s) at excision was considered the diagnostic gold standard. Tumour resection was performed at the Royal Adelaide Hospital (RAH), The Queen Elizabeth Hospital (TQEH), Port Pirie Hospital, Whyalla Hospital and other smaller medical centres in South Australia.

There are numerous subtypes of basal cell carcinoma previously established in the literature.[28, 31, 301] Although there have been multiple aggressive basal cell carcinoma subtypes identified (including infiltrative, micronodular, compositive, morpheoform, sclerosing, infundibulocystic), more recently, it has been suggested that a simplified basal cell carcinoma classification including only superficial, nodular and aggressive should be used better optimise management.[302] Therefore, non-aggressive basal cell carcinomas

were defined as being either nodular or superficial, whilst the remainder were classified as aggressive basal cell carcinoma. Specimens with squamous differentiation were also considered aggressive basal cell carcinomas.[28] The presence of at least one aggressive subtype in a mixed tumour identified it as a mixed aggressive subtype.

Continuous variables were reported as median and range as appropriate. Biopsy dimensions were compared using the Mann-Whitney U test. Statistical tests were performed using SPSS Version 16 (SPSS Inc, Chicago, Illinois) and two sided p values <0.05 were considered significant. Ethics approval was obtained for this study from the local institutional review board.

2.3 Results

2.3.1 General Characteristics

A total of 174 patients with primary periocular basal cell carcinoma were identified and analysed. Punch biopsies were utilised in 41% of cases, while the remaining patients underwent shave or incision biopsies. The biopsy type was not recorded in 49% of cases.

There were 103 cases (59%) of purely nodular basal cell carcinoma, 13 (7%) superficial basal cell carcinomas, 13 (7%) aggressive basal cell carcinoma, 33 cases (20%) of aggressive mixed basal cell carcinoma and 12 cases (6%) of non-aggressive mixed basal cell carcinoma. Of the 45 cases (26%) of mixed basal cell carcinoma, 38 specimens (22%) included two subtypes and the remaining 7 (4%) had three different subtypes. Squamous differentiation was identified in 7 specimens (4%).

2.3.2 Correlation between Biopsy and Excision

Table 3 summarises the basal cell carcinoma subtype(s) identified in the initial biopsy compared to at excision. The overall concordance rate was 54%, with 94 of the 174 initial biopsies resulting in the same findings as the excision report. Initial biopsy was able to correctly identify a nodular component in 86% of cases, compared with 27% for superficial basal cell carcinomas.

There were 51 cases (29%) of basal cell carcinoma which included aggressive subtypes, and of these, 26 initial biopsies (52%) failed to detect an aggressive component. Of the 43 biopsies which identified an aggressive

histological subtype, 19 (44%) were found to contain only non-aggressive subtypes upon excision, although this did not take into account limitations of sampling, or initial biopsies during which the aggressive subtype was completely removed.

We found that an aggressive histological subtype was present in 73% of all mixed basal cell carcinomas, and of these 33 cases, the initial biopsy was able to detect an aggressive component in 45% of cases. The most common combination encountered was nodular with infiltrative, which was correctly identified in just 14%, with over half of initial biopsies detecting only the nodular component (Table 4).

2.3.4 Biopsy and Excision Dimensions

Biopsy dimensions ranged from 0.5-10mm, whilst the median depth for biopsies was 2mm (mean 2.1mm, range 0.5-6mm). For excisional specimens, dimensions ranged from 1-65mm and median depth was 4.0mm (mean 4.7mm, range 1-35mm).

More than 80% of excisional specimens measured 10mm or greater (142 cases, 81.6%) while more than 20% of excisions measured 20mm or greater (39 cases, 22.4%). Over a third of tumours measuring 20mm or greater had an associated aggressive component (18 specimens, 35.3%).

There were 5 biopsies with dimensions of 8mm or more, and the corresponding excision specimens for these cases ranged from 17-65mm.

There was no significant difference in the maximum biopsy length and depth between specimens with an accurate initial biopsy and those without (p=NS). However, lesions with an inaccurate initial biopsy were associated with excisional lesions deeper than basal cell carcinomas correctly biopsied initially (p=0.018).

2.4 Discussion

2.4.1 Summary of Findings

Our series of 174 cases of periocular basal cell carcinoma is the first study examining the diagnostic accuracy of biopsy for basal cell carcinoma subtype at excision exclusively in this region. We found an overall concordance rate of 54%, although there were significant differences between different subtypes and mixed lesions. In total there were 51 cases (29%) of basal cell carcinoma which included aggressive subtypes, and initial biopsies failed to detect the aggressive component in 52%.

2.4.2 Characteristics of Basal Cell Carcinoma

Basal cell carcinoma represents the most common form of skin cancer in Australia, Europe and the US, accounting for 90% of all eyelid malignancies.[26] While basal cell carcinomas rarely metastasise, they can be locally destructive leading to significant morbidity especially when located in the periocular area.[27] Histologically aggressive basal cell carcinoma (infiltrative, micronodular and basosquamous subtypes) are associated with higher rates of recurrence, as well as increased risk of perineural and perivascular invasion.[28] The rise of non-surgical treatment options for non-aggressive basal cell carcinoma has provided a wider range of treatment options for patients, who are classically elderly and may often prefer conservative management. However, surgical treatment remains the most effective management of basal cell carcinoma, especially in basal cell carcinoma containing aggressive histology and therefore correct initial biopsy is essential to identify high-risk patients who are not suitable for non-surgical treatments.

In our study, 29% of cases contained an aggressive basal cell carcinoma subtype and 52% of initial biopsies failed to detect an aggressive component. This has significant implications on outcomes, with the potential for patients with aggressive disease receiving treatment based upon a biopsy which reveals only the less aggressive component. Our findings suggest that in patients undergoing non-surgical management, careful follow-up should be performed to detect recurrences as initial biopsies can miss an aggressive histological subtype.

2.4.2 Diagnostic Accuracy of Biopsy

There are few studies in the literature investigating the diagnostic accuracy of biopsy for basal cell carcinoma histological subtype, and none have investigated periocular basal cell carcinomas exclusively. Previous studies have demonstrated a concordance rate ranging from 60% to 82% for basal cell carcinomas in general, with the accuracy rate between different modalities of biopsy being similar.[303-307] Furthermore, previous reports have revealed concordance rates ranging from 27-69% for various aggressive basal cell carcinoma subtypes,[306] and similarly, we found that biopsy was able to identify an aggressive basal cell carcinoma subtype in 48% of aggressive basal cell carcinomas. Higher rates of biopsy accuracy have been reported for superficial and nodular basal cell carcinoma, with up to 90% of superficial basal cell carcinomas shown to be diagnosed correctly with initial biopsy.[303, 306] Contrastingly, we found that accuracy with biopsy was highest for nodular basal cell carcinomas, with 86% of our cases being identified correctly initially, while superficial basal cell carcinoma had an initial biopsy accuracy rate of

just 27%. Additional factors which may influence accuracy rates include the size of the biopsy relative to the lesion, the selection of biopsy location and biopsy technique. Although we found no statistical differences in the maximum dimensions and depth for lesions with a correct initial biopsy compared with those without, further studies investigating biopsy accuracy for periocular basal cell carcinomas are required to better assess concordance rates.

The accuracy of biopsy for mixed type basal cell carcinoma has been reported to be as low as 10%, and mixed basal cell carcinoma have been shown to account for up to 54% of all cases.[303, 306] In our study, mixed basal cell carcinoma were present in 26% of patients and represented the second most common type of basal cell carcinoma after nodular. We found similarly low rates of accuracy of biopsy for mixed basal cell carcinoma, with only 11% of biopsies correctly identifying a mixed histological subtype. However, in some cases initial biopsy may have completely removed one or more histological subtypes. In keeping with previous reports,[304] we found that an aggressive histological subtype was present in 73% of all mixed basal cell carcinomas. Initial biopsy was able to identify an aggressive component in 45% of our mixed basal cell carcinoma cases, which is significantly lower than previous reports where punch biopsy was able to diagnose the most aggressive component in 84.4% of cases.[305]

2.4.3 Reporting of Margins

Although not encountered in the pathology reports we reviewed, it is well described that terms such as “close” or “narrow” alone have little value in the reporting of histological margins as they are open to widely varying interpretations.[120] These terms result in significant treatment dilemmas when applied to the delicate periocular region, where there is minimal tissue to spare and significant morbidity associated with larger eyelid defects. Notably, we found that over 80% of excisional samples measured 10mm or more in length and over 20% measured 20mm or more. The eyelid tarsus tissue is the most difficult tissue to substitute in the eyelid and is known to measure around 25mm in length. This has major implications for functional reconstruction given the significant proportion of tumours occupying more than 50% of the eyelid and the fact that over a third of tumours measuring 20mm or greater were found to contain an aggressive histological subtype warranting larger safety margins during excision.

Objective reporting should include measurement of the narrowest histological safety margin in both lateral and deep margins as recommended in pathological reporting guidelines such as those of the Royal College of Pathologists.[31] However, even with a measured margin the correlation between small histological safety margins and recurrence rates is not well defined in the literature. There are only two previous studies correlating histologic safety margins with recurrences in patients with periocular basal cell carcinoma. In 1968, Pascal et al.[308] found that 12% of basal cell carcinomas recurred if tumour was present within 1 high-power field (approximately

0.44mm) compared with 1.2% if the margin clearance was larger during a follow-up period of 10 years. However, only 10% of the 143 tumours in this study involved periocular region, and recurrences were not analysed by histologic subtype. In a recent study of 101 periocular basal cell carcinomas, Auw-Haedrich and colleagues found that 16.67% of 18 tumours with a histologic safety margin of <0.2mm recurred, compared with 1.37% for the 72 cases with a histologic safety margin >0.2mm during a mean follow-up time of 7 years.[309] However, for low-risk basal cell carcinoma subtypes, histologic safety margins of <0.2mm were sufficient to prevent recurrences in all 84 cases. Furthermore, when recurrences were compared using high-power field parameters, the recurrence rate for a safety margin <0.44mm was 16.67%, compared with 1.37% for margins >0.44mm.[309]

For basal cell carcinomas in general, Dixon and colleagues demonstrated that resection margin distance and growth pattern were the most important prognostic indicators of recurrence.[293] They found that high-risk basal cell carcinoma subtypes with a histological clearance margin of <0.38mm were associated with the lowest predicted probability of non-recurrence at 18%, while low-risk basal cell carcinoma subtypes with clearance margins of >0.75mm had the lowest predicted probability of recurrence at 4%.[293] In contrast Kyrgidis et al.[310] found that rates of recurrence in 61 patients with 'close' margins <1mm were similar to 1316 cases with negative margins in head and neck basal cell carcinoma. However, the average follow-up in this study was only 4 years and it is unknown if any of these cases involved the periocular region.

The lack of evidence base regarding histological safety margins, the effect of additional risk factors for recurrence such as histological subtype and patient factors are a few of the issues that preclude proscriptive statements on the management of narrow margins. However, it would appear that histological safety margins of <0.5mm (approximately within one high powered field) are associated with a significant recurrence rate which may be up to 15% or more in the presence of an aggressive histological subtype. Hence, margins <0.5mm should prompt strong consideration of either re-excision or close observation following discussion with the patient. It should be remembered that while 80% of recurrences are within the initial 5 years, approximately 20% recur up to 10 years. A lower threshold for re-excision may be warranted for those with additional risk factors such as high risk subtype, large size or a recurrent tumour.

2.5 Study Limitations

Our study has a number of limitations which warrant recognition. Despite all pathology specimens being reported by consultant pathologists, we were unable to review the pathology of each specimen using specific standardised criteria for histological subtypes. Secondly, there was no standardised biopsy technique or size, with the size of punch biopsies varying in size and depth depending upon the type of biopsy tool utilised. Additionally, although previous studies have demonstrated comparable accuracy between the various biopsy techniques,[307] further prospective studies of each individual biopsy modality are required to further assess the accuracy rates. Furthermore, selection bias may exist, as it is possible that some basal cell carcinomas were treated by alternative options other than excision. However, the usual practice in the centres referring specimens to the IMVS is to manage periocular basal cell carcinoma with excision. It is also possible that smaller periocular basal cell carcinomas may have been treated by excisional biopsy alone and in mixed basal cell carcinoma cases, initial biopsy may have completely removed one or more histological subtypes. In addition, some patients would have been referred for excision following biopsy performed elsewhere.

2.6 Conclusion

Correct basal cell carcinoma subtype classification is crucial to ensure appropriate management and histopathologic examination with a biopsy remains the most commonly used method to determine subtype. In our study of 174 periocular basal cell carcinomas, we have found that the overall concordance between basal cell carcinoma histological subtypes identified at biopsy compared to excision was 54%. Mixed histology was present in 26% and an aggressive subtype was present in 73% of mixed basal cell carcinomas. Overall 29% of basal cell carcinomas contained an aggressive subtype, and 52% of initial biopsies failed to detect an aggressive histological component. Hence, clinicians should be aware that biopsy may not always reflect the presence of more aggressive histological subtypes due to sampling error and possibly other factors such as biopsy type and size. This may have implications for the selection of patients with periocular basal cell carcinoma for destructive treatment modalities.

Table 3: Basal Cell Carcinoma Subtypes at Initial Biopsy and Excision

Histological Subtype	Identified at Biopsy N(%)	Identified at Excision N(%)
Nodular	119 (68)	103 (59)
Superficial	12 (7)	13 (7)
Mixed Nodular and Superficial	0 (0)	12 (7)
Aggressive[1]	43 (25)	51 (29)

1. This included any sample, including mixed basal cell carcinomas

Table 4: **Biopsy Compared to Excision for Periocular Basal Cell Carcinoma of Mixed Histology**

Basal Cell Carcinoma Combinations	**Biopsy**	**%**
Nodular + Infiltrative (n=21)	Correctly Identified Nodular Infiltrative Micronodular	14 57 19 10
Nodular + Superficial (n=12)	Nodular Superficial Nodular + Infiltrative	75 16 8
Nodular + Infiltrative + Superficial (n=4)	Infiltrative Nodular Nodular + Infiltrative	50 25 25
Infiltrative + Superficial (n=3)	Nodular	100
Nodular + Micronodular (n=2)	Nodular	100
Infiltrative + Superficial + Micronodular (n=2)	Infiltrative Infiltrative + Micronodular	50 50
Nodular + Superficial + Micronodular (n=1)	Nodular	100

CHAPTER 3: SQUAMOUS CELL CARCINOMA

3.1 Introduction

Squamous cell carcinoma is the second most common cutaneous malignancy after basal cell carcinoma and accounts for 5-10% of periocular cutaneous tumours.[92, 93] Established risk factors include ultraviolet light exposure, toxin exposure, radiation, immunosuppression, pre-existing chronic skin lesions, albinism and several genetic skin disorders.[94, 95] Periocular squamous cell carcinoma can be associated with significant morbidity as these tumours are locally invasive and can metastasise. Surgical excision remains the mainstay of treatment, but there are also a number of non-surgical options. It is imperative that high-risk cases are identified early and appropriately managed.

The American Joint Committee on Cancer (AJCC) and International Union for Cancer Control TNM staging system for eyelid carcinoma, 7th edition, was established to provide a standardised approach to staging (see Chapter 1).[132] The periocular region represents a particularly unique anatomic location due to the proximity of nearby vital ocular structures and has generally been associated with higher risk disease, thereby necessitating its own specific staging. Several changes to the 6th edition of the AJCC staging were made to allow for finer discrimination between tumour size and extent of invasion.[133] Despite the recent updated staging, there has only been one previous study utilising this staging system for squamous cell carcinoma. Nasser and colleagues retrospectively reviewed 65 periocular squamous cell carcinomas (22% recurrent at presentation) found that tumours with T-stage > T2b were

associated with regional nodal metastasis; however, it is unclear what proportion of these were recurrent tumours.[136] Given the lack of prior studies, it is unknown if these findings are truly representative and thus we aimed to further investigate the correlation between AJCC TNM 7th edition staging system and outcomes in patients with periocular squamous cell carcinoma in order to determine its usefulness in initial clinical assessment.[311]

3.2 Methods

We conducted a multi-centre, non-comparative case series of all patients with periocular squamous cell carcinoma and squamous cell carcinoma in situ presenting to three centres within Australia. Consecutive patients with a histological diagnosis of invasive or in situ squamous cell carcinoma presenting to the Royal Adelaide Hospital and Royal Victorian Eye and Ear Hospital between 2000 and 2012 inclusive, and North Shore Medical Centre between 1995 and 2012 inclusive, were included.

3.2.1 Data Collection

A chart review was undertaken for all cases. The following information was retrieved: demographic data (age, gender), clinical data (primary vs recurrent, tumour location, tumour size, predisposing factors, clinical evidence of regional nodal, and distant metastatic disease), radiological data (modality, presence of metastatic disease), histology data (tumour depth, degree of differentiation, perineural invasion (perineural invasion), treatment data (treatment, complications, tumour recurrence, follow-up duration). Each case was staged according to initial clinical presentation using the AJCC TNM 7th edition staging system for eyelid carcinoma. A structured phone interview was performed for all cases. The Birth, Death and Marriage Registries in SA, NSW and VIC were utilised to obtain mortality data for cases not contactable by telephone. Institutional review board/ethics committee approval was obtained for this study from all centres.

3.2.2 Statistical Analyses

The relationship between T-stage and perineural invasion was studied using binary logistic regression models. Cox proportional hazards regression methods were used to study the impact of T-stage, immunosuppression, histologic differentiation, recurrence state, treatment modality and perineural invasion on recurrence. Continuous variables were reported as median and range. Statistical tests were performed using SPSS Version 16 (SPSS Inc, Chicago, Illinois) and a p value of $p<0.05$ was considered significant.

3.3 Results

3.3.1 Squamous Cell Carcinoma in Situ (Bowen Disease)

There were 35 patients who had squamous cell carcinoma in situ or Bowen disease. The median age at presentation was 73 years (range 49 to 84 years). Thirteen patients were female (37.1%) while the remaining 22 were male (62.9%). The median follow-up period was 20.9 months (range 0.2 to 96 months). There were 7 patients (28%) with a history of immunosuppression from medications, chemotherapy or co-morbid conditions (e.g. malignancy).

There were five cases which were recurrent (14.3%) and one case was residual (2.9%). The right eyelid was involved in 20 cases (57.1%) and left eyelid involved in 15 cases (42.9%). The lower lid was the most commonly involved location in 20 patients (57.1%), followed by the medial canthus (7 patients, 20%), upper lid (6 patients, 17.1%) and lateral canthus (2 patients, 5.7%). Tumour size ranged from 3 to 25mm with median largest tumour diameter measuring 11.0mm and over half of all cases involved tumours measuring 10mm or greater (19 cases, 54.2%).

All patients were treated for their squamous cell carcinoma in situ. There were 16 patients who underwent wide local excision (45.7%) and 16 patients who underwent Mohs excision (45.7%). The remaining 3 patients had excisional biopsies (8.6%). Two patients had residual disease requiring re-excision and a further two patients developed recurrences (5.7%). One recurrence occurred in a patient presenting with a primary tumour measuring 5mm who was initially treated with a biopsy excision and was later successfully treated

with topical 5-fluorouracil. The second case was associated with a recurrent tumour of the medial canthus measuring 10mm initially managed with wide local excision which was treated with cryotherapy. This patient then went on to develop a further two recurrences, treated initially with Mitomycin C and then imiquimod.

3.3.2 Invasive Squamous Cell Carcinoma

A total of 254 patients with invasive squamous cell carcinoma were reviewed, of which 90 (35.4%) were treated in South Australia, 88 (34.7%) in New South Wales, and 76 (29.9%) in Victoria. There were 95 female (37.4%) and 159 male patients (69.4%). The median age at presentation was 73 years (range 28 to 102 years). The median follow-up period was 40 months (range 0.3 to 416 months). There were 14 patients (5.5%) with a history of immunosuppression from medications, chemotherapy or co-morbid conditions (malignancy, diabetes, cardiovascular disease, renal disease).

Twenty-five patients presented with recurrent tumours (9.8%). The TNM stages of our patients are summarised in Table 5. All but two patients presented with disease localized to the eyelid, and over one third had T-stage T2a tumours. One patient with a 15mm T2b lower lid lesion had both liver and duodenal metastases present on computed tomography (CT) imaging. This patient had previously undergone chemotherapy one year prior for mesothelioma and later developed a local recurrence 7 months after initial squamous cell carcinoma excision with margin control. Another patient

presented with a T3b tumour associated with both regional nodal and distant metastatic disease at presentation.

3.3.3 Metastatic Disease

All patients underwent physical examination of their lymph nodes at the time of diagnosis, and 5 patients (2.0%) underwent further investigation for regional nodal metastasis, with three receiving CT scans, two undergoing ultrasounds and one patient had a sentinel lymph node biopsy. One patient had a magnetic resonance imaging scan in addition to a CT. Regional nodal metastasis occurred in two patients, both of whom presented with recurrent squamous cell carcinomas. One patient presented with a T3bN1M1 tumour and developed a further recurrence associated with new regional nodal metastasis, whilst the second presented with a T3bN0M0 tumour and later developed a local recurrence associated with regional nodal metastasis. Two patients (0.8%) had evidence of distant metastatic disease at presentation, both of whom had recurrent tumours at stage T2b and T3b.

3.3.4 Perineural Invasion and Histological Data

Perineural invasion was present histologically in 21 cases (8.3%), of which 16 (7.0%) were primary tumours and 5 (20%) were recurrent. Of these 21 cases, initial clinical TNM stage prior to histological examination was different in 12 cases (T1 in 2 cases, T2a in 4 cases and T2b in 6 cases), all of which were upstaged to T3a following histological examination. There was clinical evidence of perineural invasion in two cases (hyperaesthesia on examination), both of whom had T3a tumours. We found that perineural

invasion was significantly associated with both a higher clinical T-stage at presentation (i.e. prior to histological investigation of perineural invasion) ($p<0.001$), and recurrent tumours ($p<0.001$).

Histologically, squamous cell carcinomas were well differentiated in 74 cases (29.1%), moderately differentiated in 103 (40.6%) and poorly differentiated in 49 (19.3%). Differentiation was not reported in 49 cases (19.3%). The tumour thickness was reported in just 54 cases (21.3%) and ranged from 0.5-30mm with a median thickness of 3mm.

3.3.5 Management

In total, 251 patients underwent treatment for their squamous cell carcinoma. Two patients with T2aN0M0 tumours and one patient with a T2bN0M0 squamous cell carcinoma elected not to undergo treatment due to co-morbid life-limiting conditions. Treatment modalities included Mohs excision: 79 (31.1%), wide local excision and paraffin section: 55 (21.7%), wide local excision and frozen section: 49 (19.3%), excision alone: 62 (24.4%). One patient (0.4%) was treated with topical imiquimod, one patient underwent photodynamic therapy, two patients (0.8%) had radiotherapy alone, and two patient required orbital exenterations. There were 10 patients (3.9%) who were treated with adjuvant radiotherapy and one patient treated with adjuvant chemotherapy.

3.3.6 Follow-Up

Overall, 17 cases (6.8%) developed local recurrences (Table 6) occurring at a median time of 18.5 months following initial treatment (range 3 to 132 months). The recurrence rate for primary tumours was 5.3% compared with 20% for recurrent tumours. Recurrence rates were similar between the main surgical treatment modalities (p=NS): wide local excision and frozen section control 4.2%, wide local excision without margin control 4.6%, Mohs 5.5% and wide local excision with paraffin section 5.5%. We did, however, find that higher T-stage was significantly associated with local recurrence (p=0.035) and that recurrent tumours were themselves more likely to recur (p=0.019). The recurrence rates for each T-stage at presentation were as follows; T1: 4.1%, T2a: 4.3%, T2b: 9.8%, T3a: 9.7% and T3b: 33.3%. However, we could not identify a T-stage threshold below which there was no risk of recurrence, as evidenced by our three cases of T1 primary tumours which recurred. Of these three cases, two were treated initially with wide local excision and one underwent wide local excision with paraffin section with recurrences occurring at 41, 57 and 108 months following initial treatment. We found that immunosuppression and poorly differentiated squamous cell carcinoma were not associated with an increased risk of recurrence but there was a trend for patients with histologically confirmed perineural invasion to have an increased risk of tumour recurrence (p=0.099).

Upon review of the Death Registry in all three states, we identified four patients (1.6%) who died of invasive or metastatic squamous cell carcinoma. Two of these patients had presented with primary T2b tumours, whilst two had

presented with recurrent tumours at T2a and T3b, both of which were then associated with further local recurrences.

3.4 Discussion

3.4.1 Summary of Findings

This multi-centre study represents, to the best of our knowledge, the largest case series of eyelid squamous cell carcinomas utilising the most up-to-date AJCC TNM 7th edition staging system for eyelid carcinoma. We found low rates of disease specific mortality (1.6%) and regional nodal metastasis (0% of primary tumours, 12% of recurrent tumours). We also found that higher T-stage was significantly associated with local recurrence, but there was no T-stage below which there was no risk of recurrence.

3.4.2 Previous Reports

There has been only one previous study of periocular squamous cell carcinomas utilising the AJCC TNM 7th edition staging system which included 51 primary and 14 recurrent periocular squamous cell carcinomas. The authors found that regional nodal metastases occurred in 6% of cases and was only observed in patients presenting with T2b tumours or higher; however, it is unclear how many cases of nodal metastasis involved recurrent tumours.[136] Reported rates of regional nodal metastasis in periocular squamous cell carcinoma vary from 1.3% to 24%.[117, 312, 313] Previously reported risk factors include recurrent or large tumours (>20mm), deeply invasive tumours, poorly differentiated tumours, immunosuppression and those with perineural invasion.[314] We had no cases of regional nodal metastasis within our cases of primary squamous cell carcinoma, but had three cases of nodal metastasis (12%) within our recurrent patients. Only 2.0% of our patients underwent investigation for nodal metastasis, with

sentinel lymph node biopsy used in just one case. The use of sentinel lymph node biopsy has previously been recommended for tumours >20mm, recurrent tumours or those with perineural invasion.[315] Our findings suggest that it may be worth considering the use of sentinel lymph node biopsy, or close nodal surveillance in recurrent cases.

3.4.3 Factors Associated with Recurrence and Metastases

Perineural invasion in periocular squamous cell carcinoma is associated with an increased risk of local recurrence and both regional nodal and distant metastases.[96, 110] Reported rates of perineural invasion in periocular squamous cell carcinoma vary significantly. Malhotra et al.[104] found perineural invasion in 4.3% of 79 cases from the Australian Mohs Database, whilst Donaldson and colleagues reported perineural invasion in 7.8% of 51 cases.[94] More recently, Nasser et al.[136] reported that perineural invasion was present in 25% of their cases and that it was significantly associated with higher T-stage tumours; however, it is unclear how many of these cases were recurrent tumours. We also found tumours with histologically confirmed perineural invasion tended to have a higher clinical T-stage at presentation. In addition, we demonstrated that recurrent tumours were also associated with a statistically significant increased risk of perineural invasion and we also found a trend for patients with histologically confirmed perineural invasion to have an increased risk of tumour recurrence.

We found an overall local recurrence rate of 6.8% during a median follow up of 35 months, with similar recurrence rates between the various surgical

treatment modalities. The Australian Mohs Database found a local recurrence rate of 3.6% for periocular squamous cell carcinomas during a median follow-up of 73 months,[104] and Leibovitch and colleagues reported a similarly low 5 year recurrence rate of 3.9% for 1263 patients treated with Mohs excision.[105] Nasser et al.[136] reported a higher local recurrence rate of 10.8% for periocular squamous cell carcinomas which included one recurrent case and T-stages ranging from T2a to T3a. Numerous factors have been associated with increased risk of local recurrence including increased tumour size, thickness, or depth, poor histologic differentiation, perineural invasion, locally recurrent tumours and immunosuppression.[96-98] We also found that recurrent tumours are more likely to be associated with further recurrences, and the risk is compounded by higher T-stage at presentation. When considering recurrence rate for T-stage at presentation, we found that T1 and T2a had similar recurrence rates at 4.1 and 4.3%, which doubled to 9.8 and 9.7% for T2b and T3a respectively, with T3b tumours having a 33.3% recurrence rate. Although we did find that higher T stage was associated with increasing risk of local recurrence, these figures suggest that this risk does not increase incrementally with each T-stage, and there may be additional factors not encompassed within the TNM staging also playing a significant role in the risk of recurrence. Although tumour thickness is known to be significant risk factor for recurrence and metastasis, we found that it was not recorded in the majority of histopathological reports. Furthermore, although we did not find that immunosuppressed patients had a higher risk of recurrence, this may be due to our low rates of immunosuppression amongst our patient population.

3.4.4 Squamous Cell Carcinoma in Situ (Bowen Disease)

Bowen disease or squamous cell carcinoma in situ is a slow-growing erythematous plaque first described in 1912 by JT Bowen.[316] The head and neck region is commonly involved and the associated risk of progression into an invasive squamous cell carcinoma has been reported to be 3 to 8% in untreated cases.[317, 318] Furthermore, in cases which do progress to invasive disease, studies have demonstrated more aggressive behaviour with a 3 to 5% risk of metastases.[317] Given the malignant potential, standard surgical excision or Mohs excision remains the treatment of choice, especially in the periocular region where other therapies such as photodynamic therapy, laser ablation, cryotherapy and topical 5-fluorouracil and imiquimod can be associated with significant local side effects and higher rates of local recurrence.[319]

Compared to standard excision without margin control which has a reported 5-year recurrence rate of 19%,[320] Mohs microsurgery was found to have a significantly lower 5-year recurrence rate of 6.3% in a large, prospective multi-centre study conducted by Leibotvitch and colleagues.[321] The authors highlighted the importance of margin-controlled excision, with 20% of their cases demonstrating subclinical tumour extension, as suggested that this may account for failure of other modalities reliant upon only clinical margins. This is also particularly of concern in the eyelid region, where lash follicles and pilosebaceous glands can extend deep into the eyelid. None of our cases progressed to invasive disease and we only recorded two cases of recurrence, although this is likely attributable to our smaller patient numbers.

Notably, the majority of our cases of squamous cell carcinoma in situ measured 10mm or greater clinically, and when taking into account subclinical extension and safety margins, does leave a significant eyelid defect following excision. The implications of this are discussed further in the following chapters.

3.5 Study Limitations

Although this study revealed useful insights into periocular squamous cell carcinoma, we acknowledge several limitations. Our multi-centre study included centres with differing treatment protocols, hence making correlation of TNM stage and outcome difficult. This does however reflect the lack of standardised treatment approaches to eyelid squamous cell carcinoma and encourages future studies utilising standard reporting with TNM staging. Furthermore, although inclusion dates would ideally be consistent between all centres to prevent selection bias, we felt there were added advantages of a larger sample size and statistical power with minimal selection bias. Additionally, although we were able to utilise the Death Registry to determine mortality rates, further recurrences may have occurred in patients not identified within the registry who were lost to follow-up. Lastly, our calculation of the local recurrence rate may have been confounded by the inherent difficulty of distinguishing between recurrent squamous cell carcinoma and new squamous cell carcinoma arising in a similar anatomical location. Two of our cases were considered to have developed local recurrence more than 5 years after treatment; however, it is unusual to have such a long disease-free interval,[98] and it is possible that tumours were de novo (non-recurrent) squamous cell carcinoma. Further prospective studies utilizing the AJCC 7th edition TNM staging are required to better evaluate the association between TNM stage and nodal metastasis.

3.6 Conclusion

In summary, we have found that higher T-stage at presentation and recurrent tumours were both associated with an increased risk of local recurrence. Our findings suggest it may be reasonable to consider sentinel nodal biopsy or at least careful surveillance of recurrent or high T-stage tumours. Recurrent tumours had a four-fold increased risk of recurrence compared to primary tumours. However, recurrence rates did not increase incrementally with each T-stage, suggesting that additional factors may significantly impact the risk of recurrence. Importantly, we could not identify a T-stage threshold below which there is no risk of recurrence, and therefore clinicians should remain mindful of the potential for low T-stage tumours to recur.

Table 5: TNM Stages for Patients with Periocular Squamous Cell Carcinoma

TNM Stage	All Patients (%)	Primary (%) N=229	Recurrent (%) N=25
T1N0M0	74 (29.1)	71 (31.0)	3 (12.0)
T2aN0M0	92 (36.2)	86 (37.6)	6 (24.0)
T2bN0M0	50 (19.7)	41 (17.9)	8 (32.0)
T3aN0M0	31 (12.2)	26 (11.4)	5 (20.0)
T3bN0M0	5 (2.0)	4 (1.7)	1 (4.0)
T2bN0M1	1 (0.4)	1 (0.4)	-
T3bN1M1	1 (0.4)	-	1 (4.0)

Table 6: Recurrent Cases of Periocular Squamous Cell Carcinoma

TNM stage	Primary/ Recurrent	Perineural invasion	Treatment Modality	Time to Recurrence (Months)	Recurrence treatment	Follow-up	Further Recurrences (Months from initial treatment)
T1	P	N	wide local excision	57	wide local excision	108	
T1	P	N	wide local excision + paraffin section	108	Nil	144	
T1	P	N	wide local excision	41	wide local excision	87	

T2a	P	N	wide local excision + frozen section	4	wide local excision	13	
T2a	P	N	Mohs	132	Mohs	132	
T2a	P	N	wide local excision + paraffin section	24	wide local excision	24	
T2a	R	N	wide local excision + paraffin section	6	Radiotherapy	6	
T2b	P	N	wide local excision + paraffin section	60	wide local excision	120	
T2b	P	N	Mohs	7	Nil	19	

T2b	P	N	Mohs	10	wide local excision	63	Y (18 and 31)
T2b	R	N	Mohs	29	Mohs	97	
T2b	R	N	wide local excision + paraffin section	18	Nil	77	
T3a	P	Y	Mohs	13	wide local excision	102	Y (25)
T3a	P	N	Mohs	6	wide local excision + Radiotherapy	135	
T3a	P	Y	wide local excision + frozen section	3	Exenteration	5	

T3b	R	Y	Exenteration	24	Radiotherapy	36	
T3b	R	N	Radiotherapy	3	Radiotherapy	60	

CHAPTER 4: BIOMECHANICAL STUDIES OF THE EYELID TARSUS

4.1 Introduction

The tarsus is a fibrocartilaginous layer of the eyelid which is essential to function and appearance. The tarsus is responsible for both structural support and physical form, and thus its adequate replacement during eyelid reconstruction is vital to final outcomes. In the upper eyelid, the tarsal plate measures approximately 25mm in length, with maximal central height of 10mm. Large full thickness eyelid defects unable to be closed directly require reconstruction of both the anterior and posterior lamellae, which include the tarsal plate and palpebral conjunctiva (see anatomic figure in Chapter 1).[144] Although tarsal repair is vital for functional eyelid reconstruction, this remains limited by the complexity of tarsus tissue and lack of suitable tarsal substitutes.

As discussed above, reconstruction of the eyelid is most commonly required following skin cancer excision. Basal cell carcinoma and squamous cell carcinoma are the two most common eyelid cancers in Australia, with a mean age at presentation between 61 and 67 years.[94, 104, 322] Significant morbidity can result from poorly reconstructed eyelids, leading to complications including corneal exposure and keratopathy, issues with eyelid position, watery or dry eye and poor cosmetic outcome. These issues can be particularly troubling in the elderly population who often rely on their vision for independence. Presently there are no reconstruction methods that are

completely satisfactory, and a new solution is required to achieve the desired outcomes for patients.[147, 148, 151]

Tissue engineering aims to produce functional substitutes to repair defects, and the use of engineered three-dimensional biomaterial constructs to reconstruct or repair living tissue has been widely investigated over the last two decades.[169-171] However, it is first vital to understand the mechanics of the tissue to be engineered in its native state in order to design suitable scaffolds for tissue engineering. To the best of our knowledge, there has yet to be a study investigating the normal biomechanical properties of eyelid tarsus tissue. We aimed to better understand the viscoelastic behaviour of normal human tarsus tissue by undertaking biomechanical testing on fresh samples of human tarsus following surgical removal.[323]

4.2 Methods

4.2.1 Sample Selection

Ten samples of healthy tarsus tissue were obtained from ten patients undergoing various eyelid procedures involving tissue removal at the Royal Adelaide Hospital. Of these patients, 7 were male and 3 were female. The median age was 71.5 years (range 63 to 86). The removal of healthy eyelid tissue in all cases was in keeping with standard practice and this tissue would otherwise have been discarded if not used in our study. Ethics approval was obtained from the Royal Adelaide Hospital and the Southern Adelaide Clinical Human Research Ethics Committees and all patients provided informed consent. All tissue samples studied were disease free, and pre-operative assessment of eyelid laxity was normal in all cases. The tarsus tissue layer was dissected from the eyelid into approximately 5mm wide x 1.5mm thick samples. All samples were placed in a solution of phosphate buffered saline (PBS) at room temperature and transported to the laboratory for biomechanical testing immediately following harvest.

4.2.2 CellScale BioTester

All tarsus samples were tested ‘fresh’ within 2 hours of excision and were not frozen or stored prior. Each sample of tarsus tissue was initially trimmed into a rectangular shape using a scalpel. Uniaxial tensile tests were performed using a CellScale BioTester 5000 (CellScale, Waterloo, Canada), a micromechanical testing system specially designed for biological materials (Figure 2). Custom-made tissue clamps were used to secure the tissue sample, which was fixed to two opposing actuator arms. In keeping with the

anatomical alignment of the tarsus, the loading axis was aligned in the mediolateral direction, which represents the primary direction of tension on the eyelid, as the tarsoligamentous sling between the medial and lateral orbital rims supports the eyelid against gravity, resulting in minimal tension in the vertical direction. This is also evidenced by the horizontal tension lines in the eyelid and the general surgical principle of repairing defects using incisions parallel to relaxed tension lines.[324] The clamped samples, oriented horizontally, were lowered into a PBS bath maintained at 37±1°C for the entire duration of the experiment (Figure 3). The overhead charge-coupled device (CCD) camera was used to measure sample length, width and thickness of the tissue. The camera was calibrated prior to the experiment by photographing a steel plate with dimensions measured separately with a micrometre. Length and width were measured from above, while thickness was assessed by placing the sample on a mounting plate in its hydrated state, turning the plate on its side and imaging it under slight tension. The average thickness was calculated from five evenly spaced thickness measurements taken along the length of the sample.

4.2.3 Biomechanical Parameters

A tensile preload of 50mN was applied for 10 minutes before the sample was subjected to uniaxial tension under linear ramp displacement control. The preload was applied to place the tissue in tension and avoid a large unloaded region in the stress-strain curve. Creep did not occur as the test commenced as soon as the preload value was reached, and the use of 50mN preload was based upon previously established studies using annulus fibrosus tissue and

pilot studies with tarsus samples.[325] Maximum strain was 30% of the original tissue length, as this allowed the sample to reach the linear portion of the stress-strain response without slipping out of the clamps, as determined by pilot tests. Thirty dynamic cycles were performed at a strain rate of 1%/s using a triangular waveform. The test parameters were controlled using the LabJoy 2.0 software (CellScale, Waterloo, Canada) which collected data at a sampling rate of 10 Hz. The BioTester's overhead CCD camera captured images at a rate of 1 Hz. The raw data were processed with MATLAB R2010b (MathWorks, Natick, USA) using a custom-written program. Non-contact surface strains were calculated by tracking the relative displacements of two visible features close to mid-substance on the sample surface using the CCD camera. Non-contact strains were calculated to provide a more accurate measurement of tissue strain in the middle of the sample, since the measurement of strain based on gripper displacement distance may have included local slippage of the tissue.

Pilot tests revealed that 15 cycles were sufficient for the tarsus to demonstrate a steady-state hysteresis. As such, the last cycle was used for the calculation of the viscoelastic properties: initial modulus, final modulus, and extensibility (Figure 4). Phase angle, a measure of energy absorption, was calculated from the last 10 cycles between the input strain and measured stress using the cross spectral density estimate function (Matlab: CSD m).[326] The initial modulus represented the stiffness of the toe region, and was calculated from the initial region of the stress-strain curve. The final modulus represented the stiffness of the linear region, and was calculated from the upper, linear portion

of the stress-strain curve, as this best represented the elastic region of the material. Both moduli were calculated using moving cell linear regression to identify the most linear region having the largest coefficient of determination.[206] The strain at which the two moduli lines intersected represented the extensibility, a parameter that describes the transition strain from the toe region to the linear region and the uncrimping of collagen fibres.

4.3 Results

Of the tarsus samples tested, the mean (SD) width was 5.51mm (1.45mm) whilst mean thickness was 1.6mm (0.51mm). The mean toe modulus was 0.14 (0.10) MPa, elastic modulus was 1.73 (0.61) MPa, with an extensibility of 15.8 (2.1) %, and phase angle of 6.4° (2.4)° (Table 7). After adjusting for the initial tissue slack, the maximum strain ranged from 23.8% to 30.0%. At maximum strain, the linear region of the stress-strain curve was reached without the sample slipping out of the clamps. This would otherwise have been demonstrated by sharp drop on the stress-strain curve (Figure 4).

4.4 Discussion

4.4.1 Summary and Relevance

Our study is the first to investigate not only the viscoelastic properties of human tarsus tissue, but also the properties of fresh tissue harvested without prior storage or freezing. The tarsus tissue has previously been described as a specialised connective tissue with both fibrous and cartilaginous properties due to its unique composition. The viscoelasticity of human tarsus is determined by structural content and architecture, which is known to include collagen types I, III and VI, aggrecan, versican, tenascin, cartilage oligomeric matrix protein and a variety of glycosaminoglycans.[6] The collagen content of tarsus provides elasticity,[203] while the stiffness of tarsus has been attributed to the aggrecan content.[6] Aggrecan has been previously shown to have an estimated nanomechanical compressive stiffness of approximately 1 Pa at strains of <20% (toe region), which increased linearly from 0.1 to 1.5 MPa at strains >40% (corresponding to 40-80 mg/ml aggrecan).[204] Thus, the stiffness of tarsus tissue in tension at the microscale is many orders of magnitude greater than aggrecan alone in the toe region, and of approximately similar magnitude in the linear region, although at lower extensibility. This suggests that tarsus stiffness is attributable to the interactions between collagen and proteoglycans.

4.4.2 Alternate Tarsus Substitutes and Their Biomechanical Properties

Reconstruction of full thickness eyelid defects, in particular the upper eyelid, remains a considerable challenge due to the complexity of tissues involved. In order to achieve a functional outcome, the reconstructed eyelid should be

mobile, such that it can allow for blinking, but also should be rigid enough to allow for stabilisation of the eyelid margin in order to prevent nearby structures abrading the cornea.[145] As discussed previously in Chapter 1, where tarsal grafts are unsuitable due to defect size or patient factors, commonly used tarsoconjunctival substitutes for eyelid reconstruction include the hard palate mucoperiosteum, nasal septal chondromucosa and auricular cartilage.[151] To the best of our knowledge, there are no previous biomechanical studies investigating hard palate or nasal septa. Previous studies on auricular cartilage have found that it contains glycosaminoglycan, collagen and elastin.[205] Although tarsus tissue is also composed of both glycosaminoglycan and collagen, it is not known to contain elastin, which differentiates auricular cartilage from other subtypes of cartilage and significantly alters its biomechanical properties.[205, 206] Collagen and elastin are known to exhibit differing biomechanical properties, with the tensile modulus of collagen being approximately 1000 times greater than elastin, and its extensibility being only 13% compared to 150% for elastin.[203] Previous studies have also utilised preserved sclera as a tarsus substitute with some success.[148] However, there are significant differences in the biomechanical properties of the two tissues, with the stiffness of sclera previously shown to vary range from 2.8-3.3MPa[207] and elastic modulus averaging 2.9 +/- 1.4MPa with a maximum strain of approximately 20%.[198]

There have been many previous studies investigating the biomechanical properties of human skin both *in vivo* and *in vitro*, and there is significant variation between studies due to numerous factors including, but not limited

to: differences in specimen location, age, biological variability and test conditions. As such, there is a wide range of values reported, which is summarised well by Ni Annaidh and colleagues[327] who performed uniaxial tensile tests using a universal tensile test machine on 56 samples of excised human skin from the back and found a ultimate tensile strength of 21.6 +/- 0.88MPa, mean failure strain of 54% +/- 17%, mean initial slope of 1.18 +/- 0.88MPa and mean elastic modulus of 83.3 +/- 34.9 MPa. These findings contrast with *in vivo* studies of forearm skin using an 'extensionmeter' conducted by Khatyr and colleagues,[328] who found an average elastic modulus of 0.13-0.66MPa.

The measured phase angles of normal tarsus tissue (Table 7) were consistent with those reported for soft collagenous tissues such as the medial collateral ligament (approximately 6° - 14°[329]), and the human intervertebral disc (3°-11°[326]). These low phase angles suggest that the damping response of tarsus tissue is relatively small, and can be considered to be a more elastic-like response instead of a more viscous response. In contrast to collagenous ligaments and tendons, proteoglycans and aggrecan concentrates tend to demonstrate substantially more viscous energy dissipation behaviour. At physiologic ionic strength, concentrated solutions of purified aggrecan aggregate exhibited predominantly elastic behavior at small shear strains.[330] Over a frequency sweep from 0.01 rad/s – 100 rad/s at 10% strain, the phase angle ranged from approximately 27° between 0.01 rad/s and 0.1 rad/s to reach a maximum of approximately 56° between 10 rad/s and 100 rad/s.[330] Further viscous behavior was found in proteoglycan concentrates, with the

phase angle of pure proteoglycans solutions at high concentrations (10 to 50 mg/mL) ranging from 50° to 75°.[331-333]

4.3.3 Application in Tissue Engineering

Where natural tarsal substitutes are limited in their composition and mechanical properties, tissue engineering allows for flexibility of structure and design. Various natural and synthetic polymers have been shown to demonstrate differing elastic moduli and stiffness, which can be altered using a number of techniques.[334-336] Porosity has been shown to significantly affect macroscopic mechanical properties in addition to cell adhesion and proliferation.[210, 337] Moroni and colleagues demonstrated that with increasing porosity and/or by altering scaffold architecture, the dynamic stiffness of the resultant scaffold could be varied from 0.186 +/- 0.005 MPa to 13.7 +/- 2.6 MPa for 3D fibre-deposited polyethyleneoxide-terephtalate and polybutylene-terephtelate scaffolds.[336] The tarsus has excellent potential to be engineered as it is thin and highly vascular, both of which helps to facilitate healing and nutrient delivery. Therefore, tissue engineering offers a promising technique to produce a tarsal substitute most biomechanically similar to natural human tarsus.

4.5 Study Limitations

Although our study offers novel insights into the biomechanics of human tarsus tissue, we acknowledge several limitations. Firstly, our results are limited by our small sample size, which reflected the difficulties in co-ordinating testing of fresh human tissue within 2 hours of excision. Additionally, our study consisted of older patients, as this population tends to develop conditions which necessitate the excision of normal eyelid tissue. However, the primary applicability of engineered tarsus would be in the older population, given this is the group most frequently requiring lid reconstruction, and our data is thus directly applicable as we would be seeking to replicate the normal tarsus in this older population. We were also unable to conduct biaxial tensile testing to explore the tarsus across two orthogonal axes due to limitations in sample size. It would be virtually impossible to obtain healthy tarsus samples of the size required for such tests, and importantly, when considering the primary tension of the normal eyelid is along the horizontal axis (the direction tested) with virtually no tension in the vertical direction, we therefore feel our results remain directly clinically applicable. Finally, it is likely that the measured stress may be higher than the ‘natural stress’, although in order to perform *in vivo* measurements, we would require tarsal samples larger than practically possible to obtain. Additional studies using fresh human eyelid tarsus would be of value and are required to further develop our findings.

4.6 Conclusion

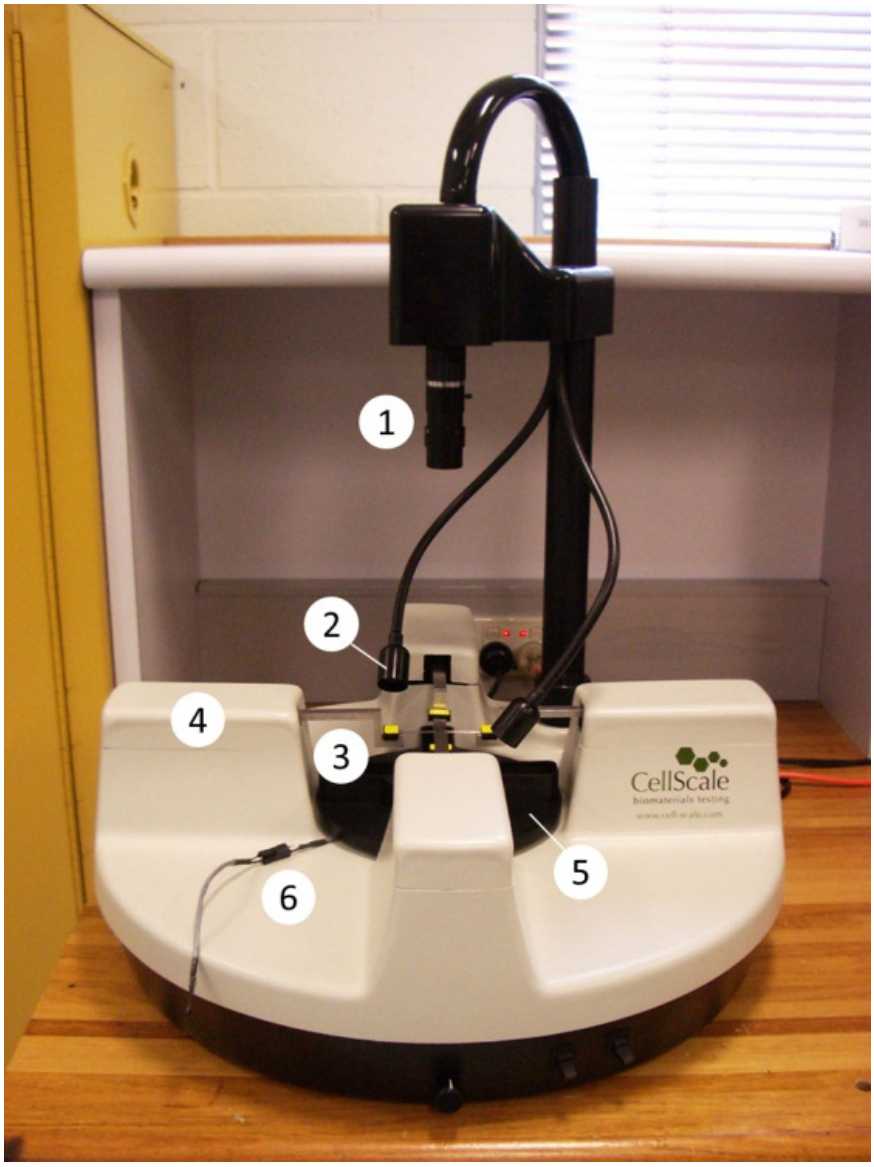
In summary, our findings represent the first biomechanical study of the human tarsus using fresh tissue specimens within 2 hours of harvest. We have found that natural human tarsus has a mean elastic modulus of 1.73 MPa with maximum strain ranging from 23.8% to 30.0%. These results establish a benchmark for native tarsus tissue, which has been used for our tissue engineering studies described in the chapters below.

Table 7: Biomechanical Properties of Tarsus Tissue

Sample dimensions, actual applied strain, initial and final moduli, extensibility, and phase angle of human eyelid tarsus tissue

Sample Number	Width [mm]	Thickness [mm]	Actual Strain [%]	Initial Modulus [MPa]	Final Modulus [MPa]	Extensibility [%]	Phase Angle [°]
1	8.17	2.79	27.3	0.015	1.11	18.73	5.02
2	5.04	1.67	25.8	0.060	1.32	16.18	4.78
3	6.14	1.53	28.2	0.298	2.69	11.51	3.88
4	6.59	1.41	30.0	0.237	2.41	16.08	12.10
5	6.61	1.19	27.3	0.076	1.86	15.03	5.25
6	4.23	1.34	26.8	0.244	1.70	16.68	5.49
7	3.49	0.97	26.8	0.239	2.47	15.16	6.30
8	5.89	1.93	26.8	0.122	1.38	15.68	8.03
9	5.08	1.81	23.8	0.045	1.43	14.27	8.35
10	3.86	1.33	28.7	0.063	0.91	18.73	5.15
Mean (SD)	**5.51 (1.45)**	**1.60 (0.51)**	**27.2 (1.7)**	**0.140 (0.103)**	**1.73 (0.61)**	**15.81 (2.11)**	**6.44 (2.44)**

Figure 2: The CellScale BioTester



1. An overhead charge-coupled device (CCD) camera, which monitors the sample as it is being stretched.
2. Overhead lamps to illuminate the sample.
3. Four actuator arms fitted with custom-made tissue clamps that hold the sample in place. As uniaxial testing was performed in this study, only two of the four actuator arms were required.

4. Load cells for measuring the force on the sample. The setup in this research used 23 N load cells (±0.1% error), which were housed in protective compartments on the sides of the device.
5. A water bath, which sits on top of a heated platform.
6. A temperature gauge, which is placed in the water bath to automatically monitor and control the temperature of the testing environment.

Figure 3: Tarsus Biomechanical Testing

A tarsus sample being tested fresh using the CellScale BioTester. The tarsus sample (1) is clamped in the mediolateral (arrow) direction. Scale bar represents 1 mm.

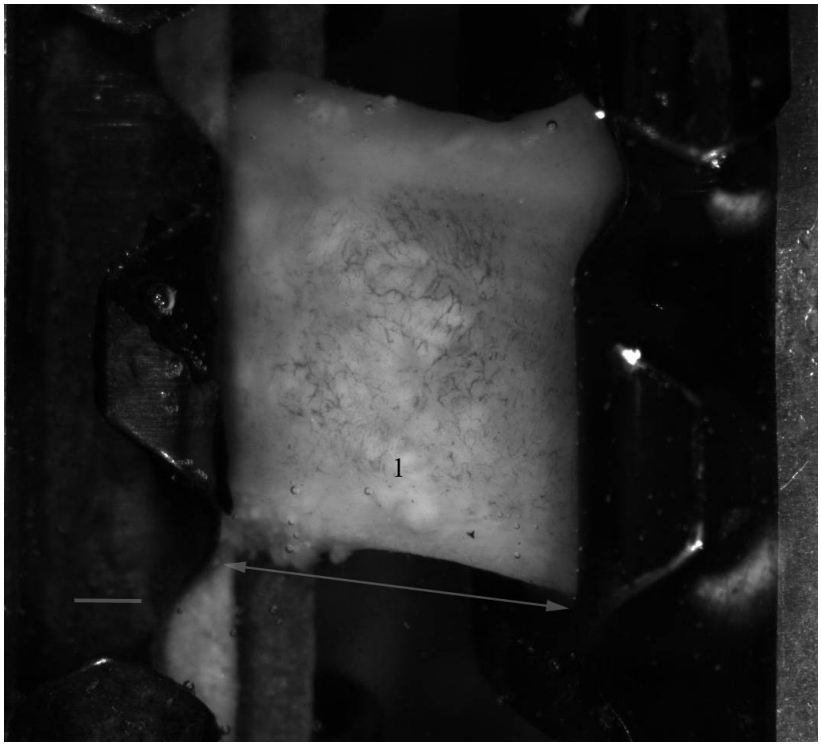
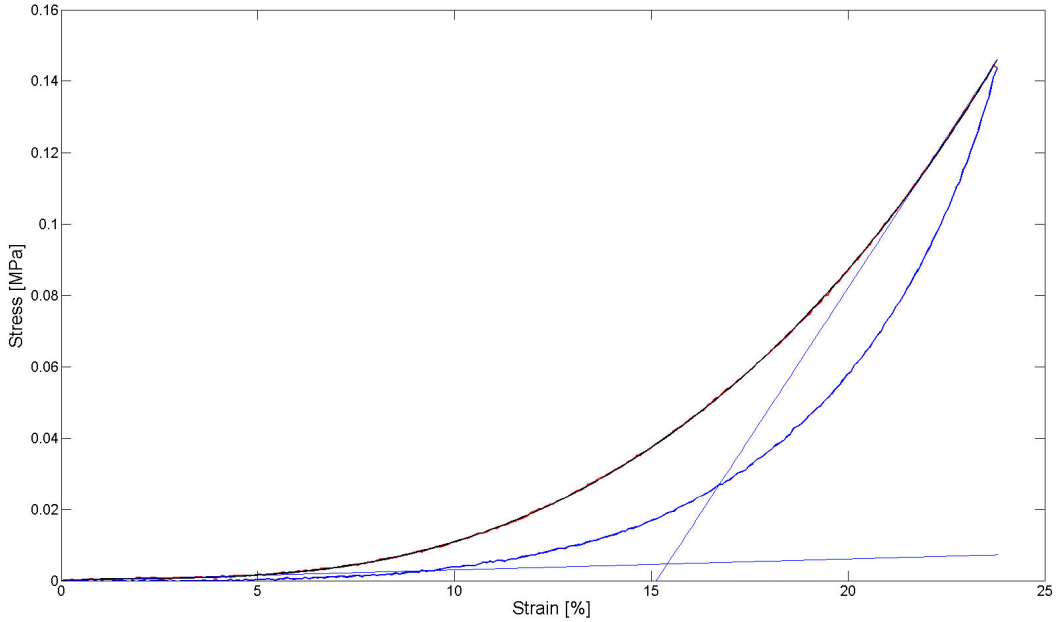


Figure 4: Stress-Strain Curve for Tarsus Tissue

A typical stress-strain curve showing the nonlinear viscoelastic load-unload response of the tissue. The initial modulus represents the stiffness of the toe region, the final modulus represents the stiffness of the linear region. Extensibility is the strain at the intersection of the two stiffness lines.



CHAPTER 5: SCAFFOLD DESIGN FOR BIOENGINEERED TARSUS

5.1 Introduction

As discussed in earlier chapters, eyelid reconstruction remains among the most challenging areas of oculoplastic reconstructive surgery and the most common indication is following skin cancer removal. Where tumours involve the full thickness and large portion of the eyelid, functional outcomes depend upon the ability to source a suitable substitute for the tarsus tissue. Natural tarsus is a specialised tissue which consists of fibroblast cells surrounded by an extra-cellular matrix with types I and III collagen, as well as aggrecan.[6] Presently there are no tarsal substitutes which are completely satisfactory and a new solution is required to achieve better functional outcomes for patients.[147, 148]

Tissue engineering represents the future of regenerative medicine and has huge potential to advance our current reconstructive techniques by producing highly specialised and personalised tissue replacements. The tarsus tissue has excellent potential to be bioengineered given its thin structure and highly vascularised surrounding eyelid tissue. To the best of our knowledge, there is only one previous study investigating the use of polymeric scaffolds for tarsal repair.[274] This study used acellular polyhydroxyalkonaoates as synthetic tarsal substitutes in rats and found that they were successful in supporting eyelid reconstruction and fibroblast growth. However, the authors noted inflammatory tissue responses within the first two weeks which developed into a milder chronic inflammation thereafter. Furthermore, this study was conducted prior to any published data on the normal biomechanical properties

of human tarsus tissue and thus it is unclear if the scaffold would be biomechanically suitable. Having established the biomechanical properties of fresh human tarsal tissue as above,[323] we describe the developed a novel bioengineered chitosan scaffold with biomechanically similar properties which has the potential to be used as a tarsal substitute during eyelid reconstruction.

5.2 Methods

5.2.1 Scaffold Material

Both synthetic and natural biopolymers may be used for tissue engineering. Synthetic polymers, such as poly(lactic-co-glycolic) acid, have tuneable properties for formation of biodegradable scaffolds and a history of clinical use. However, they are hydrophobic and so can lead to adverse tissue responses *in vivo*, including inflammation and foreign body reactions. Naturally-derived polymers such as chitosan have excellent potential as tissue engineered scaffolds for soft tissues due to their biomimetic properties.[338-342] Chitosan is derived from the exoskeleton of insects and crustaceans using N-deacetylation, and are highly porous structures, resembling glycosaminoglycans found in cartilage. Chitosan scaffolds are particularly applicable in tarsus engineering given they can be chemically similar to molecules in the native extra-cellular matrix and form hydrogels, making them more likely to be biocompatible. They are also often degraded through natural metabolic pathways in the body. Although hydrogel scaffolds tend to be relatively soft due to their high water content, their strength and stability can be enhanced through chemical cross-linking.[209]

Having experimented in initial studies with both chitosan and poly(lactic-co-glycolic) acid, we found that scaffolds created using chitosan more closely resembled the elastic modulus of human tarsus with tunable macroporous internal structure.

5.2.2 Scaffold Fabrication

Cryogelation was used to produce three dimensional macroporous chitosan hydrogel scaffolds with varying weight/volumes by cooling solutions of the polymers to below the solvent freezing point under controlled conditions. The mixture was then separated into a water-rich phase that crystallised and a polymer-rich phase that gelled to form the walls of the resulting scaffold. Cross linking agents were added to stabilise the structure before it was returned to room temperature, when the water in the pores melted and the porous scaffold is revealed, as shown schematically in Figure 5. The chitosan structures were cross-linked after solvent removal to enhance their stability. The pore size and internal architecture were regulated by controlling the processing parameters including mould geometry, freezing rates, chitosan and cross-linker concentrations.[210, 222] Previous studies suggested that a pore size of 15-160μm is sufficient for fibroblast growth and proliferation,[338] and thus we fabricated interconnected pores of size 10-100μm. At least 100 pores were measured for each sample to generate an average and standard deviation of pore size.

After initial biomechanical testing and characterisation (see below), the fabrication method was then optimised for this application with respect to the pore architecture, the bulk scaffold elasticity and tensile strength to develop structure-function relationships for the construct, relating their mechanical performance to their porous architecture.

5.2.3 Scaffold Characterisation and Preliminary Assessment

As part of preliminary studies, we have found fresh human tarsus had mean elastic modulus of 1.73 (0.61) MPa.[323] We used these results as a benchmark and the elasticity and tensile strength were tested during development of the scaffolds using the Instron Microtester to allow for optimisation of the scaffold architecture. The acellular scaffolds were constructed to exhibit slightly lower elastic modulus compared to native tarsus tissue in anticipation of cell culture, which is known to slightly alter the biomechanical properties of the resultant tissue complex.[343]

The scaffolds' stability was then assessed initially *in vitro* through aging in suitable buffer solutions or culture media for preliminary assessment. Scanning confocal microscopy was then used to characterise the scaffold architecture and thereafter used to confirm the scaffolds ability to support growth of 3T3 fibroblasts *in vitro*.

5.3 Results

5.3.1 Scaffold Characteristics and Biomechanics

Three dimensional chitosan hydrogel scaffolds of 3% and 5% w/v with tuneable internal macroporous architecture were developed. Figure 6 characterises the effect of chitosan concentration on pore size. Target pore sizes achieved using 3 and 5% w/v chitosan.

The 5% w/v chitosan cryogel scaffolds were found to exhibit biomechanical properties most suitable for tissue engineering, with an elastic modulus slightly lower but within the standard deviation range of natural natural human tarsus. Figure 7 characterises the tensile elastic modulus of 3 and 5% w/v chitosan cryogel scaffolds alongside human tarsus tissue.

5.3.2 Characterisation of Scaffolds

Figure 8 characterises the pore architecture of the 3% w/v scaffolds visualised by scanning confocal microscopy. Cross sections of the Chitosan scaffolds demonstrated varying pore sizes between 10-100μm which were successful in facilitating the attachment and proliferation of 3T3 fibroblast growth for up to 7 days (Figure 9).

5.4 Discussion

5.4.1 Summary of Findings

Our study represents the first to investigate the use of three dimensional polymeric chitosan scaffolds as an artificial eyelid tarsus extra-cellular matrix. Our scaffolds were constructed to be biomechanically similar to human tarsus and are able to support the growth of 3T3 fibroblasts *in vitro*.

5.4.2 Chitosan as Scaffold Material

Tissue engineering aims to restore pathologically altered tissue architecture by using bioengineered scaffolds and transplanted cells. Chitosan is a partially deacetylated derivative of chitin,[344] the primary structural polymer in arthropod exoskeletons, and has been widely investigated in tissue engineering, particularly in the field of orthopaedics.[341] It was approved for widespread use in 2005 as part of the HemCon bandage (HemCon Medical Technologies, Portland, Oregon) which is made from ChitoClear chitosan (Primex, Siglufjordur, Iceland) and creates an antibacterial barrier upon applications.[345] Chitosan demonstrates multiple favourable characteristics for tissue engineering, including the ability to be moulded in to various porous structures and intrinsic antibacterial nature.[341, 342] The primary enzyme responsible for degradation *in vivo* is lysozyme, and the degradation rate is inversely related to degree of crystallinity, and thus deacetylation. Animal studies have demonstrated minimal inflammatory reactions when implanted, with successful collagen deposition within the pore spaces.[346] Pore size and orientation have been shown to determine the mechanical properties of resultant scaffolds, and we found that an average pore sizes ranging from 10-

100μm were able to successfully support the growth of human orbital skin fibroblasts *in vitro* which would likely translate to ongoing proliferation *in vivo.*

5.4.3 Previous Studies of Bioengineered Tarsus

There has been only one previous study investigating the use of bioengineered scaffolds as a tarsal substitute. Zhou and colleagues studied a polyhydroxyalokonoate scaffolds implanted in rats, and compared with commercial acellular dermal matrices and blank defect controls.[274] The scaffolds were fabricated using 12% mol% hydroxyhanoate and the resultant 6wt% polyhydroxyalokonoate scaffolds were observed to consist of regular ladder-like structures with an average density of 0.069 g/mL and calculated porosity of 94.2%. Multiple micropores with a diameter of 5 μm were noted on the walls of the scaffold, which is significantly smaller than the pores within our bioengineered scaffold. We based our pore sizes on previous studies, which had shown that pores ranging from 15-160μm are sufficient for fibroblast growth and proliferation.[338] Of note, the polyhydroxyalokonoate scaffolds were found to support the least amount of fibroblast growth at each of the time-points compared to both the acellular dermal matrices and the blank implants. By 8 weeks, fibroblasts represented just under a third of the cell population of explanted eyelid samples with the bioengineered scaffold, with lymphocytes accounting for over half the cell population and macrophages making up the remainder.[274]

Additionally, these scaffolds were constructed prior to our studies on the normal biomechanical studies of the tarsus and hence it is unknown if the

scaffolds would be biomechanically similar. When examined histologically following implantation for varying weeks (range 1 to 8 weeks), the bioengineered scaffolds elicited changes suggestive of an acute, followed by chronic inflammatory response, which contrasted with minimal inflammatory changes around the acellular dermal matrices and blank defect controls.[274] These findings suggest that further refinement and optimisation of the scaffold architecture and biocompatibility would be required to improve outcomes. We elected to construct our scaffolds from chitosan, as it has been studied extensively in the past and is associated with minimal inflammatory responses when implanted. Additional methods aimed at improving biocompatibility are discussed in the following chapter.

5.5 Study Limitations

Although our study has demonstrated the feasibility of tissue engineering a biomechanically similar structure to natural human tarsus, there remain numerous uncertainties regarding the direct applicability in clinical practice. As this scaffold represents a novel structure, the safety first needs to be tested before further developments can be made. While we anticipate minimal inflammatory reaction based upon previous studies in the literature using chitosan, the response of native tissue once implanted remains to be determined. As chitosan is derived from the exoskeletons from crustaceans, there is a theoretical risk of allergic reaction to residual peptides/proteins in patients who are known to have a shellfish allergy. However, a recent study demonstrated no adverse reactions to chitosan powder or to the HemCon bandage made from chitosan in a series of nineteen patients with IgE documented skin-prick allergy to shellfish.[345] Further techniques to improve *in vivo* compatibility are discussed further below.

5.6 Conclusion

In summary, we have developed a novel bioengineered chitosan scaffold biomechanically similar to human tarsus tissue which has been found to support the growth 3T3 fibroblast cells *in vitro*. This has the potential to be used as a tarsus substitute during eyelid reconstruction, and additional studies investigating its ability to support human eyelid skin fibroblasts are discussed in the next chapter.

Figure 5: Formation of Scaffolds by Cryogelation

Schematic diagram demonstrating the formation of chitosan scaffolds by cryogelation.[209, 222]

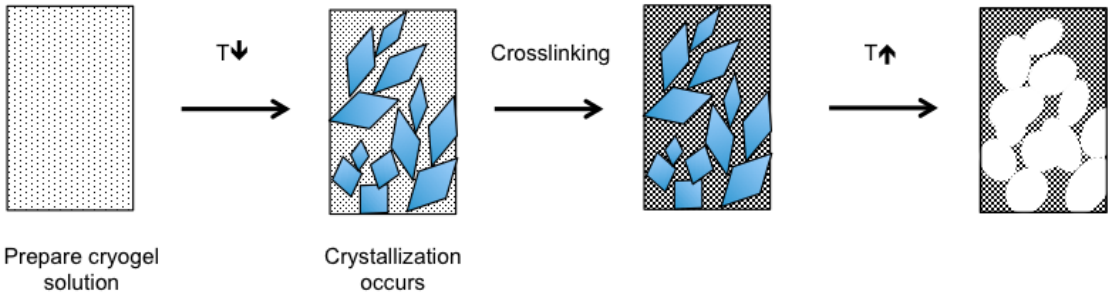


Figure 6: Effect of Chitosan Concentration of Pore Size of Chitosan Scaffolds

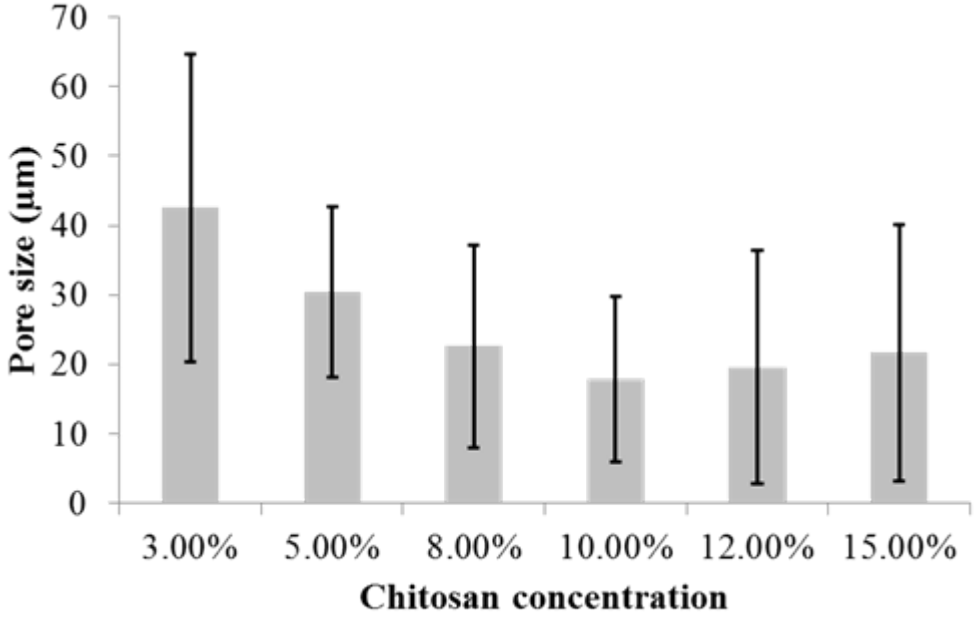


Figure 7: Tensile Elastic Modulus Scaffolds and Native Tarsus Tissue

Elastic modulus of 3 w/v% and 5 w/v% chitosan cryogel scaffolds compared with human tarsus tissue.

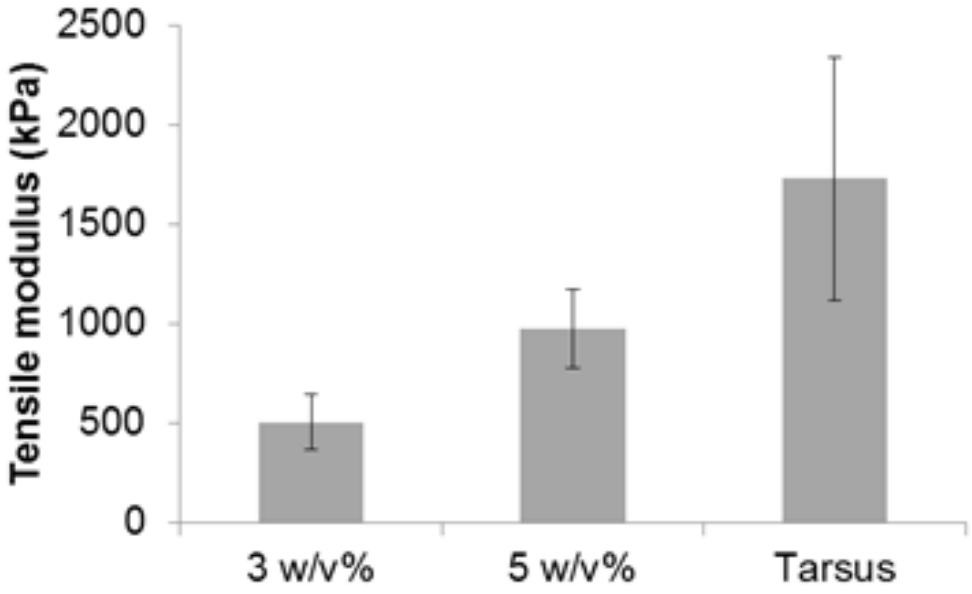


Figure 8: Pore Architecture of Chitosan Scaffolds

Pore architecture of 3% w/v chitosan scaffolds visualised by confocal microscopy. Scale bar represents 200 μm.

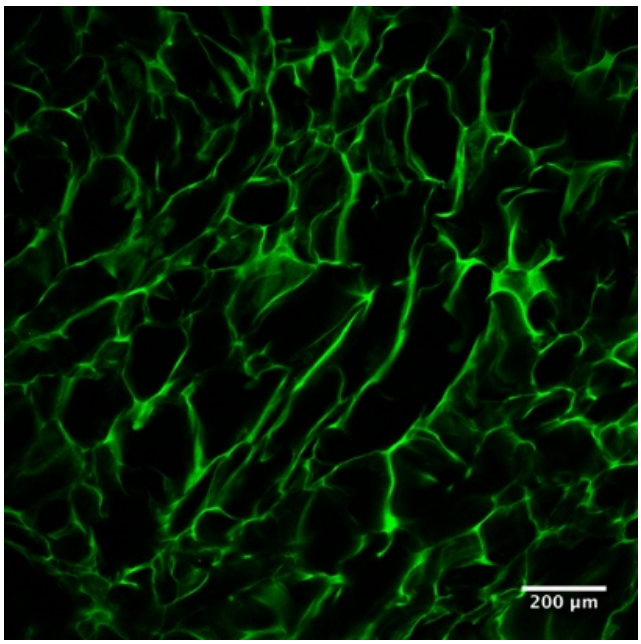
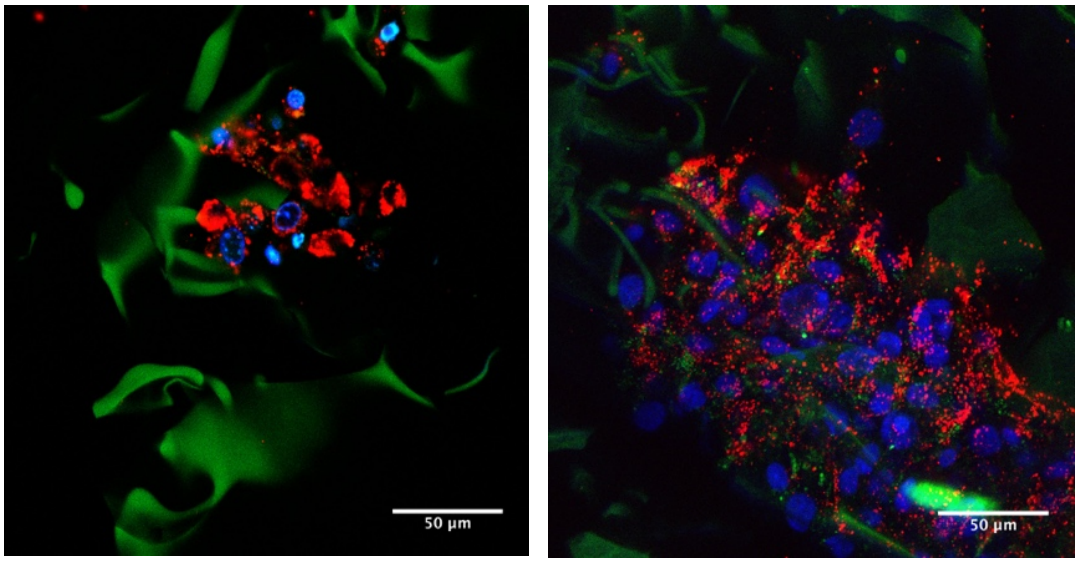


Figure 9: Chitosan Scaffolds Support Fibroblast Culture

Confocal microscopy demonstrating bioengineered chitosan scaffold surface after 1 day (left) and scaffold cross section after 7 days of 3T3 fibroblast cell culture (right). Scale bar represents 50 μm.



CHAPTER 6: FIBROBLAST CULTURE FOR BIOENGINEERED TARSUS

6.1 Introduction

The goal of tissue engineering is the development of replacement artificial constructs closely resembling native target tissue. Fundamental to this concept is the requirement for cell proliferation, differentiation and development over the bioengineered scaffold in a fashion which mimics the native and surrounding tissue. Fibroblast cells represent the fundamental cell type within the eyelid tarsus tissue which is supported by a natural extra-cellar matrix composed of different types of collagen and aggrecan.[6] These cells therefore represent the most obvious choice to culture for tissue engineering of the tarsus.

Fibroblasts are mesenchymal cells, and represent the most common connective tissue in living beings. They are easily cultured and are used extensively in tissue engineering studies for a variety of clinical applications.[347] Although fibroblasts from various anatomic sites all have similar morphology, previous DNA-microarrays studies have demonstrated that fibroblasts exhibit varying gene-expression profiles and characteristic phenotypes depending on their location.[275] Although numerous previous reports describe the successful culture of dermal fibroblasts from small skin samples, including the eyelid,[283] there has yet to be a study utilising these cells for tissue engineering purposes. We therefore aimed to culture eyelid skin fibroblasts and seed these onto our bioengineered tarsus scaffolds as part of the development of an artificial tarsus substitute.

6.2 Methods

6.2.1 Orbital Skin Fibroblast Culture

In this preliminary study, establishment of orbital skin fibroblast culture was achieved using human eyelid skin removed for various surgical procedures which would otherwise have been discarded. Patient consent was obtained and this study was approved by the Royal Adelaide Hospital ethics committee.

After removal of a piece of tissue for histological analysis via fixation in 10% neutral buffered formalin, the remaining sample was divided into 1-2mm cubes which were first washed in sterile phosphate-buffered saline (PBS; 137 mM NaCl, 5.4 mM KCl, 1.28 mM NaH_2PO4, 7 mM Na_2HPO4; pH 7.4) and then immersed in Dulbecco's modified Eagle Medium (5mM glucose, supplemented with 100U/ml Penicillin/Streptomycin, 2mM L-glutamine and 5% heat-inactivated foetal bovine serum) in a 30mm diameter petri dish. Four pieces of tissue were incubated in each dish at an orientation of 90 degrees to each other. Sterile borosilicate glass 13mm diameter coverslips were applied vertically onto the tissue pieces in order to encourage migration of fibroblasts. The tissue was then allowed to grow at 37^{o}C for 5 days, undisturbed. At this point, glass coverslips were removed and the medium changed. After a further 5 days, tissue pieces were removed and the medium changed again. We found that at this point, fibroblasts migrated from tissue onto the dishes. Within a further 4 days the fibroblasts typically reached confluence. At this point, the cells were passaged into 25cm^2 culture flasks (passage number 1). After reaching confluence again, cells were then placed into 75cm^2 culture

flasks and thereafter cells were split at a ratio of 1:4 and typically reached confluence within one week each time.

6.2.2 Characterising Differentiation

It is well known that primary cells de-differentiate in culture with increasing passage. It is imperative, therefore, that for the purposes of transplantation, fibroblasts derived from healthy patient tissue are not propagated through too many passages *in vitro*, lest they lose their defining characteristics. The rate of fibroblast de-differentiation *in vitro* was determined using characterisation by immunological and gene expression analyses. Fibroblast-specific marker proteins used included vimentin and fibroblast surface protein. Staining with alpha-smooth muscle actin was used as a marker of differentiation beyond fibroblasts to myofibroblastic cells.

6.2.3 Culture of Orbital Fibroblasts onto Bioengineered Scaffolds

Bioengineered chitosan scaffolds stored in sterile PBS at 4 degrees Celsius were dissected into discs of 5mm thickness and 13mm diameter for direct application of cells. Scaffolds were dehydrated and sterilised by passing through increasing concentrations of ethanol (25%, 50%, 75%, 100%) for 15 minutes in each dilution. To remove ethanol, scaffolds were then washed in PBS three times for 5 minutes each and exposed to UV radiation to ensure complete sterility.

For cell attachment, scaffolds were fully saturated by incubating in fibroblast Dulbecco's modified Eagle Medium, as formulated above, for 1 hour.

Fibroblasts were then sub-cultured onto scaffolds by trypsinisation at a density of 1 x 10^5 cells/ml and left to grow for 5 days. These scaffolds with cultured cells were then characterised by scanning electron microscopy imaging and confocal microscopy.

6.3 Results

6.3.1 Orbital Fibroblast Culture

We found that cells typically reached confluence after approximately 3 weeks in culture. All cells in passage 1 and passage 2 labelled with alpha-tubulin, which co-labelled with vimentin. Vimentin in turn, co-labelled with fibroblast surface protein (Figure 10). None of the cells labelled with alpha-smooth muscle actin (α-SMA), suggesting they had not yet become fibrotic or myofibroblastic. However, cells passaged through greater numbers demonstrated reduced expression of fibroblast specific markers and thus cells in passage 2 were utilised for the experiment below.

6.3.2 Culture of Fibroblasts Over Scaffolds

After establishing fibroblast confluence, cells were then cultured over our preliminary scaffolds. We found that our chitosan scaffolds were successful in supporting growth and proliferation of human orbital skin fibroblasts. Following 5 days of culture, we were able to demonstrate fibroblast growth on the scaffolds using scanning electron microscopy (Figure 11).

6.4 Discussion

6.4.1 Summary of Findings

To the best of our knowledge, our study is the first investigating the use of orbital skin fibroblast culture in tissue engineering. We have been able to demonstrate successful fibroblast culture from small pieces of eyelid skin and have used immunohistochemical staining to characterise the rate of de-differentiation. Furthermore, we have established culture of these orbital skin fibroblasts onto our three-dimensional bioengineered chitosan scaffolds which have been constructed to be biomechanically similar to human tarsus tissue. This study establishes the feasibility of this technique and has the potential to be used in the development of individualised bioengineered eyelid tarsus.

6.4.2 Previous Studies

We used similar culture techniques as previously described with lung fibroblasts derived from lung tissue from scleroderma patients at autopsy,[16, 17] and were successful in establishing and maintaining fibroblast culture using fresh eyelid skin. Although we found that cells cultured beyond passage 2 had started to de-differentiate, previous studies with lung fibroblasts utilised cells between the the second and fourth passages.[348] Furthermore, none of our cells in early passages stained for alpha-smooth muscle actin, a marker suggestive of fibrosis. However, Ludwicka and colleagues found that the lung fibroblasts expressed some markers of human smooth muscle differentiation and partially attributed this to their slightly differing biologic behaviour.[288]

In a previous study examining fibroblast source and ease of culture from various anatomic locations, Fernandes and colleagues were able to establish fibroblast growth from all sites investigated which included the eyelid, post-auricular skin and abdominal scar.[283] The authors utilised eyelid skin measuring 2mm x 2mm and were cultured using slightly different methods compared to our study. For example, the authors minced the skin samples prior to incubation in DMEM/F12, 10% of Fetal Bovine Serum (FBS, Invitrogen, Carlsbad, CA, USA), 2 mM L–glutamine (Invitrogen), 19 MEM NEAA (Invitrogen), 29 antibiotic antimycotic solution (penicillin/streptomycin/amphotericin—final concentration of 200 U/mL, 0.2 and 0.5 l g/mL, respectively—Sigma). Although they found that cell confluence for all samples reached around 90% by 2-3 weeks, it is unknown what the rate of de-differentiation was for these fibroblast cultures, which is an in important consideration for tissue engineering.

6.4.3 Applicability in Clinical Practice

There are numerous merits to utilising autologous fibroblasts which are of particular significance when applied to the delicate eyelid region. Autologous fibroblasts are associated with improved *in vivo* compatibility, no risk of rejection,[347] and furthermore have been found to result in superior cosmetic outcomes and minimal scar formation when compared to allogenic fibroblasts in dermal substitutes.[349, 350] We anticipate direct applicability in clinical practice with this technique, given the standard practice of taking a biopsy of the eyelid skin cancers to confirm the diagnosis and identify any high-risk histological features which may alter excisional margins required. We have

been able to demonstrate successful fibroblast culture from samples of skin as small as 1-2mm, which is the size of the smallest punch biopsy. It would be relatively straight-forward to take a small sample of healthy skin at the time of initial biopsy for fibroblast cell culture to occur whilst awaiting histological information. Once histology is confirmed, the scaffold could be constructed based upon the estimated defect size and the patient's own fibroblast cells cultured over scaffold to create a personalised tissue engineered tarsus tissue.

6.5 Limitations

Although our study has established the feasibility of orbital skin fibroblast culture for tissue engineering, our study is limited in its *in vitro* nature. It remains unknown how the cells would continue to proliferate, differentiate and develop once implanted *in vivo*. Additionally, the optimum volume of fibroblast seeding for greatest *in vivo* compatibility is yet to be determined, and could alter our culturing timeline. Furthermore, typically with our current culture technique, we have found cell culture typically requires 3 weeks, followed by a further week for cells to seed the scaffold. When considering this in a clinical context, although a delay between initial biopsy and excision is standard, especially for basal cell carcinomas, this may be unsuitable for patients with more aggressive tumours who need prompt excision. A staged reconstruction may be a possible alternative for these cases. Additional studies are required to evaluate the clinical applicability and these are already underway.

6.6 Conclusions

In summary, we have successfully demonstrated culture of orbital skin fibroblasts from small samples of eyelid skin tissue measuring the size of the smallest punch biopsy. These fibroblasts were found to stain strongly for fibroblast markers at the second passage of cells. Furthermore, we were able to establish confluent fibroblast growth over the surface of our bioengineered chitosan scaffolds. This has tremendous potential to be further developed and applied to clinical practice in the future as a tailor-made and personalised eyelid tarsus substitute.

Figure 10: Immunological and Gene Expression Analysis of Orbital Skin Fibroblasts

Immunological and gene expression analysis of cultured orbital skin fibroblasts at passage 1 and 2 with all cells labelling with α -tubulin and co-labelling with vimentin, the standard marker for fibroblasts. Note that cells do not label for α -SMA.

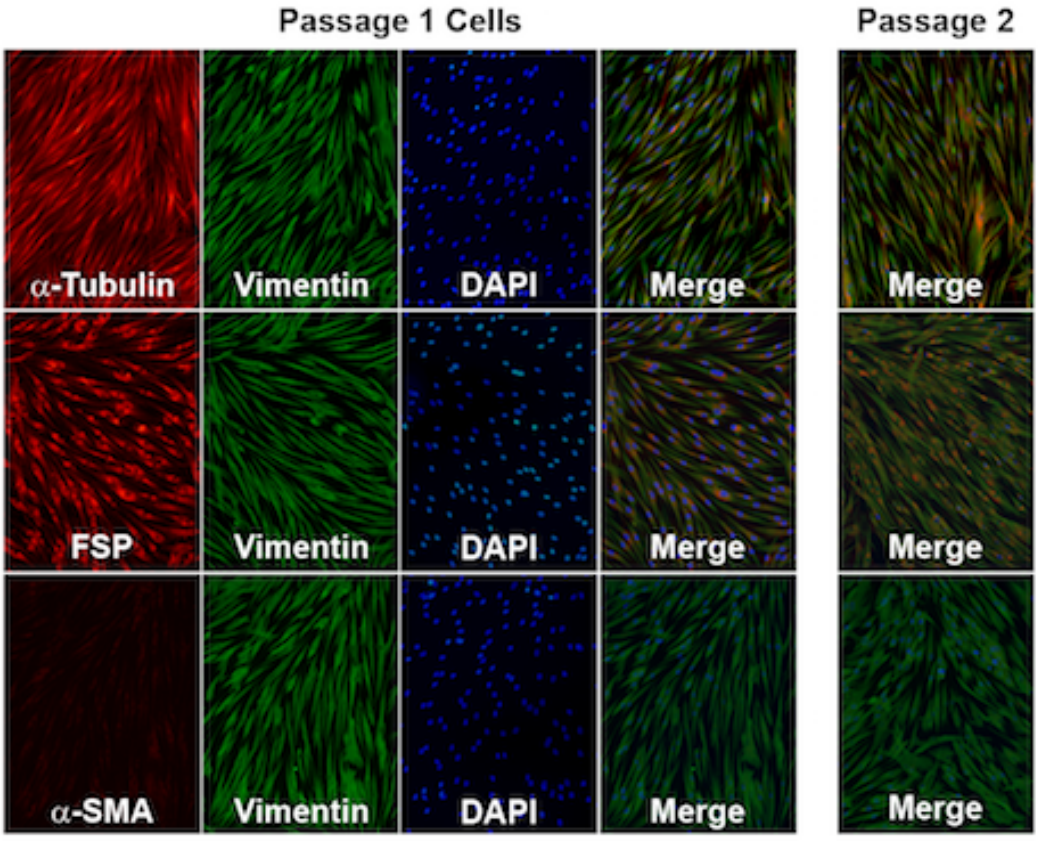
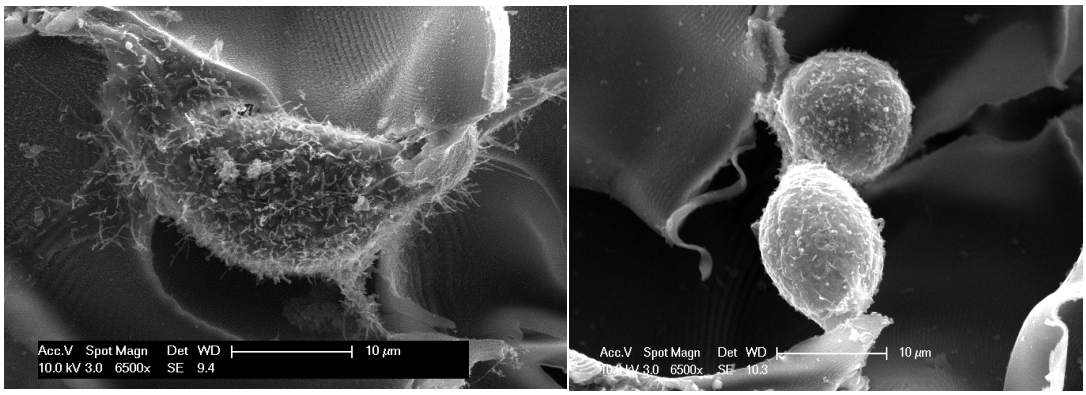


Figure 11: Scanning Electron Microscopy of Fibroblast Culture of Scaffolds

Scanning electron microscopy demonstrating fibroblast attachment over bioengineered chitosan scaffolds. Scale bar corresponds to 10 μm.



CHAPTER 7: FINAL DISCUSSION

This thesis has examined aspects related to the two most common types of skin cancer affecting the periocular region and furthermore describes the development of a bioengineered tissue for use during eyelid reconstruction. It has provided new information regarding the identification of high-risk periocular basal cell carcinomas using standard biopsy techniques and discusses the applicability and prognostic role of the AJCC TNM staging system in periocular squamous cell carcinoma. Furthermore, this thesis establishes the normal biomechanical properties of the human eyelid tarsus and directly applies this information during the development of a novel bioengineered eyelid tarsus tissue substitute created to be both biomechanically and biologically compatible. These findings provide further insights into how periocular skin cancers are diagnosed, staged and managed. Furthermore, our studies on bioengineering tarsus represent the first of its kind in the field of oculoplastic ophthalmology and has the potential to transform the way in which we approach reconstructive surgery.

In Chapter 2, we characterise the concordance of periocular basal cell carcinoma biopsies compared to excision and discuss the meanings behind margin reporting. Observations from this study include the fact that over half of initial biopsies involving mixed histology with an aggressive subtype failed to identify the aggressive component and highlights the need for careful consideration when planning treatment, especially for non-surgical management. Chapter 3 analyses the prognostic applicability of the 7th edition of the AJCC TNM staging for eyelid carcinoma for patients with periocular

squamous cell carcinoma. Although this updated staging system aimed to standardise our staging of cutaneous malignancy in the periocular region, there was only one previous study utilising this. Our study was able to demonstrate that higher T-stage tumours and recurrent tumours were both significantly associated with an increased risk of recurrence. Furthermore, there was no T-stage below which there was no risk of recurrence, highlighting the need for careful management and adequate follow-up for all patients with periocular squamous cell carcinoma. Despite the fact that squamous cell carcinoma in situ is generally associated with favourable outcomes with margin-controlled excision, we noted that the majority of tumours measured 10mm or greater which has significant implications when planning reconstruction following excision.

Eyelid reconstruction is most commonly required following periocular skin cancer excision and repair of larger defects remains a challenge due to the lack of suitable eyelid tarsus tissue substitutes. Despite numerous different autologous tissue and artificial material have been investigated in the past, none have been optimal. Although the tarsus tissue is widely recognised as a highly specialised structure, prior to studies conducted as part of thesis, the normal biomechanical properties of this tissue were unknown. In Chapter 4, we performed the first biomechanical studies on fresh healthy human eyelid tarsus tissue and characterised the mean toe modulus, elastic modulus, extensibility, phase angle and maximum strain capabilities of this unique tissue. These results were then used as a benchmark for our bioengineering studies.

Chapter 5 describes the process of creating an artificial, three-dimensional scaffold bioengineered to mimic the biomechanical properties of the extra-cellular matrix in native tarsus tissue. In this novel study, we fabricated three-dimensional chitosan scaffolds and were able to optimise tensile mechanical properties whilst also controlling pore sizes for ideal fibroblast culture. These scaffolds were shown to exhibit comparable biomechanical properties and supported the growth of 3T3 fibroblasts *in vitro*. Finally, in addition to techniques applied to the scaffold, to further improve biocompatibility, we were also able to successfully culture fibroblasts from samples of human eyelid skin and seed these onto the bioengineered scaffolds. These results are discussed in Chapter 6 and establishes the potential for a truly tailor-made tarsus tissue which could be used during eyelid reconstruction.

CHAPTER 8: FUTURE DIRECTIONS

Improving our understanding of the diagnosis, staging and management of periocular skin cancer is vital in our effort to reduce the burden of our nation's most common malignancy. Understanding the limitations of initial biopsy for histological analysis and the knowledge that recurrent periocular tumours are themselves associated with a higher risk of recurrence and aggressive behaviour highlights the need to adequately and uniformly stage disease in order to provide the most appropriate treatment and minimise risk of additional morbidity associated with recurrent disease. Importantly though, as surgical excision remains the mainstay of management for periocular skin cancer and a significant proportion of skin cancers occupy greater than 50% of the tarsal length, eyelid reconstruction with an adequate tarsus substitute is fundamental to final functional outcomes.

Having established the normal biomechanical properties of human eyelid tarsus, we have broadened our knowledge of this highly specialised tissue and this will only improve our ability to source appropriate tissue replacements. These results were used as benchmarks during development of the first bioengineered tarsus which integrates mechanical and biological compatibility. This novel tissue has the potential to improve our reconstructive outcomes and offer patients tailor-made tissue with cells cultured from their own skin. Moving forward, we hope to establish the safety of our bioengineered tissue before being able to offer this innovative reconstructive technique to patients undergoing excision of large eyelid skin cancers. Our scaffolds have the potential to be altered for numerous other applications

including the development of orbital implants for patients who require removal of their eye. We hope that the findings from this thesis represent just the beginning of bioengineering technology in the field of oculoplastic ophthalmology.

CHAPTER 9: REFERENCES

1. Staples MP, Elwood M, Burton RC, et al. Non-melanoma skin cancer in Australia: the 2002 national survey and trends since 1985. Med J Aust 2006;184(1):6-10.
2. Madan V, Lear JT, Szeimies RM. Non-melanoma skin cancer. Lancet 2010;375(9715):673-85.
3. Cancer in Australia 2010: an overview. Cancer Series No 60. Canberra: AIHW: Australian Institute of Health and Welfare, 2010.
4. Cook BE, Jr., Bartley GB. Treatment options and future prospects for the management of eyelid malignancies: an evidence-based update. Ophthalmology 2001;108(11):2088-98; quiz 99-100, 121.
5. Clarke P. Nonmelanoma skin cancers Treatment options. Australian Family Physician 2012;41(7):476-80.
6. Milz S, Neufang J, Higashiyama I, et al. An immunohistochemical study of the extracellular matrix of the tarsal plate in the upper eyelid in human beings. J Anat 2005;206(1):37-45.
7. Australian Institute of Health and Welfare & Australasian Association of Cancer Registries. Cancer in Australia: an overview, 2012. Cancer series no. 74. Cat. no. CAN 70. Canberra2012.
8. Yoshino K, Tseng SC, Pflugfelder SC. Substrate modulation of morphology, growth, and tear protein production by cultured human lacrimal gland epithelial cells. Exp Cell Res 1995;220(1):138-51.
9. Pleasance ED, Cheetham RK, Stephens PJ, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. Nature 2010;463(7278):191-6.

10. Kricker A, Armstrong BK, English DR, Heenan PJ. Does intermittent sun exposure cause basal cell carcinoma? a case-control study in Western Australia. Int J Cancer 1995;60(4):489-94.
11. Lear JT, Tan BB, Smith AG, et al. Risk factors for basal cell carcinoma in the UK: case-control study in 806 patients. J R Soc Med 1997;90(7):371-4.
12. Gallagher RP, Hill GB, Bajdik CD, et al. Sunlight exposure, pigmentary factors, and risk of nonmelanocytic skin cancer. I. Basal cell carcinoma. Arch Dermatol 1995;131(2):157-63.
13. Scotto J, Kopf AW, Urbach F. Non-melanoma skin cancer among Caucasians in four areas of the United States. Cancer 1974;34(4):1333-8.
14. Lee NG, Kim LA, Freitag SK. The role of genetics in the pathogenesis of periocular cutaneous neoplasms: implications for targeted therapy. Semin Ophthalmol 2013;28(5-6):267-74.
15. Hahn H, Wicking C, Zaphiropoulous PG, et al. Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell 1996;85(6):841-51.
16. Johnson RL, Rothman AL, Xie J, et al. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science 1996;272(5268):1668-71.
17. Tsao H. Genetics of nonmelanoma skin cancer. Arch Dermatol 2001;137(11):1486-92.

18. Hunt JL, Barnes L, Lewis JS, Jr., et al. Molecular diagnostic alterations in squamous cell carcinoma of the head and neck and potential diagnostic applications. Eur Arch Otorhinolaryngol 2014;271(2):211-23.
19. Kwa RE, Campana K, Moy RL. Biology of cutaneous squamous cell carcinoma. J Am Acad Dermatol 1992;26(1):1-26.
20. Bernstein SC, Lim KK, Brodland DG, Heidelberg KA. The many faces of squamous cell carcinoma. Dermatol Surg 1996;22(3):243-54.
21. Roewert-Huber J, Lange-Asschenfeldt B, Stockfleth E, Kerl H. Epidemiology and aetiology of basal cell carcinoma. Br J Dermatol 2007;157 Suppl 2:47-51.
22. Limawararut V, Leibovitch I, Sullivan T, Selva D. Periocular squamous cell carcinoma. Clin Experiment Ophthalmol 2007;35(2):174-85.
23. Hartevelt MM, Bavinck JN, Kootte AM, et al. Incidence of skin cancer after renal transplantation in The Netherlands. Transplantation 1990;49(3):506-9.
24. Lindelof B, Sigurgeirsson B, Gabel H, Stern RS. Incidence of skin cancer in 5356 patients following organ transplantation. Br J Dermatol 2000;143(3):513-9.
25. Mork J, Lie AK, Glattre E, et al. Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. N Engl J Med 2001;344(15):1125-31.
26. Telfer NR, Colver GB, Morton CA. Guidelines for the management of basal cell carcinoma. Br J Dermatol 2008;159(1):35-48.
27. Leibovitch I, McNab A, Sullivan T, et al. Orbital invasion by periocular basal cell carcinoma. Ophthalmology 2005;112(4):717-23.

28. Walling HW, Fosko SW, Geraminejad PA, et al. Aggressive basal cell carcinoma: presentation, pathogenesis, and management. Cancer metastasis reviews 2004;23(3-4):389-402.
29. Sun MT, Wu A, Figueira E, et al. Management of periorbital basal cell carcinoma with orbital invasion. Future Oncol 2015;11(22):3003-10.
30. Kossard S, Epstein Jr EH, Cerio R, et al. Basal cell carcinoma. Pathology and Genetics of Skin Tumours. Lyon: IARCPress, 2006.
31. Slater D, Walsh W. Dataset for the histological reporting of primary cutaneous basal cell carcinoma (2nd edition). London: The Royal College of Pathologists, 2012.
32. McCormack CJ, Kelly JW, Dorevitch AP. Differences in age and body site distribution of the histological subtypes of basal cell carcinoma. A possible indicator of differing causes. Archives of dermatology 1997;133(5):593-6.
33. Raasch BA, Buettner PG, Garbe C. Basal cell carcinoma: histological classification and body-site distribution. The British journal of dermatology 2006;155(2):401-7.
34. Scrivener Y, Grosshans E, Cribier B. Variations of basal cell carcinomas according to gender, age, location and histopathological subtype. The British journal of dermatology 2002;147(1):41-7.
35. Bastiaens MT, Hoefnagel JJ, Bruijn JA, et al. Differences in age, site distribution, and sex between nodular and superficial basal cell carcinoma indicate different types of tumors. The Journal of investigative dermatology 1998;110(6):880-4.

36. Pelucchi C, Di Landro A, Naldi L, La Vecchia C. Risk factors for histological types and anatomic sites of cutaneous basal-cell carcinoma: an italian case-control study. The Journal of investigative dermatology 2007;127(4):935-44.
37. Wu A, Sun MT, Huilgol SC, et al. Histological subtypes of periocular basal cell carcinoma. Clin Experiment Ophthalmol 2014;42(7):603-7.
38. Wong VA, Marshall JA, Whitehead KJ, et al. Management of periocular basal cell carcinoma with modified en face frozen section controlled excision. Ophthalmic plastic and reconstructive surgery 2002;18(6):430-5.
39. Ho SF, Brown L, Bamford M, et al. 5 years review of periocular basal cell carcinoma and proposed follow-up protocol. Eye 2013;27(1):78-83.
40. Neale RE, Davis M, Pandeya N, et al. Basal cell carcinoma on the trunk is associated with excessive sun exposure. Journal of the American Academy of Dermatology 2007;56(3):380-6.
41. Kaur P, Mulvaney M, Carlson JA. Basal cell carcinoma progression correlates with host immune response and stromal alterations: a histologic analysis. The American Journal of dermatopathology 2006;28(4):293-307.
42. Sexton M, Jones DB, Maloney ME. Histologic pattern analysis of basal cell carcinoma. Study of a series of 1039 consecutive neoplasms. Journal of the American Academy of Dermatology 1990;23(6 Pt 1):1118-26.
43. Wolberink EA, Pasch MC, Zeiler M, et al. High discordance between punch biopsy and excision in establishing basal cell carcinoma subtype:

analysis of 500 cases. Journal of the European Academy of Dermatology and Venereology : JEADV 2012.

44. Kamyab-Hesari K, Seirafi H, Naraghi ZS, et al. Diagnostic accuracy of punch biopsy in subtyping basal cell carcinoma. Journal of the European Academy of Dermatology and Venereology : JEADV 2012.
45. Haws AL, Rojano R, Tahan SR, Phung TL. Accuracy of biopsy sampling for subtyping basal cell carcinoma. Journal of the American Academy of Dermatology 2012;66(1):106-11.
46. Cohen PR, Schulze KE, Nelson BR. Basal cell carcinoma with mixed histology: a possible pathogenesis for recurrent skin cancer. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al] 2006;32(4):542-51.
47. Mosterd K, Thissen MR, van Marion AM, et al. Correlation between histologic findings on punch biopsy specimens and subsequent excision specimens in recurrent basal cell carcinoma. Journal of the American Academy of Dermatology 2011;64(2):323-7.
48. Roozeboom MH, Mosterd K, Winnepenninckx VJ, et al. Agreement between histological subtype on punch biopsy and surgical excision in primary basal cell carcinoma. Journal of the European Academy of Dermatology and Venereology : JEADV 2012.
49. Welsch MJ, Troiani BM, Hale L, et al. Basal cell carcinoma characteristics as predictors of depth of invasion. Journal of the American Academy of Dermatology 2012;67(1):47-53.
50. Allali J, D'Hermies F, Renard G. Basal cell carcinomas of the eyelids. Ophthalmologica Journal international d'ophtalmologie International

journal of ophthalmology Zeitschrift fur Augenheilkunde 2005;219(2):57-71.

51. Margo CE, Waltz K. Basal cell carcinoma of the eyelid and periocular skin. Survey of ophthalmology 1993;38(2):169-92.
52. Madge SN, Khine AA, Thaller VT, et al. Globe-sparing surgery for medial canthal Basal cell carcinoma with anterior orbital invasion. Ophthalmology 2010;117(11):2222-8.
53. Howard GR, Nerad JA, Carter KD, Whitaker DC. Clinical characteristics associated with orbital invasion of cutaneous basal cell and squamous cell tumors of the eyelid. American journal of ophthalmology 1992;113(2):123-33.
54. Satchi K, McNab AA. Orbital spread of eyelid and periocular cutaneous cancer. International ophthalmology clinics 2009;49(4):223-36.
55. Iuliano A, Strianese D, Uccello G, et al. Risk factors for orbital exenteration in periocular Basal cell carcinoma. American journal of ophthalmology 2012;153(2):238-41 e1.
56. Payne JW, Duke JR, Butner R, Eifrig DE. Basal cell carcinoma of the eyelids. A long-term follow-up study. Archives of ophthalmology 1969;81(4):553-8.
57. Perlman GS, Hornblass A. Basal cell carcinoma of the eyelids: a review of patients treated by surgical excision. Ophthalmic surgery 1976;7(4):23-7.
58. Prabhakaran VC, Gupta A, Huilgol SC, Selva D. Basal cell carcinoma of the eyelids. Comprehensive ophthalmology update 2007;8(1):1-14.

59. Vico P, Fourez T, Nemec E, et al. Aggressive basal cell carcinoma of head and neck areas. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology 1995;21(5):490-7.
60. Simons JN, Robinson DW, Masters FW. Malignant tumors of the orbit and periorbital structures treated by exenteration. Plastic and reconstructive surgery 1966;37(2):100-4.
61. Savage RC. Orbital exenteration and reconstruction for massive basal cell and squamous cell carcinoma of cutaneous origin. Annals of plastic surgery 1983;10(6):458-66.
62. Meads SB, Greenway HT. Basal cell carcinoma associated with orbital invasion: clinical features and treatment options. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al] 2006;32(3):442-6.
63. Mohs FE. The chemosurgical method for the microscopically controlled excision of radioresistant facial cancer. The American journal of roentgenology, radium therapy, and nuclear medicine 1955;73(1):61-9.
64. Feasel AM, Brown TJ, Bogle MA, et al. Perineural invasion of cutaneous malignancies. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al] 2001;27(6):531-42.
65. Richmond JD, Davie RM. The significance of incomplete excision in patients with basal cell carcinoma. British journal of plastic surgery 1987;40(1):63-7.
66. Rathbun JE, Beard C, Quickert MH. Evaluation of 48 cases of orbital exenteration. American journal of ophthalmology 1971;72(1):191-9.

67. Callahan A, Wilkins RB, Dowling EA. Massive orbital invasion by small malignant lesions. Southern medical journal 1975;68(3):271-8.
68. Selva D, Hale L, Bouskill K, Huilgol SC. Recurrent morphoeic basal cell carcinoma at the lateral canthus with orbitocranial invasion. The Australasian journal of dermatology 2003;44(2):126-8.
69. Williams LS, Mancuso AA, Mendenhall WM. Perineural spread of cutaneous squamous and basal cell carcinoma: CT and MR detection and its impact on patient management and prognosis. International journal of radiation oncology, biology, physics 2001;49(4):1061-9.
70. Ampil FL, Hardin JC, Peskind SP, Stucker FJ. Perineural invasion in skin cancer of the head and neck: a review of nine cases. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons 1995;53(1):34-8.
71. Ben Simon GJ, Schwarcz RM, Douglas R, et al. Orbital exenteration: one size does not fit all. American journal of ophthalmology 2005;139(1):11-7.
72. Rosen HM. Periorbital basal cell carcinoma requiring ablative craniofacial surgery. Archives of dermatology 1987;123(3):376-8.
73. Tyers AG. Orbital exenteration for invasive skin tumours. Eye 2006;20(10):1165-70.
74. Menon NG, Girotto JA, Goldberg NH, Silverman RP. Orbital reconstruction after exenteration: use of a transorbital temporal muscle flap. Annals of plastic surgery 2003;50(1):38-42.

75. Shields JA, Shields CL, Demirci H, et al. Experience with eyelid-sparing orbital exenteration: the 2000 Tullos O. Coston Lecture. Ophthalmic plastic and reconstructive surgery 2001;17(5):355-61.
76. Wulc AE, Adams JL, Dryden RM. Cerebrospinal fluid leakage complicating orbital exenteration. Archives of ophthalmology 1989;107(6):827-30.
77. de Conciliis C, Bonavolonta G. Incidence and treatment of dural exposure and CSF leak during orbital exenteration. Ophthalmic plastic and reconstructive surgery 1987;3(2):61-4.
78. Manstein ME, Manstein CH, Smith R. How accurate is frozen section for skin cancers? Annals of plastic surgery 2003;50(6):607-9.
79. Murchison AP, Walrath JD, Washington CV. Non-surgical treatments of primary, non-melanoma eyelid malignancies: a review. Clinical & experimental ophthalmology 2011;39(1):65-83; quiz 92-3.
80. Barnes EA, Breen D, Culleton S, et al. Palliative radiotherapy for non-melanoma skin cancer. Clinical oncology 2010;22(10):844-9.
81. Perez CA. Principles and practice of radiation oncology, 4th ed. Philadelphia: Lippincott Williams & Wilkins, 2004; xxiii, 2527 p.
82. Takeda A, Shigematsu N, Suzuki S, et al. Late retinal complications of radiation therapy for nasal and paranasal malignancies: relationship between irradiated-dose area and severity. International journal of radiation oncology, biology, physics 1999;44(3):599-605.
83. Rene C. Oculoplastic aspects of ocular oncology. Eye 2013;27(2):199-207.

84. Leatherbarrow B. Oculoplastic surgery, Second Edition. ed. London: Informa Healthcare, 2011; x, 584 pages.
85. Pfeiffer P, Hansen O, Rose C. Systemic cytotoxic therapy of basal cell carcinoma. A review of the literature. European journal of cancer 1990;26(1):73-7.
86. Guthrie TH, Jr., Porubsky ES, Luxenberg MN, et al. Cisplatin-based chemotherapy in advanced basal and squamous cell carcinomas of the skin: results in 28 patients including 13 patients receiving multimodality therapy. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 1990;8(2):342-6.
87. Morley M, Finger PT, Perlin M, et al. Cis-platinum chemotherapy for ocular basal cell carcinoma. The British journal of ophthalmology 1991;75(7):407-10.
88. Slutsky JB, Jones EC. Periocular cutaneous malignancies: a review of the literature. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al] 2012;38(4):552-69.
89. Neudorfer M, Merimsky O, Lazar M, Geyer O. Cisplatin and doxorubicin for invasive basal cell carcinoma of the eyelids. Annals of ophthalmology 1993;25(1):11-3.
90. Gill HS, Moscato EE, Chang AL, et al. Vismodegib for Periocular and Orbital Basal Cell Carcinoma. JAMA Ophthalmol 2013.
91. Sekulic A, Migden MR, Oro AE, et al. Efficacy and safety of vismodegib in advanced basal-cell carcinoma. N Engl J Med 2012;366(23):2171-9.
92. Reifler DM, Hornblass A. Squamous cell carcinoma of the eyelid. Surv Ophthalmol 1986;30(6):349-65.

93. Sullivan TJ. Squamous cell carcinoma of eyelid, periocular, and periorbital skin. Int Ophthalmol Clin 2009;49(4):17-24.
94. Donaldson MJ, Sullivan TJ, Whitehead KJ, Williamson RM. Squamous cell carcinoma of the eyelids. Br J Ophthalmol 2002;86(10):1161-5.
95. Maclean H, Dhillon B, Ironside J. Squamous cell carcinoma of the eyelid and the acquired immunodeficiency syndrome. Am J Ophthalmol 1996;121(2):219-21.
96. Rowe DE, Carroll RJ, Day CL, Jr. Prognostic factors for local recurrence, metastasis, and survival rates in squamous cell carcinoma of the skin, ear, and lip. Implications for treatment modality selection. J Am Acad Dermatol 1992;26(6):976-90.
97. Motley R, Kersey P, Lawrence C, et al. Multiprofessional guidelines for the management of the patient with primary cutaneous squamous cell carcinoma. Br J Plast Surg 2003;56(2):85-91.
98. Alam M, Ratner D. Cutaneous squamous-cell carcinoma. N Engl J Med 2001;344(13):975-83.
99. Cassarino DS, Derienzo DP, Barr RJ. Cutaneous squamous cell carcinoma: a comprehensive clinicopathologic classification--part two. J Cutan Pathol 2006;33(4):261-79.
100. Cassarino DS, Derienzo DP, Barr RJ. Cutaneous squamous cell carcinoma: a comprehensive clinicopathologic classification. Part one. J Cutan Pathol 2006;33(3):191-206.
101. Weedon D. Tumors of the epidermis. Skin pathology. London: Churchill Livingstone, 2002.

102. Barzilai G, Greenberg E, Cohen-Kerem R, Doweck I. Pattern of regional metastases from cutaneous squamous cell carcinoma of the head and neck. Otolaryngol Head Neck Surg 2005;132(6):852-6.
103. Broders AC. Practical points on the microscopic grading of carcinoma. NY State J Med 1932;32:667.
104. Malhotra R, Huilgol SC, Huynh NT, Selva D. The Australian Mohs database: periocular squamous cell carcinoma. Ophthalmology 2004;111(4):617-23.
105. Leibovitch I, Huilgol SC, Selva D, et al. Cutaneous squamous cell carcinoma treated with Mohs micrographic surgery in Australia I. Experience over 10 years. J Am Acad Dermatol 2005;53(2):253-60.
106. Veness MJ. Defining patients with high-risk cutaneous squamous cell carcinoma. Australas J Dermatol 2006;47(1):28-33.
107. Rudolph R, Zelac DE. Squamous cell carcinoma of the skin. Plast Reconstr Surg 2004;114(6):82e-94e.
108. Khanna M, Fortier-Riberdy G, Smoller B, Dinehart S. Reporting tumor thickness for cutaneous squamous cell carcinoma. J Cutan Pathol 2002;29(6):321-3.
109. Leibovitch I, Huilgol SC, Selva D, et al. Cutaneous squamous cell carcinoma treated with Mohs micrographic surgery in Australia II. Perineural invasion. J Am Acad Dermatol 2005;53(2):261-6.
110. McNab AA, Francis IC, Benger R, Crompton JL. Perineural spread of cutaneous squamous cell carcinoma via the orbit. Clinical features and outcome in 21 cases. Ophthalmology 1997;104(9):1457-62.

111. Goepfert H, Dichtel WJ, Medina JE, et al. Perineural invasion in squamous cell skin carcinoma of the head and neck. Am J Surg 1984;148(4):542-7.
112. Feasel AM, Brown TJ, Bogle MA, et al. Perineural invasion of cutaneous malignancies. Dermatol Surg 2001;27(6):531-42.
113. Hoxtell EO, Mandel JS, Murray SS, et al. Incidence of skin carcinoma after renal transplantation. Arch Dermatol 1977;113(4):436-8.
114. Dzubow LM, Rigel DS, Robins P. Risk factors for local recurrence of primary cutaneous squamous cell carcinomas. Treatment by microscopically controlled excision. Arch Dermatol 1982;118(11):900-2.
115. Robins P, Dzubow LM, Rigel DS. Squamous-cell carcinoma treated by Mohs' surgery: an experience with 414 cases in a period of 15 years. J Dermatol Surg Oncol 1981;7(10):800-1.
116. Brodland DG, Zitelli JA. Surgical margins for excision of primary cutaneous squamous cell carcinoma. J Am Acad Dermatol 1992;27(2 Pt 1):241-8.
117. Shulman J. Treatment of malignant tumours of the eyelids by plastic surgery. Br J Plast Surg 1962;15:37-47.
118. Francis IC, Benecke PS, Kappagoda MB. A ten-year hospital survey of eyelid cancer. Aust J Ophthalmol 1984;12(2):121-7.
119. Ghauri RR, Gunter AA, Weber RA. Frozen section analysis in the management of skin cancers. Ann Plast Surg 1999;43(2):156-60.
120. Abide J, Nahai, F., Bennett, RG. The meaning of surgical margins. Plast Reconstr Surg 1984;73:492-6.

121. Mohs FE. Micrographic surgery for the microscopically controlled excision of eyelid cancers. Arch Ophthalmol 1986;104(6):901-9.
122. Nemet AY, Deckel Y, Martin PA, et al. Management of periocular basal and squamous cell carcinoma: a series of 485 cases. Am J Ophthalmol 2006;142(2):293-7.
123. Anderson R. A warning on cryosurgery for eyelid malignancies. Arch Ophthalmol 1978;96(7):1289-90.
124. Fraunfelder FT, Zacarian SA, Wingfield DL, Limmer BL. Results of cryotherapy for eyelid malignancies. Am J Ophthalmol 1984;97(2):184-8.
125. Lederman M. Radiation treatment of cancer of the eyelids. Br J Ophthalmol 1976;60(12):794-805.
126. Fitzpatrick PJ, Thompson GA, Easterbrook WM, et al. Basal and squamous cell carcinoma of the eyelids and their treatment by radiotherapy. Int J Radiat Oncol Biol Phys 1984;10(4):449-54.
127. Veness MJ. Treatment recommendations in patients diagnosed with high-risk cutaneous squamous cell carcinoma. Australas Radiol 2005;49(5):365-76.
128. Luxenberg MN, Guthrie TH, Jr. Chemotherapy of basal cell and squamous cell carcinoma of the eyelids and periorbital tissues. Ophthalmology 1986;93(4):504-10.
129. Lewis CM, Glisson BS, Feng L, et al. A phase II study of gefitinib for aggressive cutaneous squamous cell carcinoma of the head and neck. Clin Cancer Res 2012;18(5):1435-46.

130. Yin VT, Pfeiffer ML, Esmaeli B. Targeted therapy for orbital and periocular basal cell carcinoma and squamous cell carcinoma. Ophthal Plast Reconstr Surg 2013;29(2):87-92.
131. Maubec E, Petrow P, Scheer-Senyarich I, et al. Phase II study of cetuximab as first-line single-drug therapy in patients with unresectable squamous cell carcinoma of the skin. J Clin Oncol 2011;29(25):3419-26.
132. Ainbinder DJ, Esmaeli B, Groo SC, et al. Introduction of the 7th edition eyelid carcinoma classification system from the American Joint Committee on Cancer-International Union Against Cancer staging manual. Arch Pathol Lab Med 2009;133(8):1256-61.
133. Shinder R, Ivan D, Seigler D, et al. Feasibility of using American Joint Committee on Cancer Classification criteria for staging eyelid carcinomas. Orbit 2011;30(5):202-7.
134. Buethe D, Warner C, Miedler J, Cockerell CJ. Focus Issue on Squamous Cell Carcinoma: Practical Concerns Regarding the 7th Edition AJCC Staging Guidelines. J Skin Cancer 2011;2011:156391.
135. Crawford C, Fernelius C, Young P, et al. Application of the AJCC 7th edition carcinoma of the eyelid staging system: a medical center pathology based, 15-year review. Clin Ophthalmol 2011;5:1645-8.
136. Nasser QJ, Roth KG, Warneke CL, et al. Impact of AJCC 'T' designation on risk of regional lymph node metastasis in patients with squamous carcinoma of the eyelid. Br J Ophthalmol 2014;98(4):498-501.
137. Shields JA, Demirci H, Marr BP, et al. Sebaceous carcinoma of the ocular region: a review. Surv Ophthalmol 2005;50(2):103-22.

138. Esmaeli B, Nasser QJ, Cruz H, et al. American Joint Committee on Cancer T category for eyelid sebaceous carcinoma correlates with nodal metastasis and survival. Ophthalmology 2012;119(5):1078-82.
139. Choi YJ, Jin HC, Lee MJ, et al. Prognostic value of clinical and pathologic T stages defined by the American Joint Committee on Cancer for eyelid sebaceous carcinoma in Korea. Jpn J Ophthalmol 2014;58(4):327-33.
140. Watanabe A, Sun MT, Pirbhai A, et al. Sebaceous carcinoma in Japanese patients: clinical presentation, staging and outcomes. Br J Ophthalmol 2013;97(11):1459-63.
141. Hitchcock CL, Bland KI, Laney RG, 3rd, et al. Neuroendocrine (Merkel cell) carcinoma of the skin. Its natural history, diagnosis, and treatment. Ann Surg 1988;207(2):201-7.
142. Herbert HM, Sun MT, Selva D, et al. Merkel cell carcinoma of the eyelid: management and prognosis. JAMA Ophthalmol 2014;132(2):197-204.
143. Sniegowski MC, Warneke CL, Morrison WH, et al. Correlation of American Joint Committee on Cancer T category for eyelid carcinoma with outcomes in patients with periocular Merkel cell carcinoma. Ophthal Plast Reconstr Surg 2014;30(6):480-5.
144. DiFrancesco LM, Codner MA, McCord CD. Upper eyelid reconstruction. Plast Reconstr Surg 2004;114(7):98e-107e.
145. Morley AM, deSousa JL, Selva D, Malhotra R. Techniques of upper eyelid reconstruction. Surv Ophthalmol 2010;55(3):256-71.

146. Hicks CR, Crawford GJ, Lou X, et al. Corneal replacement using a synthetic hydrogel cornea, AlphaCor: device, preliminary outcomes and complications. Eye (Lond) 2003;17(3):385-92.
147. Ito O, Suzuki S, Park S, et al. Eyelid reconstruction using a hard palate mucoperiosteal graft combined with a V-Y subcutaneously pedicled flap. Br J Plast Surg 2001;54(2):106-11.
148. Kamiya H, Kitajima Y. Successful use of preserved sclera of eyelid reconstruction. Eur J Dermatol 2003;13(3):267-71.
149. Jordan DR, Anderson RL. Eyelid reconstruction with irradiated human tarsal plate and aorta. Int Surg 1997;82(4):350-8.
150. Jordan DR, Tse DT, Anderson RL, Hansen SO. Irradiated homologous tarsal plate banking: a new alternative in eyelid reconstruction. Part II. Human data. Ophthal Plast Reconstr Surg 1990;6(3):168-76.
151. Ito R, Fujiwara M, Nagasako R. Hard palate mucoperiosteal graft for posterior lamellar reconstruction of the upper eyelid: histologic rationale. J Craniofac Surg 2007;18(3):684-90.
152. Grueterich M, Espana EM, Tseng SC. Ex vivo expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche. Surv Ophthalmol 2003;48(6):631-46.
153. Haagdorens M, Van Acker SI, Van Gerwen V, et al. Limbal Stem Cell Deficiency: Current Treatment Options and Emerging Therapies. Stem Cells Int 2016;2016:9798374.
154. Allan B. Closer to nature: new biomaterials and tissue engineering in ophthalmology. Br J Ophthalmol 1999;83(11):1235-40.

155. Huang D, Xu B, Yang X, et al. Conjunctival structural and functional reconstruction using acellular bovine pericardium graft (Normal GEN(R)) in rabbits. Graefes Arch Clin Exp Ophthalmol 2015.
156. Parodi PC, Faini G, De Biasio F, et al. Full-thickness lower eyelid reconstruction with a conchal chondro-perichondral graft and local coverage with Mio-cutaneous flaps--our divisional experience. J Oral Maxillofac Surg 2008;66(9):1826-32.
157. Zhang L, Zou D, Li S, et al. An Ultra-thin Amniotic Membrane as Carrier in Corneal Epithelium Tissue-Engineering. Sci Rep 2016;6:21021.
158. Hirayama M, Kawakita T, Tsubota K, Shimmura S. Challenges and strategies for regenerating the lacrimal gland. Ocul Surf 2015.
159. Yaqub A, Leatherbarrow B. The use of autogenous auricular cartilage in the management of upper eyelid entropion. Eye (Lond) 1997;11 (Pt 6):801-5.
160. Lai JY, Cheng HY, Ma DH. Investigation of Overrun-Processed Porous Hyaluronic Acid Carriers in Corneal Endothelial Tissue Engineering. PLoS One 2015;10(8):e0136067.
161. Zhang J, Sisley AM, Anderson AJ, et al. Characterization of a Novel Collagen Scaffold for Corneal Tissue Engineering. Tissue Eng Part C Methods 2015.
162. Nakamura T, Inatomi T, Sotozono C, et al. Ocular surface reconstruction using stem cell and tissue engineering. Prog Retin Eye Res 2016;51:187-207.
163. Kleinman HK, McGarvey ML, Liotta LA, et al. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate

proteoglycan from the EHS sarcoma. Biochemistry 1982;21(24):6188-93.

164. Jordan DR, Tse DT, Anderson RL, Hansen SO. Irradiated homologous tarsal plate banking: a new alternative in eyelid reconstruction. Part I. Technique and animal research. Ophthal Plast Reconstr Surg 1990;6(3):158-67.
165. Hodde JP, Badylak SF, Brightman AO, Voytik-Harbin SL. Glycosaminoglycan content of small intestinal submucosa: a bioscaffold for tissue replacement. Tissue Eng 1996;2(3):209-17.
166. Liao SL, Wei YH. Correction of lower lid retraction using tarSys bioengineered grafts for graves ophthalmopathy. Am J Ophthalmol 2013;156(2):387-92 e1.
167. Borrelli M, Unterlauft J, Kleinsasser N, Geerling G. Decellularized porcine derived membrane (Tarsys(R)) for correction of lower eyelid retraction. Orbit 2012;31(3):187-9.
168. Kim HJ, Grossniklaus HE, Wojno TH. A cyst-like foreign body reaction to porcine decellularized membrane (TarSys). Ophthal Plast Reconstr Surg 2014;30(4):e100-2.
169. Sun H, Liu W, Zhou G, et al. Tissue engineering of cartilage, tendon and bone. Front Med 2011;5(1):61-9.
170. O'Connor AJ, Morrison WA. Tissue Engineering. In: Gurtner GC, ed. Plastic Surgery. Amsterdam: Elsevier Inc., 2013.
171. Ladewig K, Abberton K, O'Connor AJ. Designing In vivo Bioreactors for Soft Tissue Engineering. Journal of Biomaterials and Tissue Engineering 2012;2(1):1-12.

172. Discher DE, Janmey P, Wang YL. Tissue cells feel and respond to the stiffness of their substrate. Science 2005;310(5751):1139-43.
173. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell 2006;126(4):677-89.
174. Khademhosseini A, Langer R. Microengineered hydrogels for tissue engineering. Biomaterials 2007;28(34):5087-92.
175. Butler DL, Goldstein SA, Guilak F. Functional tissue engineering: the role of biomechanics. J Biomech Eng 2000;122(6):570-5.
176. Guilak F, Butler DL, Goldstein SA, Baaijens FP. Biomechanics and mechanobiology in functional tissue engineering. J Biomech 2014;47(9):1933-40.
177. Taylor KA, Cutcliffe HC, Queen RM, et al. In vivo measurement of ACL length and relative strain during walking. J Biomech 2013;46(3):478-83.
178. Livesay GA, Rudy TW, Woo SL, et al. Evaluation of the effect of joint constraints on the in situ force distribution in the anterior cruciate ligament. J Orthop Res 1997;15(2):278-84.
179. Juncosa N, West JR, Galloway MT, et al. In vivo forces used to develop design parameters for tissue engineered implants for rabbit patellar tendon repair. J Biomech 2003;36(4):483-8.
180. Komi PV. Relevance of in vivo force measurements to human biomechanics. J Biomech 1990;23 Suppl 1:23-34.
181. Glos DL, Butler DL, Grood ES, Levy MS. In vitro evaluation of an implantable force transducer (IFT) in a patellar tendon model. J Biomech Eng 1993;115(4A):335-43.

182. Korvick DL, Cummings JF, Grood ES, et al. The use of an implantable force transducer to measure patellar tendon forces in goats. J Biomech 1996;29(4):557-61.
183. Coleman JL, Widmyer MR, Leddy HA, et al. Diurnal variations in articular cartilage thickness and strain in the human knee. J Biomech 2013;46(3):541-7.
184. Al Nazer R, Lanovaz J, Kawalilak C, et al. Direct in vivo strain measurements in human bone-a systematic literature review. J Biomech 2012;45(1):27-40.
185. De Hart J, Peters GW, Schreurs PJ, Baaijens FP. A three-dimensional computational analysis of fluid-structure interaction in the aortic valve. J Biomech 2003;36(1):103-12.
186. Marom G, Peleg M, Halevi R, et al. Fluid-structure interaction model of aortic valve with porcine-specific collagen fiber alignment in the cusps. J Biomech Eng 2013;135(10):101001-6.
187. Guilak F, Cohen DM, Estes BT, et al. Control of stem cell fate by physical interactions with the extracellular matrix. Cell Stem Cell 2009;5(1):17-26.
188. Yeatts AB, Choquette DT, Fisher JP. Bioreactors to influence stem cell fate: augmentation of mesenchymal stem cell signaling pathways via dynamic culture systems. Biochim Biophys Acta 2013;1830(2):2470-80.
189. Anderson EC, Hassan A, Barrett T, Anderson J. Observations on the pathogenicity for sheep and goats and the transmissibility of the strain of virus isolated during the rinderpest outbreak in Sri Lanka in 1987. Vet Microbiol 1990;21(4):309-18.

190. Butler DL, Goldstein SA, Guldberg RE, et al. The impact of biomechanics in tissue engineering and regenerative medicine. Tissue Eng Part B Rev 2009;15(4):477-84.
191. Huey DJ, Athanasiou KA. Tension-compression loading with chemical stimulation results in additive increases to functional properties of anatomic meniscal constructs. PLoS One 2011;6(11):e27857.
192. Huang AH, Baker BM, Ateshian GA, Mauck RL. Sliding contact loading enhances the tensile properties of mesenchymal stem cell-seeded hydrogels. Eur Cell Mater 2012;24:29-45.
193. Niklason LE, Yeh AT, Calle EA, et al. Enabling tools for engineering collagenous tissues integrating bioreactors, intravital imaging, and biomechanical modeling. Proc Natl Acad Sci U S A 2010;107(8):3335-9.
194. Butler DL, Juncosa-Melvin N, Boivin GP, et al. Functional tissue engineering for tendon repair: A multidisciplinary strategy using mesenchymal stem cells, bioscaffolds, and mechanical stimulation. J Orthop Res 2008;26(1):1-9.
195. Luce DA. Determining in vivo biomechanical properties of the cornea with an ocular response analyzer. J Cataract Refract Surg 2005;31(1):156-62.
196. Kotecha A. What biomechanical properties of the cornea are relevant for the clinician? Surv Ophthalmol 2007;52 Suppl 2:S109-14.
197. Terai N, Raiskup F, Haustein M, et al. Identification of biomechanical properties of the cornea: the ocular response analyzer. Curr Eye Res 2012;37(7):553-62.

198. Friberg TR, Lace JW. A comparison of the elastic properties of human choroid and sclera. Exp Eye Res 1988;47(3):429-36.
199. Bisplinghoff JA, McNally C, Manoogian SJ, Duma SM. Dynamic material properties of the human sclera. J Biomech 2009;42(10):1493-7.
200. Chen K, Rowley AP, Weiland JD, Humayun MS. Elastic properties of human posterior eye. J Biomed Mater Res A 2014;102(6):2001-7.
201. Chen K, Weiland JD. Anisotropic and inhomogeneous mechanical characteristics of the retina. J Biomech 2010;43(7):1417-21.
202. Chen K, Weiland JD. Mechanical properties of orbital fat and its encapsulating connective tissue. J Biomech Eng 2011;133(6):064505.
203. Gosline J, Lillie M, Carrington E, et al. Elastic proteins: biological roles and mechanical properties. Philos Trans R Soc Lond B Biol Sci 2002;357(1418):121-32.
204. Dean D, Han L, Grodzinsky AJ, Ortiz C. Compressive nanomechanics of opposing aggrecan macromolecules. J Biomech 2006;39(14):2555-65.
205. Naumann A, Dennis JE, Awadallah A, et al. Immunochemical and mechanical characterization of cartilage subtypes in rabbit. J Histochem Cytochem 2002;50(8):1049-58.
206. Smith LJ, Byers S, Costi JJ, Fazzalari NL. Elastic fibers enhance the mechanical integrity of the human lumbar anulus fibrosus in the radial direction. Ann Biomed Eng 2008;36(2):214-23.
207. Eilaghi A, Flanagan JG, Tertinegg I, et al. Biaxial mechanical testing of human sclera. J Biomech 2010;43(9):1696-701.

208. Crabb RA, Chau EP, Evans MC, et al. Biomechanical and microstructural characteristics of a collagen film-based corneal stroma equivalent. Tissue Eng 2006;12(6):1565-75.
209. Henderson TMA, Ladewig K, Haylock DN, et al. Cryogels for biomedical applications. Journal of Materials Chemistry B 2013;1:2682-95.
210. Cao Y, Davidson MR, O'Connor AJ, et al. Architecture control of three-dimensional polymeric scaffolds for soft tissue engineering. I. Establishment and validation of numerical models. J Biomed Mater Res A 2004;71(1):81-9.
211. Croll TI, O'Connor AJ, Stevens GW, Cooper-White JJ. Controllable surface modification of poly(lactic-co-glycolic acid) (PLGA) by hydrolysis or aminolysis I: physical, chemical, and theoretical aspects. Biomacromolecules 2004;5(2):463-73.
212. Clark A, Milbrandt TA, Hilt JZ, Puleo DA. Tailoring properties of microsphere-based poly(lactic-co-glycolic acid) scaffolds. J Biomed Mater Res A 2013.
213. Go DP, Palmer JA, Gras SL, O'Connor A J. Coating and release of an anti-inflammatory hormone from PLGA microspheres for tissue engineering. Journal of Biomedical Material Research part A 2012;100A(2):507-17.
214. Go DP, Gras SL, Mitra D, et al. Multilayered microspheres for the controlled release of growth factors in tissue engineering. Biomacromolecules 2011;12(5):1494-503.

215. Croll TI, O'Connor AJ, Stevens GW, Cooper-White JJ. A blank slate? Layer-by-layer deposition of hyaluronic acid and chitosan onto various surfaces. Biomacromolecules 2006;7(5):1610-22.
216. Borene ML, Barocas VH, Hubel A. Mechanical and cellular changes during compaction of a collagen-sponge-based corneal stromal equivalent. Ann Biomed Eng 2004;32(2):274-83.
217. Fagerholm P, Lagali NS, Carlsson DJ, et al. Corneal regeneration following implantation of a biomimetic tissue-engineered substitute. Clin Transl Sci 2009;2(2):162-4.
218. Lai JY, Li YT. Functional assessment of cross-linked porous gelatin hydrogels for bioengineered cell sheet carriers. Biomacromolecules 2010;11(5):1387-97.
219. Insler MS, Lopez JG. Microcarrier cell culture of neonatal human corneal endothelium. Curr Eye Res 1990;9(1):23-30.
220. Liang Y, Liu W, Han B, et al. Fabrication and characters of a corneal endothelial cells scaffold based on chitosan. J Mater Sci Mater Med 2011;22(1):175-83.
221. Cao Y, Croll TI, Cooper-White JJ, et al. Production and surface modification of polylactide-based polymeric scaffolds for soft-tissue engineering. Methods Mol Biol 2004;238:87-112.
222. Cao Y, Mitchell G, Messina A, et al. The influence of architecture on degradation and tissue ingrowth into three-dimensional poly(lactic-co-glycolic acid) scaffolds in vitro and in vivo. Biomaterials 2006;27(14):2854-64.

223. Cao Y, Croll TI, O'Connor A J, et al. Systematic selection of solvents for the fabrication of 3D combined macro- and microporous polymeric scaffolds for soft tissue engineering. Journal of Biomaterials Science: Polymer Edition 2006;14(4):369-402.
224. Liu W, Merrett K, Griffith M, et al. Recombinant human collagen for tissue engineered corneal substitutes. Biomaterials 2008;29(9):1147-58.
225. Griffith M, Jackson WB, Lagali N, et al. Artificial corneas: a regenerative medicine approach. Eye (Lond) 2009;23(10):1985-9.
226. Ozcelik B, Brown KD, Blencowe A, et al. Ultrathin chitosan-poly(ethylene glycol) hydrogel films for corneal tissue engineering. Acta Biomater 2013;9(5):6594-605.
227. Vazquez N, Chacon M, Meana A, et al. Keratin-chitosan membranes as scaffold for tissue engineering of human cornea. Histol Histopathol 2015;30(7):813-21.
228. Young TH, Wang IJ, Hu FR, Wang TJ. Fabrication of a bioengineered corneal endothelial cell sheet using chitosan/polycaprolactone blend membranes. Colloids Surf B Biointerfaces 2014;116:403-10.
229. Lai JY, Lu PL, Chen KH, et al. Effect of charge and molecular weight on the functionality of gelatin carriers for corneal endothelial cell therapy. Biomacromolecules 2006;7(6):1836-44.
230. Lai JY, Chen KH, Hsiue GH. Tissue-engineered human corneal endothelial cell sheet transplantation in a rabbit model using functional biomaterials. Transplantation 2007;84(10):1222-32.

231. Takezawa T, Ozaki K, Nitani A, et al. Collagen vitrigel: a novel scaffold that can facilitate a three-dimensional culture for reconstructing organoids. Cell Transplant 2004;13(4):463-73.
232. Takezawa T, Nishikawa K, Wang PC. Development of a human corneal epithelium model utilizing a collagen vitrigel membrane and the changes of its barrier function induced by exposing eye irritant chemicals. Toxicol In Vitro 2011;25(6):1237-41.
233. McIntosh Ambrose W, Salahuddin A, So S, et al. Collagen Vitrigel membranes for the in vitro reconstruction of separate corneal epithelial, stromal, and endothelial cell layers. J Biomed Mater Res B Appl Biomater 2009;90(2):818-31.
234. Tan XW, Thompson B, Konstantopoulos A, et al. Application of Graphene as Candidate Biomaterial for Synthetic Keratoprosthesis Skirt. Invest Ophthalmol Vis Sci 2015;56(11):6605-11.
235. Chen HS, Ritch R, Krupin T, Hsu WC. Control of filtering bleb structure through tissue bioengineering: An animal model. Invest Ophthalmol Vis Sci 2006;47(12):5310-4.
236. Dietlein TS, Jordan J, Lueke C, Krieglstein GK. Modern concepts in antiglaucomatous implant surgery. Graefes Arch Clin Exp Ophthalmol 2008;246(12):1653-64.
237. He M, Wang W, Zhang X, Huang W. Ologen implant versus mitomycin C for trabeculectomy: a systematic review and meta-analysis. PLoS One 2014;9(1):e85782.

238. Cillino S, Casuccio A, Di Pace F, et al. Biodegradable collagen matrix implant versus mitomycin-C in trabeculectomy: five-year follow-up. BMC Ophthalmol 2016;16(1):24.
239. Hsu WC, Spilker MH, Yannas IV, Rubin PA. Inhibition of conjunctival scarring and contraction by a porous collagen-glycosaminoglycan implant. Invest Ophthalmol Vis Sci 2000;41(9):2404-11.
240. Lee SY, Oh JH, Kim JC, et al. In vivo conjunctival reconstruction using modified PLGA grafts for decreased scar formation and contraction. Biomaterials 2003;24(27):5049-59.
241. Lu Q, Al-Sheikh O, Elisseeff JH, Grant MP. Biomaterials and Tissue Engineering Strategies for Conjunctival Reconstruction and Dry Eye Treatment. Middle East Afr J Ophthalmol 2015;22(4):428-34.
242. Zhou H, Lu Q, Guo Q, et al. Vitrified collagen-based conjunctival equivalent for ocular surface reconstruction. Biomaterials 2014;35(26):7398-406.
243. Hann LE, Tatro JB, Sullivan DA. Morphology and function of lacrimal gland acinar cells in primary culture. Invest Ophthalmol Vis Sci 1989;30(1):145-58.
244. Stevenson D, Schechter JE, Nakamuro T, et al. A new model system for studying lacrimal physiology using cultured lacrimal gland acinar cells on Matrigel rafts. Adv Exp Med Biol 2002;506(Pt A):159-63.
245. Yoshino K. Establishment of a human lacrimal gland epithelial culture system with in vivo mimicry and its substrate modulation. Cornea 2000;19(3 Suppl):S26-36.

246. Selvam S, Thomas PB, Trousdale MD, et al. Tissue-engineered tear secretory system: functional lacrimal gland acinar cells cultured on matrix protein-coated substrata. J Biomed Mater Res B Appl Biomater 2007;80(1):192-200.
247. Huh D, Matthews BD, Mammoto A, et al. Reconstituting organ-level lung functions on a chip. Science 2010;328(5986):1662-8.
248. Kim HJ, Ingber DE. Gut-on-a-Chip microenvironment induces human intestinal cells to undergo villus differentiation. Integr Biol (Camb) 2013;5(9):1130-40.
249. Lee SA, No da Y, Kang E, et al. Spheroid-based three-dimensional liver-on-a-chip to investigate hepatocyte-hepatic stellate cell interactions and flow effects. Lab Chip 2013;13(18):3529-37.
250. Rigat-Brugarolas LG, Elizalde-Torrent A, Bernabeu M, et al. A functional microengineered model of the human splenon-on-a-chip. Lab Chip 2014;14(10):1715-24.
251. Torisawa YS, Spina CS, Mammoto T, et al. Bone marrow-on-a-chip replicates hematopoietic niche physiology in vitro. Nat Methods 2014;11(6):663-9.
252. Chung SH, Lee JH, Yoon JH, et al. Multi-layered culture of primary human conjunctival epithelial cells producing MUC5AC. Exp Eye Res 2007;85(2):226-33.
253. Hirayama M, Ogawa M, Oshima M, et al. Functional lacrimal gland regeneration by transplantation of a bioengineered organ germ. Nat Commun 2013;4:2497.

254. Gunarajah DR, Samman N. Biomaterials for repair of orbital floor blowout fractures: a systematic review. J Oral Maxillofac Surg 2013;71(3):550-70.
255. Al-Sukhun J, Tornwall J, Lindqvist C, Kontio R. Bioresorbable poly-L/DL-lactide (P[L/DL]LA 70/30) plates are reliable for repairing large inferior orbital wall bony defects: a pilot study. J Oral Maxillofac Surg 2006;64(1):47-55.
256. Baino F. Biomaterials and implants for orbital floor repair. Acta Biomater 2011;7(9):3248-66.
257. Kontio R, Ruuttila P, Lindroos L, et al. Biodegradable polydioxanone and poly(l/d)lactide implants: an experimental study on peri-implant tissue response. Int J Oral Maxillofac Surg 2005;34(7):766-76.
258. Al-Sukhun J, Lindqvist C. A comparative study of 2 implants used to repair inferior orbital wall bony defects: autogenous bone graft versus bioresorbable poly-L/DL-Lactide [P(L/DL)LA 70/30] plate. J Oral Maxillofac Surg 2006;64(7):1038-48.
259. Zhou J, Lin H, Fang T, et al. The repair of large segmental bone defects in the rabbit with vascularized tissue engineered bone. Biomaterials 2010;31(6):1171-9.
260. Stephan SJ, Tholpady SS, Gross B, et al. Injectable tissue-engineered bone repair of a rat calvarial defect. Laryngoscope 2010;120(5):895-901.
261. Wang S, Zhang Z, Xia L, et al. Systematic evaluation of a tissue-engineered bone for maxillary sinus augmentation in large animal canine model. Bone 2010;46(1):91-100.

262. Leach JK, Mooney DJ. Bone engineering by controlled delivery of osteoinductive molecules and cells. Expert Opin Biol Ther 2004;4(7):1015-27.
263. Wozney JM, Rosen V. Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. Clin Orthop Relat Res 1998(346):26-37.
264. Betz MW, Caccamese JF, Coletti DP, et al. Challenges associated with regeneration of orbital floor bone. Tissue Eng Part B Rev 2010;16(5):541-50.
265. Termaat MF, Den Boer FC, Bakker FC, et al. Bone morphogenetic proteins. Development and clinical efficacy in the treatment of fractures and bone defects. J Bone Joint Surg Am 2005;87(6):1367-78.
266. Betz MW, Caccamese JF, Coletti DP, et al. Tissue response and orbital floor regeneration using cyclic acetal hydrogels. J Biomed Mater Res A 2009;90(3):819-29.
267. Malizos KN, Papatheodorou LK. The healing potential of the periosteum molecular aspects. Injury 2005;36 Suppl 3:S13-9.
268. Rohner D, Hutmacher DW, Cheng TK, et al. In vivo efficacy of bone-marrow-coated polycaprolactone scaffolds for the reconstruction of orbital defects in the pig. J Biomed Mater Res B Appl Biomater 2003;66(2):574-80.
269. Henkel J, Woodruff MA, Epari DR, et al. Bone Regeneration Based on Tissue Engineering Conceptions - A 21st Century Perspective. Bone Res 2013;1(3):216-48.

270. Hutmacher DW, Cool S. Concepts of scaffold-based tissue engineering--the rationale to use solid free-form fabrication techniques. J Cell Mol Med 2007;11(4):654-69.
271. Schantz JT, Teoh SH, Lim TC, et al. Repair of calvarial defects with customized tissue-engineered bone grafts I. Evaluation of osteogenesis in a three-dimensional culture system. Tissue Eng 2003;9 Suppl 1:S113-26.
272. Schantz JT, Hutmacher DW, Lam CX, et al. Repair of calvarial defects with customised tissue-engineered bone grafts II. Evaluation of cellular efficiency and efficacy in vivo. Tissue Eng 2003;9 Suppl 1:S127-39.
273. Dean D, Wolfe MS, Ahmad Y, et al. Effect of transforming growth factor beta 2 on marrow-infused foam poly(propylene fumarate) tissue-engineered constructs for the repair of critical-size cranial defects in rabbits. Tissue Eng 2005;11(5-6):923-39.
274. Zhou J, Peng SW, Wang YY, et al. The use of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) scaffolds for tarsal repair in eyelid reconstruction in the rat. Biomaterials 2010;31(29):7512-8.
275. Chang HY, Chi JT, Dudoit S, et al. Diversity, topographic differentiation, and positional memory in human fibroblasts. Proc Natl Acad Sci U S A 2002;99(20):12877-82.
276. Nolte SV, Xu W, Rennekampff HO, Rodemann HP. Diversity of fibroblasts--a review on implications for skin tissue engineering. Cells Tissues Organs 2008;187(3):165-76.
277. Parsonage G, Filer AD, Haworth O, et al. A stromal address code defined by fibroblasts. Trends Immunol 2005;26(3):150-6.

278. Tomasek JJ, Gabbiani G, Hinz B, et al. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 2002;3(5):349-63.
279. Jones HW, Jr., McKusick VA, Harper PS, Wuu KD. George Otto Gey. (1899-1970). The HeLa cell and a reappraisal of its origin. Obstet Gynecol 1971;38(6):945-9.
280. Todaro GJ, Green H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol 1963;17:299-313.
281. Kawamura T, Suzuki J, Wang YV, et al. Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature 2009;460(7259):1140-4.
282. Whibley C, Odell AF, Nedelko T, et al. Wild-type and Hupki (human p53 knock-in) murine embryonic fibroblasts: p53/ARF pathway disruption in spontaneous escape from senescence. J Biol Chem 2010;285(15):11326-35.
283. Fernandes IR, Russo FB, Pignatari GC, et al. Fibroblast sources: Where can we get them? Cytotechnology 2016;68(2):223-8.
284. Bogatkevich GS, Highland KB, Akter T, Silver RM. The PPARgamma Agonist Rosiglitazone Is Antifibrotic for Scleroderma Lung Fibroblasts: Mechanisms of Action and Differential Racial Effects. Pulm Med 2012;2012:545172.
285. Bogatkevich GS, Ludwicka-Bradley A, Highland KB, et al. Impairment of the antifibrotic effect of hepatocyte growth factor in lung fibroblasts from

African Americans: possible role in systemic sclerosis. Arthritis Rheum 2007;56(7):2432-42.

286. Bogatkevich GS, Ludwicka-Bradley A, Highland KB, et al. Down-regulation of collagen and connective tissue growth factor expression with hepatocyte growth factor in lung fibroblasts from white scleroderma patients via two signaling pathways. Arthritis Rheum 2007;56(10):3468-77.
287. Bogatkevich GS, Ludwicka-Bradley A, Silver RM. Dabigatran, a direct thrombin inhibitor, demonstrates antifibrotic effects on lung fibroblasts. Arthritis Rheum 2009;60(11):3455-64.
288. Ludwicka A, Trojanowska M, Smith EA, et al. Growth and characterization of fibroblasts obtained from bronchoalveolar lavage of patients with scleroderma. J Rheumatol 1992;19(11):1716-23.
289. Rinn JL, Bondre C, Gladstone HB, et al. Anatomic demarcation by positional variation in fibroblast gene expression programs. PLoS Genet 2006;2(7):e119.
290. Li H, Roos JC, Rose GE, et al. Eyelid and Sternum Fibroblasts Differ in Their Contraction Potential and Responses to Inflammatory Cytokines. Plast Reconstr Surg Glob Open 2015;3(7):e448.
291. Kim HS, Kim SE, Evans GR, Park SH. The usability of the upper eyelid crease approach for correction of medial orbital wall blowout fracture. Plast Reconstr Surg 2012;130(4):898-905.
292. Brissett AE, Sherris DA. Scar contractures, hypertrophic scars, and keloids. Facial Plast Surg 2001;17(4):263-72.

293. Dixon AY, Lee SH, McGregor DH. Histologic features predictive of basal cell carcinoma recurrence: results of a multivariate analysis. J Cutan Pathol 1993;20(2):137-42.
294. Dixon AY, Lee SH, McGregor DH. Histologic evolution of basal cell carcinoma recurrence. Am J Dermatopathol 1991;13(3):241-7.
295. Dixon AY, Lee SH, McGregor DH. Factors predictive of recurrence of basal cell carcinoma. Am J Dermatopathol 1989;11(3):222-32.
296. Longo C, Lallas A, Kyrgidis A, et al. Classifying distinct basal cell carcinoma subtype by means of dermatoscopy and reflectance confocal microscopy. J Am Acad Dermatol 2014;71(4):716-24 e1.
297. Lallas A, Tzellos T, Kyrgidis A, et al. Accuracy of dermoscopic criteria for discriminating superficial from other subtypes of basal cell carcinoma. J Am Acad Dermatol 2014;70(2):303-11.
298. Lien MH, Sondak VK. Nonsurgical treatment options for Basal cell carcinoma. J Skin Cancer 2011;2011:571734.
299. Sun MT, Wu A, Huilgol SC, Selva D. Accuracy of Biopsy in Subtyping Periocular Basal Cell Carcinoma. Ophthal Plast Reconstr Surg 2015;31(6):449-51.
300. Sun MT, Figueira E, Huilgol SC, Selva D. Minimum histological safety margins in periocular basal cell carcinoma. Br J Ophthalmol 2014;98(5):706.
301. Raasch BA, Buettner PG, Garbe C. Basal cell carcinoma: histological classification and body-site distribution. Br J Dermatol 2006;155(2):401-7.

302. Nedved D, Tonkovic-Capin V, Hunt E, et al. Diagnostic concordance rates in the subtyping of basal cell carcinoma by different dermatopathologists. J Cutan Pathol 2014;41(1):9-13.
303. Haws AL, Rojano R, Tahan SR, Phung TL. Accuracy of biopsy sampling for subtyping basal cell carcinoma. J Am Acad Dermatol 2012;66(1):106-11.
304. Kamyab-Hesari K, Seirafi H, Naraghi ZS, et al. Diagnostic accuracy of punch biopsy in subtyping basal cell carcinoma. J Eur Acad Dermatol Venereol 2012.
305. Roozeboom MH, Mosterd K, Winnepenninckx VJ, et al. Agreement between histological subtype on punch biopsy and surgical excision in primary basal cell carcinoma. J Eur Acad Dermatol Venereol 2012.
306. Wolberink EA, Pasch MC, Zeiler M, et al. High discordance between punch biopsy and excision in establishing basal cell carcinoma subtype: analysis of 500 cases. J Eur Acad Dermatol Venereol 2012.
307. Russell EB, Carrington PR, Smoller BR. Basal cell carcinoma: a comparison of shave biopsy versus punch biopsy techniques in subtype diagnosis. J Am Acad Dermatol 1999;41(1):69-71.
308. Pascal R, Hobby, LW., Lattes, R.. Crikelair, GF. Prognosis of ‘incompletely excised’ versus ‘completely excised’ basal cell carcinoma. Plast Reconstr Surg 1968;41:328-32.
309. Auw-Haedrich C, Frick, S., Boehringer, D., Mittelviefhaus, H. Histologic safety margin in basal cell carcinoma of the eyelid: correlation with recurrence rate. Ophthalmology 2009;116(4):802-6.

310. Kyrgidis A, Vahtsevanos, K., Tzellos, TG., Xirou, P., Kitikidou, K., Antoniades, K., Zouboulis, CC., Triaridis, S. Clinical, histological and demographic predictors for recurrence and second primary tumours of head and neck basal cell carcinoma. A 1062 patient-cohort study from a tertiary cancer referral hospital. Eur J Dermatol 2010;20(3):276-82.
311. Sun MT, Andrew NH, O'Donnell B, et al. Periocular Squamous Cell Carcinoma: TNM Staging and Recurrence. Ophthalmology 2015;122(7):1512-6.
312. Faustina M, Diba R, Ahmadi MA, Esmaeli B. Patterns of regional and distant metastasis in patients with eyelid and periocular squamous cell carcinoma. Ophthalmology 2004;111(10):1930-2.
313. Ridenhour CE, Spratt JS, Jr. Epidermoid carcinoma of the skin involving the parotid gland. Am J Surg 1966;112(4):504-7.
314. Thosani MK, Schneck G, Jones EC. Periocular squamous cell carcinoma. Dermatol Surg 2008;34(5):585-99.
315. Maalouf TJ, Dolivet G, Angioi KS, et al. Sentinel lymph node biopsy in patients with conjunctival and eyelid cancers: experience in 17 patients. Ophthal Plast Reconstr Surg 2012;28(1):30-4.
316. Bowen JT. Centennial paper. May 1912 (J Cutan Dis Syph 1912;30:241-255). Precancerous dermatoses: a study of two cases of chronic atypical epithelial proliferation. By John T. Bowen, M.D., Boston. Arch Dermatol 1983;119(3):243-60.
317. Kao GF. Carcinoma arising in Bowen's disease. Arch Dermatol 1986;122(10):1124-6.

318. Arlette JP, Trotter MJ. Squamous cell carcinoma in situ of the skin: history, presentation, biology and treatment. Australas J Dermatol 2004;45(1):1-9; quiz 10.
319. Malhotra R, James CL, Selva D, et al. The Australian Mohs database: periocular squamous intraepidermal carcinoma. Ophthalmology 2004;111(10):1925-9.
320. Cox NH, Eedy DJ, Morton CA. Guidelines for management of Bowen's disease. British Association of Dermatologists. Br J Dermatol 1999;141(4):633-41.
321. Leibovitch I, Huilgol SC, Selva D, et al. Cutaneous squamous carcinoma in situ (Bowen's disease): treatment with Mohs micrographic surgery. J Am Acad Dermatol 2005;52(6):997-1002.
322. Malhotra R, Huilgol SC, Huynh NT, Selva D. The Australian Mohs database, part I: periocular basal cell carcinoma experience over 7 years. Ophthalmology 2004;111(4):624-30.
323. Sun MT, Pham DT, O'Connor AJ, et al. The Biomechanics of eyelid tarsus tissue. J Biomech 2015;48(12):3455-9.
324. Rosser P, McCormick A. Horizontal skin wound closure in full thickness eyelid excision. Clin Experiment Ophthalmol 2008;36(8):709-11.
325. Costi JJ, Pham DT, Shapter JG. Microtensile Properties of Individual Fibre Bundles in Healthy and Degenerative Human Annulus Fibrosus. Spine Society of Australia 24th Annual Scientific Meeting. Perth, Australia 2013.
326. Costi JJ, Stokes IA, Gardner-Morse MG, Iatridis JC. Frequency-dependent behavior of the intervertebral disc in response to each of six

degree of freedom dynamic loading: solid phase and fluid phase contributions. Spine 2008;33(16):1731-8.

327. Ni Annaidh A, Bruyere K, Destrade M, et al. Characterization of the anisotropic mechanical properties of excised human skin. J Mech Behav Biomed Mater 2012;5(1):139-48.
328. Khatyr F, Imberdis C, Vescovo P, et al. Model of the viscoelastic behaviour of skin in vivo and study of anisotropy. Skin Res Technol 2004;10(2):96-103.
329. Bonifasi-Lista C, Lake SP, Small MS, Weiss JA. Viscoelastic properties of the human medial collateral ligament under longitudinal, transverse and shear loading. J Orthop Res 2005;23(1):67-76.
330. Meechai N, Jamieson AM, Blackwell J, Carrino DA. Nonlinear viscoelasticity of concentrated solutions of aggrecan aggregate. Biomacromolecules 2001;2(3):780-7.
331. Hardingham TE, Muir H, Kwan MK, et al. Viscoelastic properties of proteoglycan solutions with varying proportions present as aggregates. J Orthop Res 1987;5(1):36-46.
332. Mow VC, Mak AF, Lai WM, et al. Viscoelastic properties of proteoglycan subunits and aggregates in varying solution concentrations. J Biomech 1984;17(5):325-38.
333. Zhu W, Lai WM, Mow VC. The density and strength of proteoglycan-proteoglycan interaction sites in concentrated solutions. J Biomech 1991;24(11):1007-18.
334. Rowlands AS, Lim SA, Martin D, Cooper-White JJ. Polyurethane/poly(lactic-co-glycolic) acid composite scaffolds fabricated

by thermally induced phase separation. Biomaterials 2007;28(12):2109-21.

335. Hollister SJ. Porous scaffold design for tissue engineering. Nat Mater 2005;4(7):518-24.
336. Moroni L, de Wijn JR, van Blitterswijk CA. 3D fiber-deposited scaffolds for tissue engineering: influence of pores geometry and architecture on dynamic mechanical properties. Biomaterials 2006;27(7):974-85.
337. Boffito M, Bernardi E, Sartori S, et al. A mechanical characterization of polymer scaffolds and films at the macroscale and nanoscale. J Biomed Mater Res A 2014.
338. Adekogbe I, Ghanem A. Fabrication and characterization of DTBP-crosslinked chitosan scaffolds for skin tissue engineering. Biomaterials 2005;26(35):7241-50.
339. Berger J, Reist M, Mayer JM, et al. Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. Eur J Pharm Biopharm 2004;57(1):35-52.
340. Koyano T, Minoura N, Nagura M, Kobayashi K. Attachment and growth of cultured fibroblast cells on PVA/chitosan-blended hydrogels. J Biomed Mater Res 1998;39(3):486-90.
341. Di Martino A, Sittinger M, Risbud MV. Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. Biomaterials 2005;26(30):5983-90.
342. Madihally SV, Matthew HW. Porous chitosan scaffolds for tissue engineering. Biomaterials 1999;20(12):1133-42.

343. Arahira T, Todo M. Variation of mechanical behavior of beta-TCP/collagen two phase composite scaffold with mesenchymal stem cell in vitro. J Mech Behav Biomed Mater 2016;61:464-74.
344. Chandy T, Sharma CP. Chitosan--as a biomaterial. Biomater Artif Cells Artif Organs 1990;18(1):1-24.
345. Waibel KH, Haney B, Moore M, et al. Safety of chitosan bandages in shellfish allergic patients. Mil Med 2011;176(10):1153-6.
346. VandeVord PJ, Matthew HW, DeSilva SP, et al. Evaluation of the biocompatibility of a chitosan scaffold in mice. J Biomed Mater Res 2002;59(3):585-90.
347. Wong T, McGrath JA, Navsaria H. The role of fibroblasts in tissue engineering and regeneration. Br J Dermatol 2007;156(6):1149-55.
348. Bogatkevich GS, Tourkina E, Silver RM, Ludwicka-Bradley A. Thrombin differentiates normal lung fibroblasts to a myofibroblast phenotype via the proteolytically activated receptor-1 and a protein kinase C-dependent pathway. J Biol Chem 2001;276(48):45184-92.
349. Lamme EN, van Leeuwen RT, Mekkes JR, Middelkoop E. Allogeneic fibroblasts in dermal substitutes induce inflammation and scar formation. Wound Repair Regen 2002;10(3):152-60.
350. Morimoto N, Saso Y, Tomihata K, et al. Viability and function of autologous and allogeneic fibroblasts seeded in dermal substitutes after implantation. J Surg Res 2005;125(1):56-67.

“Do or Do Not. There is no Try.”

Yoda