TURUN YLIOPISTO
UNIVERSITY OF TURKU

CLASSICAL PATHWAY OF THE COMPLEMENT SYSTEM IN CUTANEOUS SQUAMOUS CELL CARCINOMA

Kristina Viiklepp

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. D OSA – TOM. 1683 | MEDICA – ODONTOLOGICA | TURKU 2022

TURUN
YLIOPISTO
UNIVERSITY
OF TURKU

CLASSICAL PATHWAY OF THE COMPLEMENT SYSTEM IN CUTANEOUS SQUAMOUS CELL CARCINOMA

Kristina Viiklepp

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. D OSA – TOM. 1683 | MEDICA – ODONTOLOGICA | TURKU 2022

University of Turku

Faculty of Medicine, Institute of Clinical Medicine
Department of Dermatology and Venereology, University of Turku, Finland
FICAN West Cancer Centre Laboratory, University of Turku and Turku University Hospital and MediCity Research Laboratory, University of Turku, Turku, Finland
Doctoral Programme in Clinical Research (DPCR)

Supervised by

Professor Veli-Matti Kähäri, MD, PhD
Department of Dermatology and Venereology
University of Turku and Turku University Hospital
Turku, Finland

Docent Liisa Nissinen, PhD
Department of Dermatology and Venereology
University of Turku
Turku, Finland

Docent Pilvi Riihilä, MD, PhD
Department of Dermatology and Venereology
University of Turku and Turku University Hospital
Turku, Finland

Reviewed by

Professor Kaisa Tasanen-Määttä, MD, PhD
Department of Dermatology and Venereology
University of Oulu
Oulu, Finland

Docent Sakari Jokiranta, MD, PhD
Medicum
Department of Bacteriology and Immunology
University of Helsinki
Helsinki, Finland

Opponent

Professor Külli Kingo, PhD, MD
Department of Dermatology and Venereology
University of Tartu
Tartu, Estonia

The originality of this publication has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

Cover Image: Kristina Viiklepp

ISBN 978-951-29-9119-8 (PRINT)
ISBN 978-951-29-9120-4 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama, Turku, Finland 2022

”En tiedä edes mistä pitäis alottaa
Ei kukaan muu voi tietää meidän koko tarinaa”
-Pyhimys

To Karolina and Kristjan

UNIVERSITY OF TURKU
Faculty of Medicine, Institute of Clinical Medicine
Department of Dermatology and Venereology
KRISTINA VIIKLEPP: Classical pathway of the complement system in cutaneous squamous cell carcinoma
Doctoral Dissertation, 156 pp.
Doctoral Programme in Clinical Research (DPCR)
December 2022

ABSTRACT

Keratinocyte-derived cutaneous squamous cell carcinoma (cSCC) is the most common metastatic skin cancer and it causes approximately 20% of all skin cancer-related deaths. The incidence of cSCC is increasing worldwide. Usually the first phase of cSCC is actinic keratosis (AK), which can progress to local cSCC (Bowen's disease, *in situ* cSCC, cSCCIS). cSCCIS can finally progress to invasive and metastatic cSCC with poor prognosis. Inflammatory cells and factors are a part of the cancer microenvironment. Alterations in the microenvironment of premalignant lesions are required for their progression to invasive and metastatic cSCC. The complement system is an important part of innate immunity and regulates inflammatory processes and is activated via three major pathways: classical, lectin or the alternative pathway.

In this study, the role of the classical pathway components (C1q, C1r and C1s) of the complement system in the progression of cSCC was studied. High expression of C1r and C1s was seen in cSCC cells in culture. Immunohistochemistry (IHC) showed the expression of C1r and C1s specifically in tumor cells *in vivo.* IHC analysis revealed higher expression of C1q specifically in the tumor microenvironment in stromal fibroblasts and in macrophages compared to AK or cSCCIS *in vivo*. Knockdown of C1r or C1s and knockout of C1r decreased viability, migration and invasion of cSCC cells in culture. Knockout of C1r inhibited expression of MMP-1, -13, -10 and -12 by cSCC cells and MMP-13 production, degradation of collagen and invasion in cSCC xenografts in SCID mice *in vivo*. Knockdown of C1r or C1s and knockout of C1r inhibited growth of cSCC xenografts *in vivo*. In these tumors, C1r or C1s knockdown or C1r knockout decreased the number of proliferating cells and CD34 positive blood vessels and increased the number of apoptotic cells.

This study provides new evidence for the roles of C1q, C1r and C1s in tumor progression, demonstrating these as molecular biomarkers and putative therapeutic targets for cSCC.

KEYWORDS: cutaneous squamous cell carcinoma, complement system, complement component C1q, serine proteinase C1r, serine proteinase C1s

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Kliininen laitos
Iho- ja sukupuolitautioppi
KRISTINA VIIKLEPP: Komplementin klassinen reitti okasolusyövässä
Väitöskirja 156 s.
Turun kliininen tohtoriohjelma (TKT)
Joulukuu 2022

TIIVISTELMÄ

Keratinosyyttiperäinen okasolusyöpä (ihon levyepiteelisyöpä) on yleisin metastasoituva ihosyöpä ja se aiheuttaa 20% ihosyöpäkuolemista. Okasolusyövän ilmaantuvuus lisääntyy maailmanlaajuisesti. Okasolusyövän esiaste on aktiininen keratoosi, joka voi kehittyä paikalliseksi levyepiteelisyöväksi (Bowenin tauti). Tämä voi muuttua edelleen leviäväksi ja metastaattiseksi okasolusyöväksi. Tulehdussolut ja –tekijät kuuluvat syöpien mikroympäristöön. Muutokset esiasteisten ja paikallisesti levinneiden kasvaimien mikroympäristössä, kuten tyvikalvon rakenteen muuttuminen, tulehduksen lisääntyminen ja altistuminen mikrobirakenteille, lisäävät okasolusyövän kasvua. Komplementtijärjestelmä on tärkeä osa ihmisen luonnollista immuniteettia ja se säätelee tulehduksellisia prosesseja. Se voi aktivoitua kolmea eri reaktiotietä: klassinen, vaihtoehtoinen ja lektiini-aktivaatiotie.

Tässä väitöskirjassa on tutkittu komplementin klassisen aktivaatiotien komponentteja (C1q, C1r ja C1s) okasolusyövän kehittymisessä viljelmässä ja *in vivo*. Korkeat C1r ja C1s tasot havaittiin okasolusyövän soluissa viljelmässä. Lisäksi havaittiin immunohistokemiallisella analyysilla, että C1r ja C1s ilmentyvät erityisesti okasolusyöpäsoluissa *in vivo*. Immunohistokemiallinen analyysi paljasti myös, että C1q ilmeni erityisesti kasvaimen mikroympäristössä makrofageissa ja aktivoituneissa fibroblasteissa *in vivo*. C1r:n ja C1s:n hiljentäminen sekä C1r:n poistaminen vähensi okasolusyövän kasvainsolujen elinkykyä ja liikkumista viljelmässä. C1r:n poistaminen vähensi kasvainsolujen MMP-1, -13, 10 ja -12 tuotantoa ja MMP-13 tuotantoa, kollageenin hajotusta ja invaasiota *in vivo* SCID-hiirissä kasvatetuissa ksenograftikasvaimissa. C1r:n ja C1s:n hiljentäminen sekä C1r:n poistaminen vähensi okasolusyövän kasvua ja vähensi lisääntyvien solujen jakautumista, CD34-positiivisten verisuonten määrää sekä lisäsi apoptoottisten solujen määrää.

Tässä tutkimuksessa osoitetaan C1q, C1r ja C1s rooli okasolusyövän kehittymisessä. Komplementin klassisen aktivaatiotien komponentit C1q, C1r ja C1s voivat mahdollisesti toimia merkkitekijöinä ja hoitokohteina ihon okasolusyövälle.

AVAINSANAT: okasolusyöpä, komplementtijärjestelmä, komplementin–komponentti C1q, seriiniproteaasi C1r, seriiniproteaasi C1s

TURU ÜLIKOOL
Arstiteaduskond
Kliiniline instituut
Dermatoloogia ja veneroloogia teaduskond
KRISTINA VIIKLEPP: Komplemendi klassikaline rada naha lamerakulises kartsinoomis
Doktoritöö, 156 lk.
Turu kliiniline doktoriõppekava
Detsember 2022

KOKKUVÕTE

Naha lamerakk-kartsinoom (NLK), mis tuleneb epiteliaalsetest keratinosüütidest, on kõige levinum metastaase tekitav nahavähi vorm, mis põhjustab 20% nahavähi surmajuhtumitest. NLK esinemissagedus kasvab kogu maailmas. NLKle eelneb tavaliselt aktiiniline keratoos (AK), mis võib areneda edasi lokaalseks lamerakuliseks kartsinoomiks (Boweni tõbi), sellisel juhul esinevad pahaloomulised kasvajarakud ainult epidermises ega läbista basaalmembraani. Haigus võib edasi areneda invasiivseks ja metastaatiliseks NLKks. Põletikulised rakud on osa vähkkasvajate mikrokeskkonnast. AK ja Boweni tõbi põhjustavad lokaalseid muudatusi mikrokeskkonnas. Muutused basaalmembraani struktuuris, suurenenud põletik ja kokkupuude mikroobsete struktuuridega suurendavad NLK teket ja kasvu. Komplemendisüsteem on üks olulisemaid kaasasündinud immuunkaitse osi, mis reguleerib põletikulisi protsesse. Komplemendisüsteem on aktiveeritav läbi kolme erineva raja: klassikalise, alternatiivse ja lektiini raja.

Käesolevas doktoritöös olen uurinud komplemendi klassikalise raja komponentide (C1q, C1r ja C1s) esinemist ja rolli NLK tekkes koekultuuris ja kasvaja koes. Koekultuuris tuvastati kõrged C1r ja C1s tasemed NLK rakkudes. Immunohistokeemilise analüüsiga leidsime, et C1r ja C1s ekspresseeruvad NLK vähirakkudes ja C1q valku ekspresseeriti spetsiifiliselt kasvaja mikrokeskkonna markofaagides ja aktiveeritud fibroblastides. Lisaks näitasin koekultuuri mudelitega, et C1r ja C1s vaigistamine ning C1r eemaldamine vähendas NLK rakkude elujõulisust ja liikuvust koekultuuris. C1r eemaldamine vähendas kasvajarakkude MMP-1, -13, -10 ja -12 ensüümide tootmist ning MMP-13 poolt ensümaatilist kollageeni lagundamist ja vähi rakkude invasiooni SCID hiirtel ksenotransplantaadi kasvajat ümbritsevatesse kudedesse. C1r ja C1s vaigistamine ning C1r eemaldamine vähendas NLK kasvu ning prolifereeruvate rakkude arvu, CD34-positiivsete veresoonte arvu ja suurendas apoptootiliste rakkude arvu kasvaja koes.

Selles uurimustöös näitan C1q, C1r ja C1s valkude rolli NLK tekkes. Komplemendi klassikalise raja komponendid C1q, C1r ja C1s võiksid olla naha lamerakk-kartsinoomi markeriteks ja ravi sihtmärkideks.

MÄRKSÕNAD: lamerakk-kartsinoom, komplemendisüsteem, komplemendi komponent C1q, seriini proteaas C1r, seriini proteaas C1s

Table of Contents

Abbreviations

6-TG	6-thioguanine
AK	actinic keratosis
AKT	protein kinase B
BCC	basal cell carcinoma
C1-INH	C1 inhibitor
C1r KO	C1r knockout
C4bp	C4b-binding protein
CD	cluster of differentiation
CHP	collagen hybridising peptide
CLL	chronic lymphocytic leukemia
CR1	complement receptor 1
CRP	C-reactive protein
CS	complement system
cSCC	cutaneous squamous cell carcinoma
cSCCIS	cutaneous squamous cell carcinoma *in situ*
DAF	decay-accelerating factor
DLE	discoid lupus erythematosus
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EB	epidermolysis bullosa
ERK	extracellular signal-related kinase
FB	complement factor B
FCS	fetal calf serum
FD	complement factor D
FH	complement factor H
FI	complement factor I
GO	gene ontology
HE	hematoxylin-eosin
HIV	human immunodeficiency virus
HPV	human papillomavirus
HRAS	Harvey rat sarcoma virus oncogene

IHC	immunohistochemistry
IL	interleukin
JAK	Janus kinase
KO	knockout
KRAS	Kirsten rat sarcoma virus oncogene
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MASP	MBL-associated serine proteinases
MBL	mannose-binding lectin
MCP	membrane cofactor protein
MMP	matrix metalloproteinase
NEAA	non-essential amino acid
NHEK	human epidermal keratinocyte
NMSC	non-melanoma skin cancers
NRAS	neuroblastoma RAS virus oncogene
OTR	organ transplant recipient
PI3K	phosphoinositide 3 kinase
RDEB	recessive dystrophic epidermolysis bullosa
RDEBSCC	recessive dystrophic epidermolysis bullosa associated SCC
PDGFRβ	platelet-derived growth factor receptor-β
RNA	ribonucleic acid
ROS	reactive oxygen species
SCID	severe combined immunodeficient
SiRNA	small interfering RNAs
STAT3	signal transducer and activator of transcription 3
TMA	tissue microarray
TNF	tumor necrosis factor
UV	ultraviolet
VG	Van Gieson
WT	wild type
XP	xeroderma pigmentosum

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Riihilä P, Viiklepp K, Nissinen L, Farshchian M, Kallajoki M, Kivisaari A, Meri S, Peltonen J, Peltonen S, Kähäri VM. Tumour-cell-derived complement components C1r and C1s promote growth of cutaneous squamous cell carcinoma. *Br J Dermatol*, 2020;182:658-670.

II Viiklepp K, Nissinen L, Ojalill M, Riihilä P, Kallajoki M, Meri S, Heino J, Kähäri VM. C1r upregulates production of matrix metalloproteinase-13 and promotes invasion of cutaneous squamous cell carcinoma. *J Invest Dermatol*, 2022;142:1478-1488.

III Viiklepp K, Nissinen L, Kallajoki M, Meri S, Kähäri VM, Riihilä P. The expression of C1q by macrophages and stromal fibroblasts in tumor microenvironment is associated with progression of cutaneous squamous cell carcinom (manuscript).

1 Introduction

Skin cancers are the most frequently diagnosed cancers, with growing incidence globally due to cumulative ultraviolet (UV) exposure and ageing of the population. Skin cancers are divided into two groups: melanocyte-derived melanomas and keratinocyte-derived non-melanoma skin cancers (NMSCs). NMSCs are divided into basal cell carcinoma (BCC; about 80% of NMSCs) and cutaneous squamous cell carcinoma (cSCC; about 20% of NMSCs). In this research the focus is on cSCC. Globally, cSCC is the most common metastatic skin cancer and the second most common skin cancer in the Caucasian population. About 20% of skin cancer-related deaths are caused by cSCC.

Usually cSCC progresses from actinic keratosis (AK) via cSCC *in situ* to invasive and metastatic cSCC. In addition to ultraviolet radiation, chronic cutaneous ulcers and immunosuppression are significant risk factors for cSCC. Inflammation is involved in several types of cancers. Inflammatory cells are present in the tumor microenvironment of cSCC. The complement system plays a crucial role in the innate and adaptive immune system and regulates inflammation. The complement system can be activated via three pathways: classical, alternative and the lectin pathways. All these pathways lead to activation of C3, which in turn activates the lytic pathway and results in target cell lysis. The classical pathway is typically activated by alteration in the tertiary structure of C1q in the C1 complex. Changes of C1q structure lead to autolytic activation of C1r, which activates C1s. Then C1s cleaves two serum proteins C2 and C4.

Most cSCCs are surgically removable with a good prognosis and the cure rate is higher than 90%. However, sometimes recurrences and metastases can occur. The estimated metastasis rate is 3-5% for primary cSCC and the prognosis of metastatic cSCC is poor, as more than 70% will die from the disease within 3 years. Thus it is very important to identify high-risk cSCCs and find molecular markers to predict cSCC aggressiveness and metastasis. In this study, the roles of complement system component C1q, serine proteinases C1r and C1s in the progression of cSCC have been studied. The role of the classical pathway of the complement system in cSCC tumor growth was also studied *in vivo*.

2 Review of the Literature

2.1 Cutaneous squamous cell carcinoma

2.1.1 Overview

Skin cancers are the most common cancers worldwide (Rollison et al, 2021). According to epidemiological and prognostic differences, skin cancers are divided into two groups: melanocyte-derived melanomas and NMSCs (Tejera-Vaquerizo et al, 2016). NMSCs, in turn, are divided into BCCs, SCCs and rare skin cancers (e.g. cutaneous lymphomas, sarcomas, adnexal tumours, Merkel-cell carcinomas, and other rare primary cutaneous neoplasms) (Lomas et al, 2012; Madan et al, 2010). Although BCC and SCC have many similarities (e.g. they both are keratinocyte-derived skin cancers), they also have a lot of differences, including incidence rates, clinical course, management strategies and etiology (Lomas et al, 2012). NMSCs consist of about 80% BCCs and about 20% cSCCs (Albert and Weinstock, 2003; Lee and Miller, 2009; Lomas et al, 2012). Other NMSCs are less common and differ in their cell type, behaviour, and epidemiological features compared to BCC and cSCC (Albert and Weinstock, 2003).

Although the mortality of BCC is extremely low (less than 0.1%), it is the most common human cancer globally and usually causes significant financial burden to health systems, and may also cause significant morbidity as most BCC lesions occur on visible areas, such as the head and neck (Lewis and Weinstock, 2004; Lomas et al, 2012; Lucas et al, 2006; Madan et al, 2010).

The second most frequent skin malignancy is cSCC, which is the most common metastatic skin cancer in the world in the Caucasian population (Brougham and Tan, 2014; Corchado-Cobos et al, 2020; Palazzo et al, 2020; Que et al, 2018). cSCC is much more aggressive than BCC (Lee and Miller, 2009). The incidence of cSCC has been dramatically increasing globally in recent decades. The risk of developing cSCC during one's lifetime grows in parallel to the lengthening of the mean length in lifespan. (Strippoli et al, 2021) Lifetime incidence of cSCC is 7 to 11% (Bergón-Sendín et al, 2021). Patients aged 75 years or older have a 5-10 times higher risk to get cSCC than younger people. Men also have a higher risk than women for developing cSCC due to higher occupational

exposure, e.g. usually working more outside than women. (Armstrong and Kricker, 2001; Foote et al, 2001; McCarthy et al, 1999; Thomas-Ahner et al, 2007) The risk of developing cSCC relates to lifetime accumulation of UV solar radiation (Strippoli et al, 2021). The exact incidence of cSCC is probably underestimated because it is difficult to verify the exact number of cases; countries register cSCC cases significantly differently, or have poor registration, not considering the multiplicity of cSCC (Lomas et al, 2012).

2.1.2 Epidemiology of cSCC

In Finland cSCC is the sixth most common cancer in men (5.7% of all cancers) and the fifth most common cancer in women (5.3% of all cancers). cSCC is the fourth most common cancer in over 70 years old men and women. In 2020 there were 1916 new cases of cSCC in Finland; 1032 men and 884 women. 44 patients, 33 men and 11 women, died from cSCC in 2020 in Finland. Men's 5-year survival rate of cSCC was 94.28% and women's 94.91% in the years 2018-2020. (Finnish Cancer Registry, 2021; Sinikumpu et al, 2022)

2.1.3 Progression of cSCC and premalignant forms of cSCC

The premalignant form of cSCC is AK (also called solar or senile keratosis). AKs represent proliferation of transformed, neoplastic keratinocytes and are confined to the epidermis and have the potential risk of malignant transformation. (Cockerell, 2000; Heaphy and Ackerman, 2000; Reinehr and Bakos, 2019) AKs are categorised as pre-neoplastic lesions, but since they develop from clonal DNA modifications in keratinocytes it is believed that AK is an early form of cSCC *in situ* (cSCCIS, Bowen's disease) (Heaphy and Ackerman, 2000; Lober and Lober, 2000; Röwert-Huber et al, 2007). Increased exposure to UV radiation is the most important risk factor for AK, cSCCIS and cSCC (Anwar et al, 2004). Usually the first manifestation of cSCC is AK, which can develop into cSCCIS, invasive cSCC, and finally to metastatic SCC (Ratushny et al, 2012).

AKs are among the most frequent keratinocyte-derived precancerous lesion in humans, as more than 60% of elderly people have them (Palazzo et al, 2020; Ratushny et al, 2012). There is a 0.025-16% possibility per year that an individual AK lesion may develop into cSCC (Fernandez Figueras, 2017; Glogau, 2000; Marks et al, 1988; Quaedvlieg et al, 2006). Classically one patient has 6-8 AK lesions and for that reason there is 0.15-20% risk for one patient with several lesions to develop invasive cSCC (Glogau, 2000; Marks, 1995; Mittelbronn et al, 1988; Salasche, 2000). For example, patients with less than five AK or cSCCIS lesions have a risk of developing cSCC at less than 1% of cases, whereas patients

with more than 20 lesions have a risk of developing cSCC at about 20% of cases. cSCC develops into metastatic cSCC in 0.5-3.3% of cases. (Ratushny et al, 2012; Knuutila et al, 2020)

Clinically AK lesions are characterized as a superficial scale on a red base, squamous, crusty and keratotic papules, macules or plaques, usually with poorly defined borders (Reygagne and Rostain, 2018). AKs can appear in different forms and attend different clinical variations, such as hyperkeratotic AKs, atrophic, pigmented lichenoid AKs, cutaneous horn, and actinic cheilitis. Generally, AK is classified as mild, moderate and severe. A mild AK lesion is slightly palpable and usually lightly red; a moderate lesion is simply felt, seen and moderately thick; a severe AK lesion is very thick and easy to feel and see. Severe AK can clinically resemble cSCCIS but with a thicker hyperkeratosis, resembling crust. (Ratushny et al, 2012; Reinehr and Bakos, 2019) (**Figure 1**)

Usually cSCCIS is a constant reddish-brown plaque or patch of dry/scaly skin which grows slowly. This lesion can be flat or a little bit raised. Typically, cSCCIS lesions have no symptoms, but occasionally they may itch, be tender, have a crust, or bleed. Sometimes the lesions of cSCCIS can be warty/verrucous, pigmented or fissured. (Palaniappan and Karthikeyan, 2022)

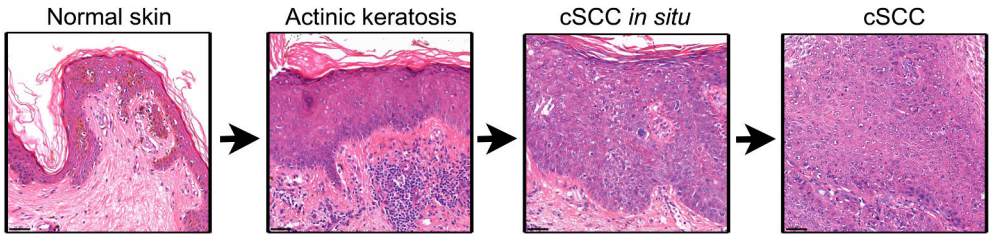

Figure 1. Histological presentation of normal skin, actinic keratosis, Bowen's disease (cSCC *in situ*) and cutaneous squamous cell carcinoma (cSCC). Scale bar = 50 µm (Stainings by P. Riihilä; photographs by K. Viiklepp)

AK is represented at the histological level by dysplasia and consists of keratinocytes with atypical nuclei that are expanded, asymmetrical, and hyperchromatic. AK is frequently accompanied by skin solar elastosis, a flimsy granular layer, parakeratosis, buds of nontypical epidermis reaching toward the papillary dermis, and inflammation. There is also disordered growth in AK, which disrupts keratinocyte differentiation and is the reason for parakeratosis. To understand the severity of epidermal dysplasia, a three-tiered grading scale (I-III) is used in histopathology. Keratinocytic intraepidermal neoplasia I: the basal keratinocyte's cellular atypia is confined to the lower third of the epidermis; II: atypical keratinocytes occupy the lower ⅔ of the epidermis; III: atypical

keratinocytes are seen throughout the epidermis and this stage is also equal to carcinoma *in situ.* In AK, restricted epidermal atypia has incomplete disruption of the differentiation program, whereas a more absolute disruption of differentiation is related to cSCCIS. cSCCIS does not invade the basement membrane but atypical keratinocytes are detected at all levels of epidermis. (Cockerell, 2000; Ratushny et al, 2012)

2.1.4 Clinical signs and histopathology of cSCC

Cutaneous SCC will usually develop in chronically photo-exposed skin areas, e.g. face, bald scalp, neck, shoulders, back of the hands, extensor forearms, shins and cervix of neck (Reinehr and Bakos, 2019). Clinically, the colour of cSCC varies from flesh toned to erythematous with variable degrees of crusting, scale, hyperkeratosis, induration and ulceration. Sometimes there are also telangiectasias with or without spontaneous bleeding. cSCC can be plaque-like in some cases with significant induration and/or subcutaneous spread considerable on palpation; flat and nodular. Sometimes cSCC may be painful and/or tender, which can be a sign of perineural invasion; but usually it is painless. Lesions of cSCC often grow rapidly. Complications of cSCC are pain, local invasion, metastases and death. (Howell and Ramsey, 2021) Metastatic cSCC presents as multiple nodular lesions in skin or internal organs (Ratushny et al, 2012; Reinehr and Bakos, 2019). It is important to palpate regional lymph nodes to examine metastasis. When metastasis is suspected, additional imaging examinations are needed and sentinel lymph node biopsy is performed if necessary. (Howell and Ramsey, 2021) When a metastasis appears in regional lymph nodes they are usually matted, solid and adhered to the overlying skin (Madan et al, 2010).

cSCC is remarkable for asymmetrical cords, sheets and nest sheets of neoplastic keratinocytes that penetrate from epidermis to dermis. Tumor cells are unshaped, large and histopathologically have abnormal mitosis. Depending on histopathology, cSCCs are categorized into well, moderately and poorly differentiated. Lesion thickness is very important in predicting the risk of metastasis. cSCC has a much higher risk of metastasizing when the thickness of the lesion is over 4 mm. (Kueder-Pajares et al, 2018; Raone et al, 2018) Other higher risk factors for metastatic cSCC are presence of the lesion on the ear, the lower lip or the temple area; perineural invasion, poorly differentiated lesion, primary tumor diameter over 20 mm and tumor invasion beyond subcutaneous fat. Other possible findings are lymphocytes and histiocytes containing inflammatory cell infiltrates at the tumor site. (Baum et al, 2018; Howell and Ramsey, 2021; Knuutila et al, 2020; Thompson et al, 2016)

For patients with suspicion of cSCC or cSCCIS it is important to take a skin biopsy. cSCC can resemble other skin cancers (e.g. BCC or melanoma), AK or cSCCIS. Some burns, scar tissue and pyoderma gangrenosum may also resemble cSCC. (Howell and Ramsey, 2021)

2.1.5 Treatment of cSCC

AK and cSCCIS are non-invasive and have several various treatment options, depending on different factors, e.g. localisation, size, number of lesions. Most commonly, cryo- and photodynamic therapy with aminolevulinic acid or methyl aminolevulinic acid are in use. In cryotherapy, liquid nitrogen (-196°C) is used. Topical agents are largely in use, as is imiquimod cream. Also diclofenac, 5-fluorouracil cream and carbon dioxide laser are used for the treatment of AK. Surgery is sometimes used for cSCCIS treatment. Preventative treatments for AK and cSCCIS utilise topical tretinoids. (Ferrandiz et al, 2012; Morton et al, 2019)

The majority of primary cSCCs are indolent, small and surgically resectable with sufficiently wide margins, good prognosis and cure rates higher than 90%. Margins of 4-5 mm are sufficient when tumor diameter is less than 2 cm. Margins should be wider than 6 mm when tumor diameter is more than 2 cm or it is located in high-risk areas, e.g. scalp, lips, nose, ears, eyelids. (Brougham et al, 2012; Madan et al, 2010) Sometimes, following operation of cSCC, local recurrence of the tumor occurs and can be fatal, with a metastasis rate of 25-45%, underlining the importance of checking margins of the primary tumor excision (Baum et al, 2018; Johnson et al, 1992; Rowe et al, 1992). If the patient is not suitable for operation, primary radiation therapy is an alternative choice. Surgically untreatable tumors with depth of tumor invasion more than 2–6 mm, extensive perineural invasion, large nerve involvement or in metastatic cases, surgery should be combined with another treatment, e.g. radiation therapy or adjuvant chemotherapy. (Baum et al, 2018; Brantsch et al, 2008; Haisma et al, 2016) One treatment option is Mohs micrographic surgery for cSCC on the head and neck areas of immunosuppressed patients, SCC with aggressive histological features, SCC greater than or equal to 2 mm of depth, or recurrent SCC. During Mohs surgery thin layers of skin is cut away and is checked carefully for signs of cSCC until there are no marks of cancer. (Howell and Ramsey, 2021)

Although metastatic cSCC is not common, its prognosis is poor. Up to 5% of cSCC cases develop local or distant metastases and then the survival rate drops dramatically; the 3-year survival rate is 30%. (Bergón-Sendín et al, 2021; Corchado-Cobos et al, 2020; Knuutila et al, 2020)

Treatment for advanced cSCC is challenging (Kramb et al, 2022). Adjuvant chemoradiation shows better recurrence-free survival than radiation therapy alone

in metastatic cSCC (Tanvetyanon et al, 2015). Unfortunately, current treatment possibilities for metastatic cSCC are unestablished and limited. The only approved systemic therapy for patients with locally advanced or metastatic cSCC who are not candidates for surgery or radiation is cemiplimab. Cemiplimab is a programmed cell death protein-1 blocking monoclonal antibody and an immune checkpoint inhibitor. Cemiplimab was approved for treatment for advanced cSCC by the European Medicines Agency in 2019 and by the Food and Drug Administration in 2018. The EGFR inhibitor, cetuximab, has also been reported for systemic off label treatment for advanced cSCC. Cetuximab may be combined with chemotherapy or radiation therapy. Cetuximab is useful in the treatment of cSCC but remains a risk for cancer cells acquiring drug resistance, remission rates are high and an adverse event profile provides challenges. (Migden et al, 2018; Stratigos et al, 2020)

Patients with cSCC are advised to protect their skin from further UV damage by using sunscreen, protective clothes and avoiding the sun during periods of maximum intensity (in summer and spring; the middle of the day). Regular check-ups are also very important, e.g patients with a few cSCC or AK in their history may be followed every 6 to 12 months, but patients with several or aggressive cSCC need to be followed more often. (Howell and Ramsey, 2021)

2.1.6 Prognosis of cSCC

Usually small cSCC lesions can be excised and are not fatal but may add to morbidity depending on their location, thickness and diameter of tumor, and degree of differentiation. Usually cSCC on the lip or the ear is more aggressive, recurring more often and metastasising in 10-25% of cases. Tumors thicker than 4-6 mm, more likely recur locally. Recurrence of cSCC is 10-18% more likely with larger lesions, despite wide excision. Tumors with a diameter over 2 cm recur two times more often than smaller lesions. cSCC has poor prognosis when penetrating into subcutaneous fat or with perineural invasion. The 5-year survival rate is poor, less than 40%, in advanced cSCC patients. Large lesions around the neck and head region need complex surgery, which can lead to poor cosmetic results. (Dalal et al, 2018; Higgins et al, 2018)

2.1.7 Risk factors for cSCC

Chronic UV radiation exposure is the major environmental risk factor for cSCC and is one reason why the risk of developing cSCC increases with the ageing of the population. In addition to AK and cSCCIS, other potentional risk factors are chronic inflammation, immunosuppression, chronic cutaneous ulcers, advanced age, fair skin, environmental exposures, human papillomavirus (HPV) infection,

medications, smoking, male sex, hereditary blistering skin disorders (especially recessive dystrophic epidermolysis bullosa – RDEB). (Rollison et al, 2021; Rosso et al, 1996; Strippoli et al, 2021) (**Figure 2**)

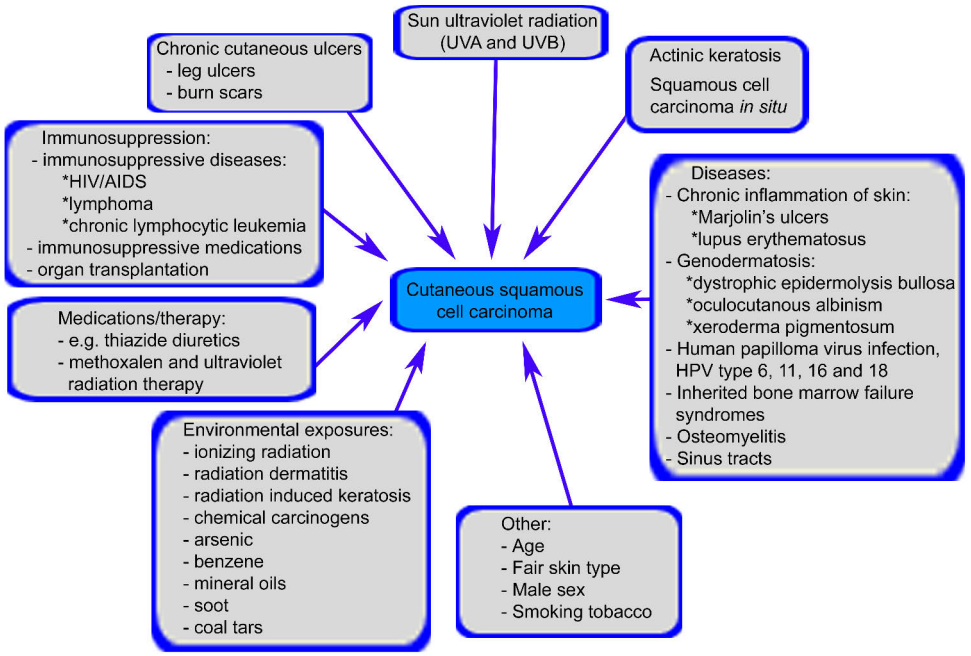

Figure 2. Risk factors for developing cutaneous squamous cell carcinoma. The references are found in the section 2.1.7.1-2.1.7.5.

2.1.7.1 UV radiation

UV radiation consists of UVA, UVB and UVC radiation. UVA (315-400 nm) radiation raises the risk of cSCC by causing random DNA damage via a photo-oxidative-stress-mediated mechanism, creating reactive oxygen species (ROS). ROS form pre-mutagenic adducts by interacting with lipids, proteins and DNA. To prevent the harmful effects of pre-mutagenic adducts requires many DNA repair systems. UVA is not as mutagenic as UVB. (Madan et al, 2010; Sinha and Häder, 2002) UVC (100-280 nm) is fully absorbed by the Earth's ozone layer because it has the shortest wavelength (Sinha and Häder, 2002).

DNA and RNA are directly damaged by UVB (280-315 nm) radiation and it causes the formation of cyclobutane-pyrimidine dimers and 6-4 pyrimidine-pyrimidine adducts, which deform the DNA helix and prevent transcription and replication (Sinha and Häder, 2002). Due to their structure, certain genomic

positions are especially vulnerable to UVB-induced DNA damage. (Benjamin and Ananthaswamy, 2007; Benjamin et al, 2008)

UV radiation can cause systemic immune suppression and antigen-specific immune tolerance because of the induction of regulatory T cells. T cells have an immunosuppressive effect. (Rollison et al, 2021) UV radiation also causes the inhibition of macrophage migration (Heise et al, 2012).

Several sunburn incidents during one's lifetime, especially during childhood, are associated with progression of cSCC. Also, due to extended life expectancy, increased cumulative UV radiation exposure, and lifestyle factors (e.g. increased travelling to the south in the winter time) cause an increased risk for cSCC. Usually cSCC develops on sun-exposed skin. Awareness about the harmful effects of UV radiation and protection of the skin has increased, but total sun avoidance is impossible. (de Vries et al, 2012; Lee and Miller, 2009; Mahé et al, 2011; Traianou et al, 2012)

The risk of UV radiation also depends on skin type. The Fitzpatrick skin type scale is used to categorise the constitutional amount of melanin pigment in the skin (white, brown, or black skin) and the reaction of the skin to sunlight exposure (tanning). For example, Fitzpatrick type 1 is white or pale skin that does not tan and burns easily, and Fitzpatrick type 6 is very pigmented skin that never or rarely burns. Fitzpatrick type 1 skin type has a much higher risk of getting cSCC, compared to the Fitzpatrick type 6 skin type. (Fitzpatrick, 1988; Li et al, 2022)

2.1.7.2 Immunosuppression

Immunosuppressed patients have a significantly increased risk of developing cSCC. Immunosuppression can be caused by diseases, e.g. chronic lymphocytic leukemia (CLL), human immunodeficiency virus (HIV), or drugs. (Ziegler et al, 1994)

CLL, low-grade lymphoproliferative malignancy, is described as clonal proliferation of functionally incompetent B cells. The risk for developing cSCC is 5-8 times higher for patients with CLL than without. (Brewer et al, 2015; Levi et al, 1996; Que et al, 2018) The 5-year metastasis rate of cSCC for CLL patients is 18%, with a standardised mortality ratio of 17.0, as for sporadic cSCC the metastasis rate is 3-5% (Knuutila et al, 2020; Royle et al, 2011). An immunosuppressive environment is promoted by monoclonal leukemic B cells downregulating CD154 in activated T cells. CD154 has a very important role in stimulating monocytes, B cells and dendritic cells to proliferate and differentiate. (Cantwell et al, 1997) CLL patients also have higher levels of interleukin-2 (IL-2) receptors. T-regulatory cells secrete IL-2 receptors and bind free IL-2, which decreases its availability. (Lindqvist et al, 2010) CLL patients also have other

immune deficits, for example functional defects in helper B cells and impaired phagocytosis (Riches et al, 2012).

The HIV-infected patients have a risk of developing cSCC 5-times higher that a person without HIV infection (Collins et al, 2019). Nowadays, patients who have antiretroviral therapy, live much longer with low-grade CD4 lymphopenia. CD4 lymphopenia usually leads to an increased risk of malignancy. Cell-mediated immunity defects are caused by CD4 lymphopenia but also by the dysfunction in the activation of CD8, cytokine profile changes, decreased apoptosis, and decreased antigen presentation. (Chang et al, 2017; Wilkins et al, 2006)

Some drugs are related to an increased risk of developing cSCC by inadvertently activating immunosuppression, paradoxical activation of pathways that lead to keratinocyte proliferation, and loss of apoptosis. Typical patients using long-term immunosuppressive drugs are organ transplant recipients (OTRs). About 30% of OTRs developed NMSCs, most of which were cSCCs, in a long-term observational study. From transplant to first cSCC, the average time was 9.9 years. Over time the prevalence increased from 24.8% at 10 years to 53.9% at 20 years, and to 73.9% 30 years after transplant. (Harwood et al, 2013) Primary cSCC in OTRs has a bigger risk of metastases, cancer-specific death, and recurrence compared to those not receiving transplants (Garrett et al, 2016).

Calcineurin inhibitors, cyclosporine and tacrolimus, are commonly used immunosuppressive drugs. They decrease IL-2 receptor expression and IL-2 production, which leads to reduced T-cell activation. At the same time cyclosporine prevents UV-induced DNA repair mechanisms and keratinocyte apoptosis, by counteracting p53 through ATF3 and so increase the risk for developing cSCC. (Wu et al, 2010; Yarosh et al, 2005)

Commonly used immunosuppressants also include oral glucocorticoids, but the risk associated with the development of cSCC is controversial. One case-control and one planned sub-study did not find a connection between the risk of cSCC and oral steroid use (Baibergenova et al, 2012; Jensen et al, 2009). On the other hand, one cohort study discovered patients with long term oral glucocorticoid treatment had a much larger risk of developing cSCC (Sørensen et al, 2004). Another study found that OTR who had a bigger cumulative immunosuppression, e.g. combination of oral glucocorticoids, azathioprine, and cyclosporine, had four times higher risk for cSCC compared to lower doses. There was no connection between the risk of cSCC and cumulative doses of each drug alone. (Fortina et al, 2004)

Azathioprine is an immunosuppressive medication used for systemic lupus erythematosus, rheumatoid arthritis, ulcerative colitis, Crohn's disease, and in kidney transplants to prevent rejection. Azathioprine acts as an antagonist to endogenous purines, which are necessary components of RNA, DNA, and some coenzymes. This results in metabolite 6-thioguanine (6-TG) accumulation in DNA.

6-TG replaces a small amount of DNA guanine and so becomes a strong UVA chromophore. This, in turn, interacts with UVA and generates reactive oxygen species, causing protein oxidation and extensive DNA damage increasing UVB mutagenicity and harming the DNA repair proteome. Patients using azathioprine were abnormally sensitive to UVA light with an increased mutation burden (UV signature 23). (Inman et al, 2018; Lennard, 1992)

6-mercaptopurine is an immunosuppressive medication used for acute lymphocytic leukemia, acute promyelocytic leukemia, Crohn's disease, and ulcerative colitis. 6-mercaptopurine metabolism results in the incorporation of 6-TG into DNA. This increases skin sensitivity to UVA and thus high risk to develop cSCC. (Attard and Karran, 2011)

BRAF inhibitors, e.g. vemurafenib, dabrafenib, and encorafenib, are in use as monotherapies for metastatic melanoma harbouring BRAF V600E mutations. 18% of patients on vemurafenib developed cSCC in a meta-analysis of seven randomised trials, and 6-26% of patients on dabrafenib developed cSCC. (Anforth et al, 2013; Ascierto et al, 2013; Chen et al, 2019) Usually cSCC develops within 3 months of treatment with a BRAF inhibitor (Anforth et al, 2015). It is believed that the activation of the mitogen-activated protein kinase (MAPK) pathway causes subsequent extracellular signal-related kinase (ERK)-mediated transcription in BRAF keratinocytes resulting in BRAF-inhibitor-associated atypical squamous proliferation, especially with concomitant oncogenic RAS mutations (Gibney et al, 2013; Heidorn et al, 2010; Poulikakos et al, 2010; Su et al, 2012).

Janus kinase (JAK)1/2 inhibitors are family of intracellular, nonreceptor tyrosine kinases that transduce cytokine-mediated signals. JAK1/2 inhibitors are used for many diseases, e.g ruxolitinib is used in myelofibrosis, polycythemia vera, or in atopic dermatitis treatment; tofacitinib is used to treat rheumatoid arthritis, psoriatic arthritis, and ulcerative colitis; baricitinib is used to treat severe alopecia areata and rheumatoid arthritis; deucravacitinib is used to treat moderate-to-severe plaque psoriasis; upadacitinib is used to treat rheumatoid arthritis and psoriatic arthritis; fedratinib is used to treat myeloproliferative diseases including myelofibrosis, etc. JAK1/2 inhibitors are associated with the development of multiple and rapidly progressing cSCCs. The exact tumorigenesis mechanism is unknown. (Aboul-Fettouh and Nijhawan, 2018; Aleisa et al 2020; March-Rodriguez et al, 2019)

Thiazide diuretics are used in antihypertensive treatment. Thiazide diuretics have been associated with increasing the risk of skin cancers, ecpecially with cSCC by inducing photosensitivity. The risk for developing cSCC increases with longer durations and higher doses of use of thiazide diuretics. (Blakely et al, 2019; Robinson et al, 2013; Shao et al, 2022)

2.1.7.3 β-human papillomavirus (β-HPV)

HPV consist of heterogeneous subgroups. The α-papillomavirus (α-HPV) subtype is connected with mucosal SCC. The β-papillomavirus (β-HPV) subtype is a risk factor for cSCC. Usually in UV-exposed areas pre-cancerous wart-like lesions may develop into cSCC. At the same time there are multiple factors indicating that β-HPV may be a risk factor for cSCC including patients with immunodeficiency having increased rates of β-HPV infection and a much higher risk of developing cSCC, suggesting a possible causal relationship between cSCC and β-HPV (Antonsson et al, 2000; de Koning et al, 2009; Neale et al, 2013). Furthermore, studies have shown a connection between cSCC and β-HPV DNA and/or serum antibodies (Bouwes Bavinck et al, 2018). Additionally, previous studies have shown that β-HPV has a role in tumor initiation, although it is uncertain whether this extends to tumor maintenance (Howley and Pfister, 2015). In pre-cancerous lesions such as AKs, β-HPV DNA is present in high levels but lower levels are seen in cSCC lesions. Previous studies have demonstrated that HPV oncoproteins E6 and/or E7 may increase sensitivity to UV-induced oncogenesis via alterations in p53 and Notch1 signalling. (Conforti et al, 2019; Weissenborn et al, 2005) It was also found that β-HPV infects and increases the size of adult tissue stem cells, allowing cells to persist and accumulate mutations (Olivero et al, 2018; Thai et al, 2021).

2.1.7.4 Environmental exposure

Environmental risk factors include ionising radiation via diagnostic, therapeutic or environmental exposure. Ionising radiation preferentially affects the basal layer of the epidermis, leading to an increased risk of developing BCC more than cSCC. (Kishikawa et al, 2005; Lichter et al, 2000; Yoshinaga et al, 2005) Chronic arsenic exposure is a risk factor for cSCC, where cSCC can then develop also in non-sun-exposed areas. Arsenic can be found in contaminated drinking water. (Yu et al, 2006) Other risk factors for developing cSCC are exposure to aromatic hydrocarbons such as benzene, mineral oils, soot and coal tars (Pukkala et al, 2014; Stenehjem et al, 2017).

2.1.7.5 Other skin diseases

In addition to AK and cSCCIS, other skin diseases can also be risk factors for cSCC, e.g. chronical inflammatory skin disease lupus erythematosus; Marjolin's ulcers; as well as the genetic syndromes epidermolysis bullosa, albinism and xeroderma pigmentosum (XP). (Arvanitidou et al, 2018; Fernandes et al, 2015;

Kadir, 2007; Khatri, 2021; Montaudié et al, 2016; Perry and Silverberg, 2001; Xiang et al, 2019)

Epidermolysis bullosa (EB) is a group of heritable diseases described as having increased mucosal and/or skin fragility and blister formation after slight mechanical trauma. Patients with EB may develop several complications and cSCC is the most serious of them. Most cSCCs occur with the RDEB subtype, but cSCC developed also in patients with other subtypes (junctional and dominant dystrophic) of EB. RDEB is skin and mucous membrane blistering fatal disease, which is caused by mutationin the *COL7A1* gene. Collagen VII is encoded by *COL7A1*. Collagen VII is synthesized by dermal fibroblasts and epidermal keratinocytes and it is the major component of the anchoring fibrils. Anchoring fibrils give the stability to the dermal-epidermal adhesion. Loss of the structural function of *COL7A1* causes deficiency of collagen VII and so makes the skin fragile, which causes lifelong blistering, chronic wounds and is very painful. Moreover, it increases fibrosis, inflammation, bacterial colonization, progressive scarring and secondary multiorgan involvement. There is no treatment for RDEB and it is progressive. The most common cause of death among patients with RDEB is cSCC because it is usually very aggressive with high metastatic potential and develops at a younger age in EB patients than in non-EB patients. cSCC patients with RDEB are more aggressive than UV-induced cSCC. RDEB-SCC has mutations in *NOTCH1, NOTCH2, TP53, FAT1, HRAS*, and *CDKN2A* genes. (Chung and Uitto, 2010; Condorelli et al, 2019; Montaudié et al, 2016)

Discoid lupus erythematosus (DLE) is an autoimmune inflammatory disease. A usual symptom is a red rash on the face. Most frequently cSCC develops on sun-exposed skin with the period between DLE and development of cSCC about in 9.59 ± 5.6 years. The most frequently affected area was the upper lip (28.57% of cases). (Arvanitidou et al, 2018; Fernandes et al, 2015)

A rare form of cSCC is Marjolin's ulcer. They appear in chronic inflammatory areas, such as venous stasis ulcers, pressure sores and burn scars. (Kerr-Valentic et al, 2009) The risk of developing cSCC metastases is about 30% suggesting Marjolin's ulcers are much more aggressive than spontaneous cSCC (Kadir, 2007; Xiang et al, 2019).

Albinism is a hypopigmentation disorder affecting the skin, eyes, and hair. Because of absent or decreased defenses from melanin, cSCC is common in patients with albinism. (Perry and Silverberg, 2001)

XP is a rare autosomal recessive disorder. Patients with XP have DNA repair defects, leading to abnormal sensitivity of the skin to UV light, increasing the risk of cSCC. Clinically, patients develop pigmentary changes, keratosis, atrophy, and carcinomas on light-exposed skin. (Khatri, 2021)

2.1.8 Pathogenesis of cSCC

The pathogenesis of cSCC is dependent on a number of different factors (Biao et al, 2022; Sengupta and Harris, 2005). Mutations in tumor suppressor gene *TP53* lead to the growth of a precursor lesion (AK), which leads to increased genetic instability or loss of cell cycle control. Further mutations in oncogenes can cause more neoplastic properties, which can lead to metastatic cSCC. Driver mutations have been identified for cSCC in several genes, including *EGFR, NOTCH1* and *NOTCH2*. Four to six genetic changes are needed for the transition of benign epithelium to metastatic carcinoma. (**Figure 3**) (Fearon and Vogelstein, 1990; Li et al, 2015; Pickering et al, 2014) Similar driver gene mutations can be found already in normal epidermal keratinocytes in chronically sun-exposed skin (Martincorena et al, 2015). Modifications in the tumour microenvironment are needed for the progression of AK to invasive and metastatic cSCC (Nissinen et al, 2016; Piipponen et al, 2021; Riihilä et al, 2019), such as a loss of collagen XV and collagen XVIII at an early stage of cSCC progression in the basement membrane. Collagen XV accumulates in cSCC stroma in the later stages of invasive cSCC. Invasive cSCC cells are able to produce collagen XVIII. (Karppinen et al, 2016) Collagen VII suppresses cSCC vascularisation by regulating TGF-β (Martins et al, 2015). Normally, collagen XV is a tumor suppressor and provides the membrane with support and cell anchorage. Collagen XV is associated with basement membranes. (Bretaud et al, 2020) Collagen XVIII is located within the basement membrane. Collagen XVIII has an important role in the integrity of the structure of the basement membrane for endothelial and epithelial cells. (Heljasvaara et al, 2017) Collagen VII is an important component of the anchoring fibrils. Collagen VII provides stability to the dermal-epidermal adhesion on the dermal site. (Chung and Uitto, 2010)

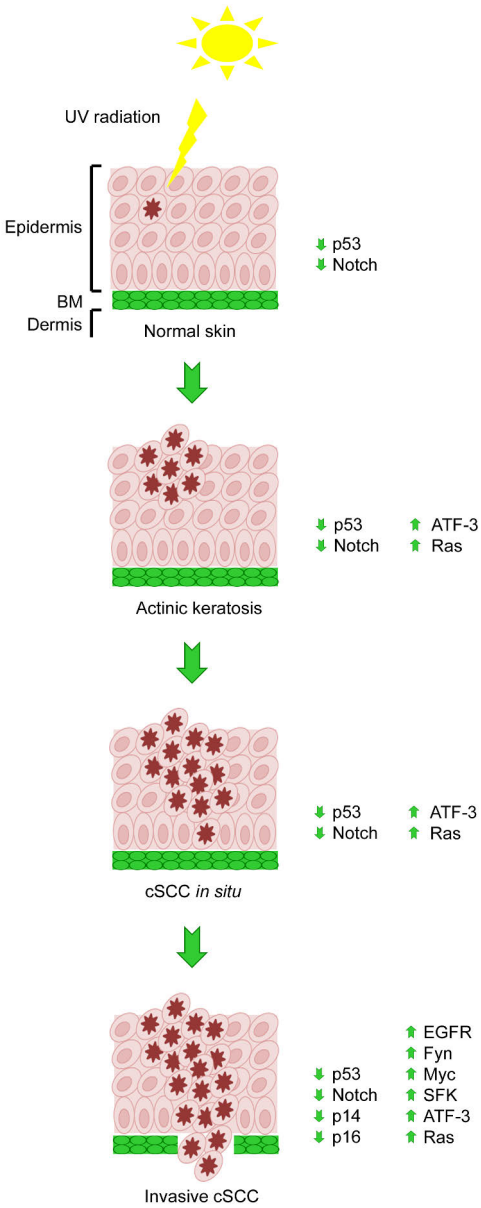

Figure 3. Pathogenesis of cutaneous squamous cell carcinoma (cSCC). Molecular alterations in the development of actinic keratosis, cutaneous squamous cell carcinoma *in situ* (cSCC *in situ*), and invasive cutaneous squamous cell carcinoma (cSCC). UV, ultraviolet; BM, basement membrane; p53, the product of tumor suppressor gene *TP53*; Notch, signalling pathway controlling cell-fate; EGFR, epidermal growth factor receptor; Fyn, tyrosine-protein kinase Fyn; Myc, transcription factor; SFK, Src-family tyrosine kinase; ATF-3, cyclic AMP-dependent transcription factor; Ras, activator of kinase pathway; p14, the product of cyclin-dependent kinase inhibitor 2A (CDKN2A); p16, the product of cyclin-dependent kinase inhibitor 2A (CDKN2A).

UVB inactivates the tumor suppressor *TP53* gene, which leads to genomic instability in keratinocytes (Kubo et al, 1994; Nelson et al, 1994; Ziegler et al, 1994). The product of *TP53* gene, p53, acts predominantly as a transcriptional factor and has a very important role in regulating nucleotide excision repair (Sengupta and Harris, 2005). *TP53* mutations take place early in cSCC progression but mutations are also found in normal keratinocytes and pre-malignant lesions

(Jonason et al, 1996; Nakazawa et al, 1994; Ren et al, 1996; Ziegler et al, 1994). The presence of *TP53* mutations in AK indicate that dysplastic lesions already have genetic mutations before developing into cSCC (Ortonne, 2002; Ziegler et al, 1994). 50% of all human cancers and nearly all skin carcinomas have *TP53* mutations (Benjamin et al, 2008). It is found that 90% of cSCC patients have a *TP53* mutation, demonstrating that the loss of p53 takes place before tumor invasion (Cho et al, 2018).

Activation of Src-family tyrosine kinases, EGFR and Fyn have been detected in human cSCC. Through a c-Jun dependent mechanism these kinases downregulate p53 mRNA and protein levels, which in turn regulates p53 function. (Kolev et al, 2008; Zhao et al, 2009) AK formation is associated with increased activation of ATF-3, EGFR, SFK and Myc; and decreased levels of inositol polyphosphate 5′-phosphatase, which could be the outcome of increased PI3K/Akt (Kim et al, 2011; Sekulic et al, 2010; Toll et al, 2009; 2010).

The Notch signalling pathway is usually affected in cSCC and changes are found in 60-80% of cSCCs (Di Nardo et al, 2020; Li et al, 2015; South et al, 2014). Notch, a highly conserved intercellular signalling mechanism, has a very important role in the development and maintenance of tissue homeostasis, and controls normal skin function (Artavanis-Tsakonas et al, 1999). Four transmembrane receptors (Notch1-4) are encoded by genes of the Notch family. Notch signalling is part of the terminal differentiation of keratinocytes in the epidermis. (Watt et al, 2008) What is fascinating is that depending on the cell context, Notch may have oncogenic or tumor-suppressive functions (Nowell and Radtke, 2017). Notch1 is involved in maintaining functionality of the skin barrier and regulates cSCC tumorigenesis by regulating the tumor microenvironment and inflammatory response. Interestingly, loss of Notch signalling is associated with carcinogenesis in keratinocytes, especially loss of Notch1 and Notch2, which are frequently mutated in cSCC. (Martincorena et al, 2015; Nicolas et al, 2003; Pickering et al, 2014; South et al, 2014; Zheng et al, 2021). In keratinocytes, Notch1 is a downstream positive target of p53 and leads to inhibition of cell growth and induced differentiation. Notch1 also supresses MRCKα and ROCK1/2 kinases and induces tumor suppressor protein p21 expression via p53, which leads to cell growth inhibition. Notch1 is a direct target for p53 in keratinocytes and *NOTCH1* mutations have already been identified in AK and cSCCIS. Notch1 mutations are detected more often in sun-exposed normal skin and prove the tumorigenic role of Notch and p53 mutations in early skin carcinogenesis. (Lefort et al, 2007). Loss of Notch signalling causes disruption of skin barrier function and generates a chronic wound-like environment (Demehri et al, 2009).

Two tumor-suppressor genes, *p16(INK4a)* and *p14(ARF)*, are encoded by the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene. Both genes control cell

cycle; p16INK4A binds to CDK4 and CDK6 prevents phosphorylation of Rb protein and G1-S phase progression, p14ARF binds to MDM2 preventing degradation of p53 and inactivation of Rb and is the reason for cell arrest. The most common mechanism of p16 and p14 inactivation is the promoter region's methylation, which is followed by mutations and loss of heterozygosity in cSCC. (Brown et al, 2004) Up to 80% of cSCCs have changes in *CDKN2A* (Di Nardo et al, 2020). It is hypothesised that ERK signalling may upregulate *CDKN2A* as a response to stress to induce aging rather than stimulating cell cycling in cSCC. (Inman et al, 2018)

The most frequently mutated genes found in human cancers, including cSCC, are *RAS* genes (Pierceall et al, 1991; Spencer et al, 1995): HRAS (Harvey rat sarcoma virus oncogene), NRAS (neuroblastoma RAS virus oncogene),and two splice variants of KRAS (Kirsten rat sarcoma virus oncogene) are a family of GTP-binding proteins that cycle between "off" and "on" states. (Khavari, 2006; Ryan and Corcoran, 2018) Oncogenic constitutive activation of the Raf/Mek/Erk1/Erk2 kinase and phosphoinositide 3 kinase-protein kinase B (PI3K-AKT) pathways can be the result of an active *RAS* mutation, leading to cell proliferation and cSCC progression (Fruman et al, 2017; Khavari, 2006). HRAS mutations have been found in 3-20% of cSCCs (Pickering et al, 2014; South et al, 2014). Activation of *RAS* alone is not sufficient to stimulate tumorigenesis in keratinocytes (D'Arcy et al, 2020). More changes are needed for cSCC formation in the presence of activated *RAS*, e.g. IκBα co-expression, *NOTCH1* deletion, or CDK4-mediated bypass of Rb cell cycle (Dajee et al, 2003; Nicolas et al, 2003; Ridky et al, 2010).

2.2 The complement system

2.2.1 Overview

The complement system (CS) is an important part of the innate and adaptive immune system, which increases the function of antibodies and phagocytes (Ling and Murali, 2019). CS functions as the first line of host protection against microbial pathogens (bacteria, viruses, parasites) by activating the immune reaction via opsonisation, recruitment of immune cells to the site of infection, destroying foreign structures and immediate cell lysis, and increases humoral immune responses. In addition, CS recognises damage associated molecular patterns of altered, damaged or apoptotic host cells. (Bulla et al, 2016; Riihilä, et al, 2019; Vignesh et al, 2017)

CS comprises more than 50 protein components, each functioning as part of a well-differentiated regulatory system, a 3-branch activation cascade, or as receptors transmitting the activation signal to multiple cellular effector functions. The

complement proteins can be found in plasma, cell surfaces and other body fluids in low concentrations. The main origin of complement components is the liver, but other cells in different tissues e.g. epidermal keratinocytes can also produce complement proteins. (Bulla et al, 2016; Morgan and Gasque, 1997; Riihilä et al, 2019; Vignesh et al, 2017)

Some complement components are serine proteinases and have roles beyond complement activation. These serine proteinases are C1r, C1s, C2, C4, C5, mannose-binding lectin (MBL)-associated serine proteinases (MASP)-1, -2, and -3, and complement factor B (FB), factor D (FD), and factor I (FI) (Forneris et al, 2012).

Usually the structure of the foreign target molecule is what activates the complement cascade, which in turn activates the complement proteins in a consecutive manner. CS has three distinct activation pathways: the antibody-dependent classical pathway and two phylogenetically older antibody-independent pathways – the alternative and lectin pathways. All three pathways lead to activation of the central component C3, which activates the lytic pathway and finally cell lysis may take place (**Figure 4**). (Mamidi et al 2017; Riihilä et al, 2019) Commonly CS is reported as a linear cascade of three unconnected pathways, but in fact all pathways are tightly connected to each other. The activation of CS activates three main biological responses. At first, C3b and iC3b, fragments of C3, provoke complement dependent cellular cytotoxicity by acting as cell surface receptors for phagocytes, e.g. macrophages and neutrophils. Second, C3a, C4a, and C5a, the activation fragments of C3, C4, and C5, act as chemotactic activators and anaphylatoxins of leukocytes and increase immune responses. Third, the lytic terminal pathway leads to membrane attack complex (MAC) formation. CS activation is strictly regulated by inhibitors to maintain homeostasis and avoid excessive consumption of components. (Janssen and Gros, 2006; Sim and Tsiftsoglou, 2004)

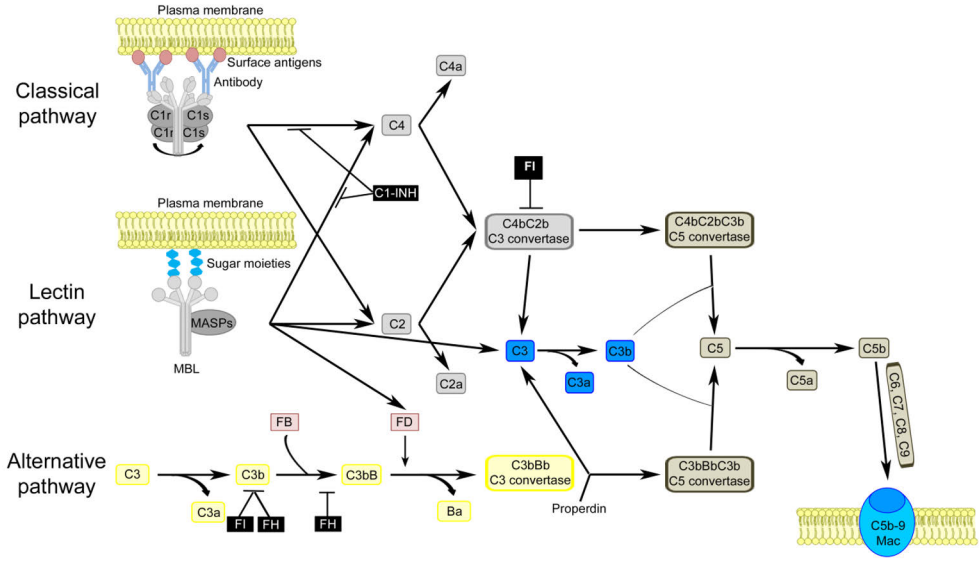

Figure 4. The complement system. The complement system has three distinct activation pathways. (1) The classical pathway is usually activated by antigen-antibody complexes on the cell surface. The C1 complex cleaves serum proteins C4 and C2. C4b binds to the target cell surface and C2b binds to a site on C4b and forms the complex C4bC2b, which is a classical pathway C3 convertase. (2) The lectin pathway is activated by connecting carbohydrate ligands on the surface of pathogens to pattern-recognising Mannose-Binding Lectins (MBLs). Activated MBL-associated serine proteinase (MASP) cleaves C4 and C2. MASP can also activate complement factor D (FD). (3) The alternative pathway is activated on any permissive surface in plasma. Continual C3 breakdown takes place spontaneously at low level in plasma. C3b is generated on the surfaces of microbes. Complement factor B (FB) attaches to C3b and complex C3bB is formed. FB is cleaved into Ba and Bb by complement factor D (FD). The fragment Bb and C3b remain linked, and C3 convertase (C3bBb) is formed. C3 convertase is stabilized by properdin. All these three pathways lead to C3 activation, finally activating the lytic pathway. C3b associates with C3 convertase and forms C5 convertase, which cleaves C5. C5b associates with C6, C7, C8, and C9 to form the terminal membrane attack complex (MAC). MAC promotes cell lysis. The activation of complement is strictly regulated by inhibitors – complement factor I (FI) and complement factor H (FH). C1 inhibitor (C1-INH) prevents C4 cleavage.

2.2.2 Classical pathway

The classical pathway of CS is activated when C1q, in complex with two copies each of the C1r and C1s serine proteinases (the C1 complex, held together non-covalently in a calcium-dependent complex), attaches to at least two surface/bound Fc regions of a complement-fixing antibody cluster (IgG and IgM), attached to an antigen on target. IgM is a pentameric molecule and is the most intensive activator of CS. For activation of CS, one single IgM molecule needs to bind its five antigen binding domains to an antigen (altogether there are ten C1q binding sites on IgM). This kind of binding causes extensive structural change, which affects the whole

molecule and generates the transition from planar to a staple conformation. Structural change leaves five subunits of complement binding sites in the constant (Fc) regions unprotected and launches complement activation. However, free IgM in plasma does not activate CS. On the other hand, multiple IgG molecules closely connected are required together on the target to set off complement activation. IgG subclasses have different complement binding sites, e.g. strong activators of CS are IgG1 and IgG3; a weak activator is IgG2 and IgG4 does not activate CS at all. Other C1 activators are also membrane blebs on apoptotic cells, serum amyloid P component or C-reactive protein (CRP). (Deban et al, 2008; Gaboriaud et al, 2004; Kojouharova et al, 2010; Mamidi et al, 2017) (**Figure 5**)

C1q is activated as a result of alterations in the tertiary structure in the C1 complex. Binding to a ligand, conformational change of C1q leads to autolytic activation of zymogen proteinase C1r, which in turn activates serine proteinase C1s. The conformation of C1 is changed when C1r and/or C1s is activated, no proteolytic cleavage fragment is released. (Kojouharova et al, 2010; Mamidi et al, 2017) C1s then cleaves two serum proteins C4 and C2. C4 is cleaved into C4a, which is an anaphylatoxin, and C4b, which binds covalently to carbohydrate residues of cell surface glycoproteins. C2 is cleaved into C2a, which diffuses away, and C2b, which binds non-covalently to a site on C4b, resulting in the complex C4bC2b (former C4b2a), which is also called the classical pathway C3 convertase. C3 convertase cleaves C3. (Deban et al, 2008; Gaboriaud et al, 2004; Mamidi et al, 2017) (**Figure 5**)

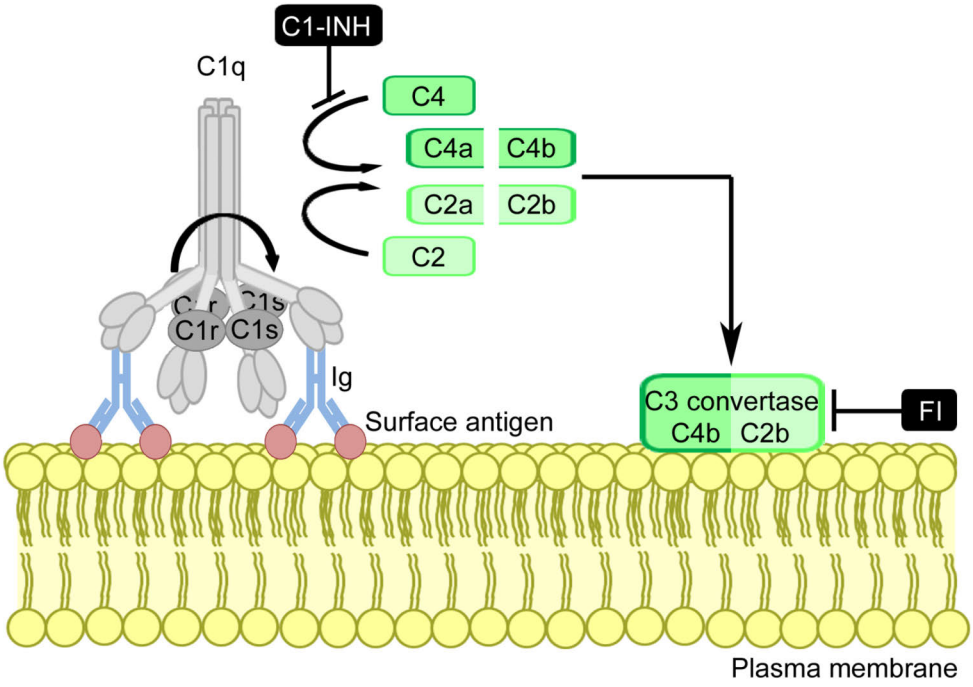

Figure 5. Activation of the classical pathway of the complement system. C1q binds to Fc regions of the complement-fixing antibody cluster (IgG or IgM) attached to a surface antigen on a target cell membrane. Conformational change of C1q leads to autolytic activation of C1r, which in turn activates C1s. C1s cleaves two serum proteins C4 and C2. C4 is cleaved into C4a and C4b, which binds to sugar residues on cell surface glycoproteins. C2 is cleaved into C2a and C2b, which binds to a site on C4b. This forms the complex C4bC2b, also called classical pathway C3 convertase. The C1 inhibitor (C1-INH) prevents the cleavage of C4. Complement factor I (FI) together with cofactors, such as C4bp, CR1, or MCP, cleaves C4b from the C3 convertase to inhibit activation of the classical pathway of the complement system.

2.2.2.1 Complement component C1q

The target identifying unit of the C1 complex is C1q. It activates the classical pathway by binding to immobilised IgM, IgG, serum amyloid P component, apoptotic cells or CRP. For CS activation at least two of the six head domains must be involved at the same time, indicating both the ability of activation by the multivalent IgM and the demand for a marginal surface density of IgG, the immune array, for activation. This last restriction decreases the risk of unsuitable complement activation on host tissues. (Kishore and Reid, 2000)

C1q is a multimere with six subunits and each subunit consisting of a carboxy-terminal globular head and a triple helical collagenous stalk. Subunits are densely linked throughout the length of the collagenous stalks, but globular head regions are separate and bouquet-like. Also each one optic six subunits consists of a trimer

of homologous chains, C1qA, C1qB and C1qC, each three 25 kDa chains (**Figure 6a**). Altogether there are 18 polypeptide chains of three different types: A, B and C, which exhibit homologous amino acid sequences and are alike in length. Each chain comprises an N-terminal region connected to each other by disulfide bonds. The genes of C1q are located on human chromosome 1p36. (Kishore and Reid, 2000) (**Figure 6b**)

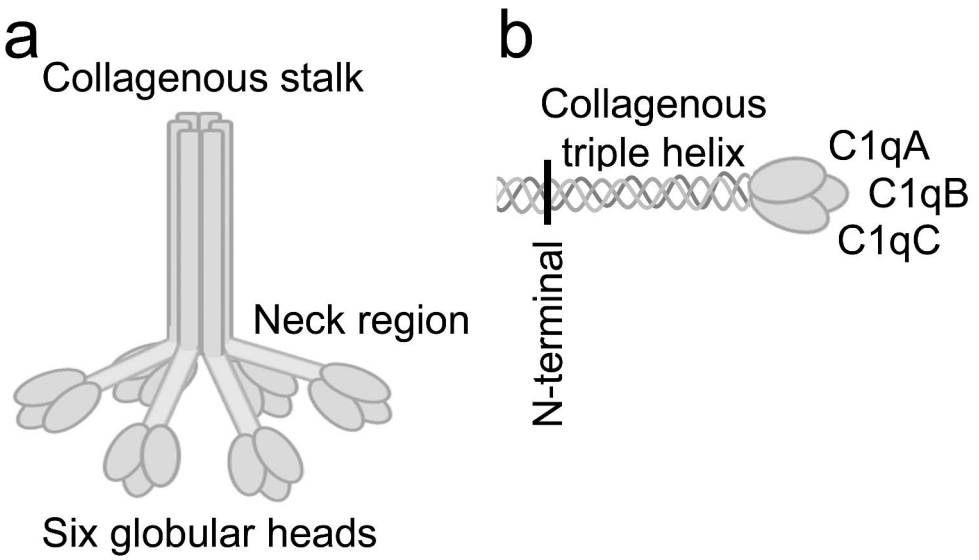

Figure 6. Structure of C1q. (a) C1q consists of six subunits, which are densely connected through a triple helical collagenous stalk, separating at the neck region. The globular heads lay out from the collagenous stalk. (b) Each globular head consists of three subunits: C1qA, C1qB and C1qC. The collagenous stalk has a short N-terminal area, where it is connected to the other collagen stalks of C1q. N-terminal area includes a cysteine residue, which has a role in formating interchain disulfide bonds.

2.2.2.2 Complement component C1r

Serine proteinase C1r is an 80 kDa single chain subcomponent of complement complex C1. Upon binding of C1q to antigen-bound antibodies, C1r is activated and cleaved into two chains: A (heavy) and B (light), which are connected by disulfide bonds. Activated C1r in turn activates C1s. The C1 complex has two C1r serine proteinases. C1r has both an interaction domain and catalytic domain. C1r is connected with the catalytic domains of C1r and C1s and is necessary for C1s activation by C1r. (**Figure 7**) (Villiers et al, 1985)

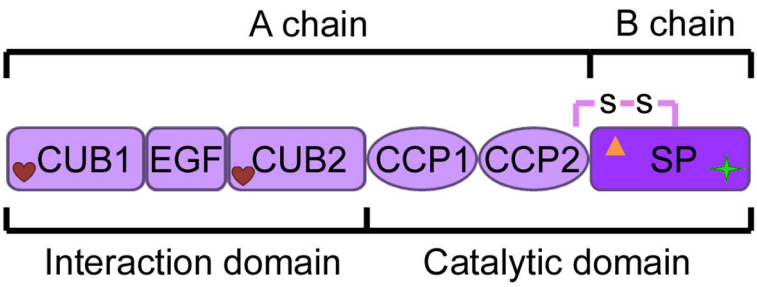

Figure 7. Schematic overview of C1r structure. C1r is a single chain component of the classical pathway. C1r consists of two N-terminal CUB domains [CUB1-2; found in the proteins: human complement proteinase C1r, embryonic sea urchin protein Uegf, and human BMP-1 (bone morphogenetic protein-1)]. CUB 1 and CUB 2 are separated by an epidermal growth factor (EGF)-like domain. In addition, it consists of two complement control modules (CCP1-2) and a C-terminal serine proteinase (SP) domain. Hearts mark C1q binding sites, a triangle an activation site, and a star the active site. CUB1-EGF-CUB2-CCP1-CCP2 is also called the A (heavy) chain and SP is the B (light) chain, connected by a disulfide bond (S-S). The interaction domain of C1r is CUB1-EGF-CUB2 and the catalytic domain is CCP1-CCP2-SP.

2.2.2.3 Complement component C1s

Serine proteinase C1s is a 77 kDa single chain subcomponent of complement complex C1, encoded by the gene *C1S*. After C1q is linked to antibodies, C1r is activated and then it cleaves C1s into two chains: A (heavy) and B (light), linked by disulfide bonds, yielding the active C1s. (**Figure 8**) Activated C1s in turn cleaves C2 and C4. (Mortensen et al, 2017)

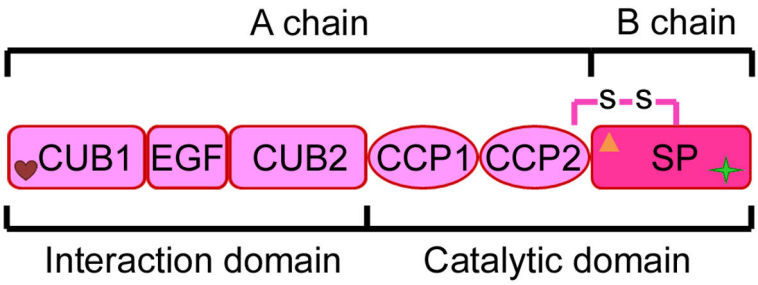

Figure 8. Schematic overview of C1s structure. C1s consists of two N-terminal CUB domains [CUB1-2; consists of the proteins: human complement proteinase C1s, embryonic sea urchin protein Uegf, and human BMP-1 (bone morphogenetic protein-1)] separated by an epidermal growth factor (EGF)-like domain. C1s also has two complement control modules (CCP1-2) and a C-terminal serine proteinase (SP) domain. A heart marks the C1q binding site, a triangle the activation site, and a star the active site. The A (heavy) chain is CUB1-EGF-CUB2-CCP1-CCP2 and B (light) chain is SP. The A and B chains are connected by a disulfide bond (S-S). The interaction domain of C1r and C1s is CUB1-EGF-CUB2 and the catalytic domain is CCP1-CCP2-SP. C1s has the same domain structure as C1r except for the missing C1q binding site in the CUB2 domain.

C1r and C1s are connected to each other in a calcium-dependent straightforward tetramer complex with the order C1s-C1r-C1r-C1s. (**Figure 9a**) (Arlaud et al, 2002) The tetramer folds upon itself in a figure-eight conformation when bound in the C1 complex. This figure-eight complex settles down between the C1q stalks, which are kept in position by ionic bonds in the middle of acidic residues in C1r or C1s. (**Figure 9b**) (Arlaud et al, 2002) C1r and C1s are homologous serine proteinases in their function and domain structure. Human *C1S* and *C1R* genes are located on chromosome 12p13. (Tosi et al, 1989)

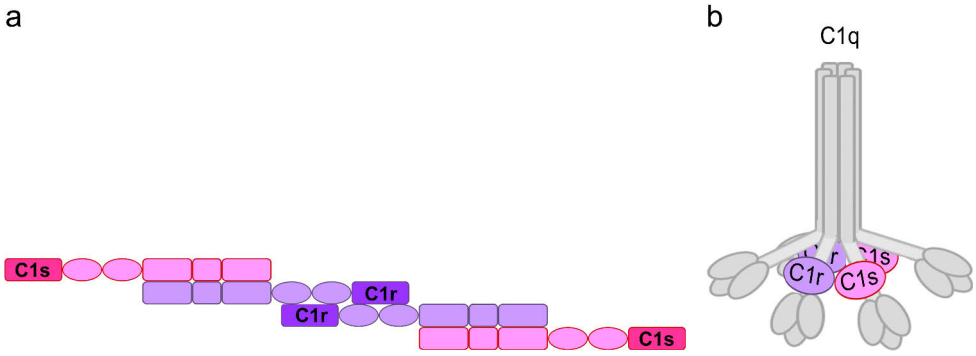

Figure 9. Model of the C1 complex. (a) C1s-C1r-C1r-C1s tetramer. Serine proteinases C1r and C1s are connected through a catalytic domain and form a C1s-C1r-C1r-C1s tetramer. (b) The C1 complex consists of C1q and two copies each of the C1r and C1s serine proteinases connected non-covalently in a calcium-dependent complex. The $C1r_2C1s_2$ complex forms a figure-of-eight conformation and links via the globular heads of C1q.

2.2.2.4 Regulation of the classical pathway

Soluble and membrane-bound regulators strictly control CS to prevent harmful destruction of surrounding tissue or to maintain homeostasis (Kojima et al, 1993). There are some soluble inhibitors of the CS, which are present in human serum. In the classical pathway the most important of those inhibitors are C1-inhibitor (C1-INH) and serine proteinase FI. (Afshar-Kharghan, 2017)

In the classical pathway, C1-INH, a soluble inhibitor, binds to C1r and C1s to prevent C4 cleavage. C3 convertase of the classical pathway is inactivated by C4b-binding protein (C4bp), which acts as a cofactor for FI. Together FI and C4bp inactivate C4b and so prevent new C3 convertase formation. (Kojima et al, 1993) C1-INH consists of two domains: a C-terminal and an N-terminal. The C-terminal domain clearly has characteristics of a serine proteinase inhibitor (serpin). The C-terminal domain is part of C1-INH that provides the inhibitory activity. The N-terminal domain has a special sequence, which has no homology with any other protein. (**Figure 10**) (Bock et al, 1986; Drouet et al, 2018)

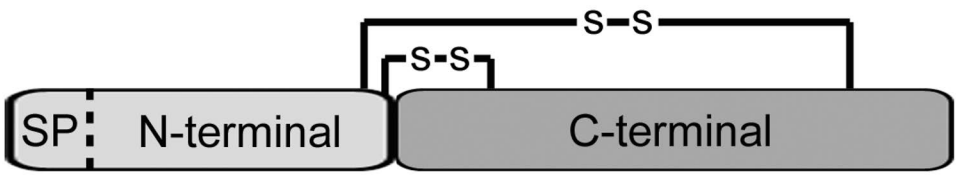

Figure 10. The schematic structure of C1-inhibitor (C1-INH). C1-INH consists of an N-terminal domain and C-terminal serpin domain. The signal peptide (SP) has 22 residues. The N-terminal and serpin domain are connected by two disulphide bonds (S-S).

FI inhibits activation of all three pathways of CS by cleaving the C4b and C3b components of the C3 and C5 convertases. To function properly FI needs a cofactor, e.g. C4bp, the complement receptor 1 (CR1), complement factor H (FH), or the membrane cofactor protein (MCP). (Afshar-Kharghan, 2017)

FI is an 88 kDa proteinase that consists of two polypeptide chains connected covalently with a disulfide bond: a 50 kDa heavy and 38 kDa light chain. (**Figure 11**) FI is produced mainly in the liver but also by skin fibroblasts, monocytes, myoblasts, endothelial cells, and keratinocytes. FI has no endogenous inhibitors and it has very limited substrate specificity. (Afshar-Kharghan, 2017)

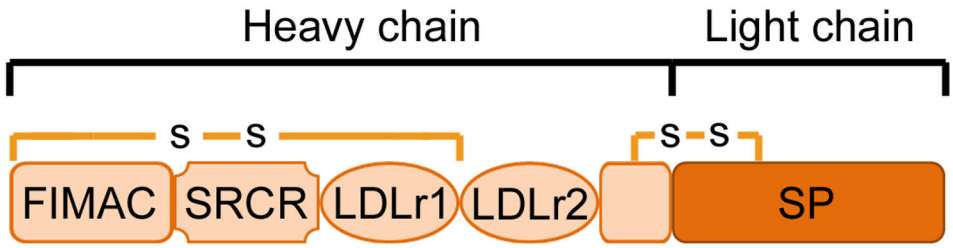

Figure 11. The schematic structure of complement factor I (FI). FI consists of the following domains: the membrane attack complex domain of FI (FIMAC), scavenger receptor cysteine-rich protein domain (SRCR), two low-density lipoprotein receptor 1 and 2 (LDLr1 and LDLr2) domains, the small region with unknown homology, and a serine proteinase domain (SP). Also two important interdomain disulfide bridges (S-S) are shown. The heavy chain consists of the first five domains and the light chain consists of the SP domain.

Membrane-bound complement regulators are CR1 (cluster of differentiation 35 (CD35)), MCP (CD46), membrane-bound regulator protectin (CD59), and decay-accelerating factor (DAF/CD55). (Liszewski et al, 2000)

CR1 inhibits C4bC2b and C3bBb complex formation on cell surfaces. The role of CR1 is the proteolytic inactivation of C4b and C3b with FI but it also acts as a decay acceleration of the convertases. CR1 also acts as a receptor for C1q, C4b, C3b and iC3b on the surface of phagocytes. CR1 is found on the surface of

eosinophils, erythrocytes, monocytes, neutrophils, glomerular podocytes, certain T cells, and B lymphocytes. (Fearon, 1979; Medicus et al, 1983; Medof et al, 1982)

Also MCP promotes FI mediated inactivation of C4b and C3b to iC4b and iC3b, respectively. MCP is expressed on most human cell membranes, except for erythrocytes. (Kojima et al, 1993)

DAF is a membrane protein and does not act as a cofactor for FI. DAF inhibits the activation of the C3 complement component by disrupting C2b or Bb from C3 convertase complexes. DAF is on nearly all cells associated with the complement components, e.g. monocytes, erythrocytes, lymphocytes, neutrophils, and platelets. (Fearon, 1979; Medicus et al, 1983; Medof et al, 1982)

2.2.3 Lectin pathway

The lectin pathway is similar to the classical pathway in that its activation also leads to C3 convertase complex formation. (**Figure 4**) Activation of the lectin pathway is initiated upon identification of carbohydrate molecules. MBL binds to mannose and ficolins (homologous to MBL), e.g. N-acetyl-glucosamine, fucose or glucose, on the surface of microorganisms, also known as pattern recognition molecules. (Sorensen et al, 2005) MBL is similar to C1q, as it consists of up to six trimeric subunits, whose pattern looks like a bouquet-like shape (Nehal and Bichakjian, 2018). Binding of MBL or other pattern recognition molecules to sugar moieties leads to activation of MBL-associated serine proteinases: MASP-1, MASP-2 and MASP-3. Only MASP-2 in the MBL-MASP-2 complex can cleave C4 to C4a and C4b, and C2 to C2a and C2b. C4b binds covalently to sugar residues on cell surface glycoproteins and C2b binds to a site on C4b. These form the complex C4bC2b. (Hajela et al, 2002) MASP-1-3 have structural analogy with C1r and C1s and form a complex, which reminds C1 complex (Afshar-Kharghan, 2017). MASP-1 and MASP-3 cleave and activate FD from the alternative pathway (Noris and Remuzzi, 2013). Once the lectin pathway is initiated, MASP-1 cleaves C2 and C3, but not C4 increasing the effectiveness of convertase formation (Garred et al, 2009; Ricklin et al, 2010; Walport, 2001; Wang et al, 2011).

The lectin pathway is also regulated by soluble and membrane-bound regulators like the classical pathway. The most important inhibitor is FI. FI cleaves C4b of the C3 convertase. The activity of FI depends on the cofactors C4bp, FH, CR1, or MCP. The C1-INH binds to MASP-2 and prevents cleavage of C4. C4bp prevents the function of the C4bC2b complex. C4bp functions as a cofactor for FI and together they inactivate C4b and thus prevent new C3 convertase formation. (Kojima et al, 1993)

2.2.4 The alternative pathway

The alternative pathway does not need any specific protein or initiating agent for activation. The alternative pathway is continuously and spontaneously activated at a low level in human plasma because of the breakdown and hydrolysis of fluid phase C3 thioester ($C3(H_2O)$) in serum. As a result, FB (a soluble component) binds to it and forms the complex $C3b(H_2O)$. Soluble serine proteinase FD cleaves FB to Bb and Ba. Fragment Bb, functioning as a serine proteinase, remains connected to C3b and this is the alternative pathway's C3 convertase, $C3b(H_20)Bb$. C3 convertase is fluid phase convertase. The half-life of C3 convertase is about 90 seconds. Properdin (complement factor P) stabilises the C3bBb complex up to 5–10 fold, increasing the accumulation of C3b on the target cell surface, which allows target cell phagocytosis via opsonisation. Fragment Bb, in an original convertase, cleaves more C3 and generates C3 convertases (C3bBb). Fragment Bb can also cleave other certain proteins besides C3, e.g. complement component C5. C3a, soluble cleavage fragment, is an anaphylatoxin, which increases inflammation. C3a promotes phagocyte chemotaxis and the degranulation of mast cells and granulocytes. C3b attaches to complexes C3bBb or C4bC2b (C3 convertases) and forms complexes C3bBbC3b or C4bC2bC3b (C5 convertases). C5 convertase cleaves C5 to C5a and C5b, and the lytic pathway is activated. (Coulthard and Woodruff, 2015; Gros et al, 2008; Kemper et al, 2010; Liszewski et al, 1996; Noris and Remuzzi, 2013; Ricklin et al, 2010; Sim and Tsiftsoglou, 2004; Xu et al, 2005; Zipfel et al, 2006)

The alternative pathway is regulated by FI. The proper function of FI needs a cofactor, e.g. FH, CR1 or MCP. FI cleaves C3b in the enzyme complex, resulting in inhibition of C3 convertase. (Liszewski et al, 2000) (**Figure 4**)

2.2.5 The lytic pathway

All three CS pathways are differently activated, but all connect at the central component C3 molecule level, which is activated through cleavage of the C3 convertases, and cleaved into C3a and C3b. C3b connects to C3 convertase and forms the C5 convertase: C4bC2bC3b from the classical or lectin pathways, and C3bBbC3b from the alternative pathway. The lytic pathway consists of complement components C5, C6, C7, C8, and C9. (**Figure 4**) C5 convertase proteolytically activates C5 and cleaves C5 into the biologically-active fragments C5a and C5b, activating the lytic pathway. As a result of its histamine-releasing ability, C5a is an anaphylatoxin and strong proinflammatory factor. C5b is a larger proteolytic fragment and launches the formation of MAC. At first C5b binds C6 and C7 to form the complex C5bC6C7, which instantly attaches to the cell surface. The C5bC6C7 complex binds C8 and approximately sixteen C9 molecules and

forms MAC (C5b-9). MAC, on the cell surface, leads eventually to cell lysis. (Afshar-Kharghan, 2017; Coulthard and Woodruff, 2015; Ricklin et al, 2010)

The lytic pathway is regulated by CD59 and the soluble regulators vitronectin (S-protein) and clusterin (SP40), which prevent MAC formation on the cell membrane by interrupting gathering of the C5b-9 complex. This removes MAC from the cell surface and prevents cytolysis (Meri et al, 1990). CD59 binds C8 and C9 and thus prevents the polymerisation of C9 into cell membrane lipid bilayers (Ricklin et al, 2010).

2.2.6 Complement system in skin

Skin is the largest organ in the human body. The major function of the skin is to protect the body from environmental risks. The skin consists of three layers, the epidermis, the dermis, and the hypodermis. The epidermis is a physical barrier and consists of 90-95% keratinocytes, and some Langerhans cells, Merkel cells, melanocytes, and resident memory CD8+ T cells. The dermis supplies the epidermis with defence and nutrition, and has a heterogeneous population of cells. Innate lymphoid cells of the dermis secrete molecules that work as innate immunity soluble factors. In the skin both adaptive and innate immune responses can be provoked. Some soluble factors included in innate immunity are CS, inflammasomes, pattern recognition receptors, cytokines, and antimicrobial peptides. Epidermal keratinocytes are capable of producing the components, inhibitors and receptors of the CS, e.g. C3, C4, FI, FH, FB, CR1, cC1qR (cell-surface calreticulin receptor for C1q), CR2 (complement receptor type 2), C5aR1 (complement C5a Receptor 1), DAF, MCP, and CD59. These components, receptors and inhibitors have a role in the host defense against microbial pathogens in skin. (Dovezenski et al, 1992; Timár et al, 2006; 2007; Terui et al, 1997)

2.2.7 Complement system in cancer

The growth and spread of cancer is strongly affected by the tumor microenvironment, which is a complicated system, consisting of e.g. inflammatory cells, tumor cells, activated fibroblasts, capillary cells, and extracellular matrix, which regulates the progression of tumor and antitumor responses. Chronic inflammation is usually found in the tumor microenvironment and increases cancer promotion, invasion, malignant transformation, and metastasis. Complement components are also found in the cancer microenvironment and CS has an important role in the progression of cancer. CS may support local chronic inflammation and destroy antibody-coated tumor cells (phagocytes destroy antibody-coated tumor cells after CS has marked those for phagocytosis), or inhibit

antitumor T cell responses, promoting the progression of cancer. These opposite effects are dependent on the structure of the tumor microenvironment, the location of complement activation, and the tumor cell sensitivity to complement attack. (Balkwill and Mantovani, 2012; Fridman et al, 2017; Grivennikov et al, 2010; Lubbers et al, 2017; Lujambio et al, 2013; Mantovani et al, 2008; Rutkowski et al, 2010a; Trinchieri, 2012) Studies over the past 10 years have shown that malignant and infiltrating tumor cells have the ability to produce a huge spectrum of complement components *in situ* (Reis et al, 2018; Roumenina et al, 2019a).

The expression of 50 complement-related genes has been found in solid tumours in 30 cancer types. There is strong heterogeneity in gene expression, but almost no heterogeneity between cancer types (Roumenina et al, 2019b). The gene encoding C3 and genes of components of the classical pathway (*C1QA, C1QB, C1QC, C1R, C1S, C2* and *C4A*) are very strongly expressed in all cancer types. Complement components from the alternative pathway (FB and FD) with high local expression of C3 have a high expression in studied tumors, except in uveal melanoma, kidney chromophobe and prostate adenocarcinoma. Interestingly, genes encoding components of the lectin pathway have low expression in the majority of cancers. (Roumenina et al, 2019b)

CS activation plays a role in chronic inflammation with skin cancers frequently appearing in close proximity to chronic inflammation. Cancer cells try to resist from the attack of the immune system to survive by taking advantage of complement inhibitors. The regulators and inhibitors of CS are strongly expressed in the majority of cancer types. Some complement components have extra indirect functions besides the activation and inhibition of the CS, which may have a role in tumor progression, e.g. serine proteinases C1r, C1s, C2, C4, MASP-1, MASP-2, MASP-1, FB, FD, FI, and C5. (Uhlen et al, 2017; Sim and Laich, 2000) C3 is upregulated in different cancers and is necessary for cancer growth and metastases. Some human cancer cells are able to produce C3. (Boire et al, 2017)

3 Aims

At present, there are no molecular markers for predicting which cSCC lesions are aggressive or metastasize rapidly. The main aim in this thesis project was to elucidate the role of the classical pathway of CS in cSCC and to find new molecular markers for the progression of cSCC. In addition, our aim is to identify possible new therapeutic targets for treatment of metastatic cSCC.

The specific aims are:

1. To investigate the expression of C1q and serine proteinases C1r and C1s in cSCC. (I-III)
2. To investigate the molecular mechanisms how C1r affects the progression and metastasis of cSCC. (I-II)
3. To investigate the molecular mechanisms how C1s affects the progression and metastasis of cSCC. (I)

4 Materials and Methods

4.1 Ethical issues (I-III)

The collection of normal skin and cSCC tissues and the use of archival tissue specimens was approved by the Ethics Committee of the Hospital District of Southwest Finland (study number 187/2006). The research was performed according to the Declaration of Helsinki. All studied patients gave informed and written consent before operation, and the research was carried out with the authorisation of Turku University Hospital (Turku, Finland). Registry research approval for the use of clinicopathological data was provided by the Scientific Steering Committee of Auria Biobank (study number AB15-9721) and the Clinical Research Centre of Turku University Hospital (study number T80/2018). All experiments with mice were performed with the approval of the State Provincial Office of Southern Finland, according to institutional guidelines. All tests with mice were performed according to guidelines of institutional animal care and with the permission of the animal test review board of the University of Turku, Finland (study number ESAVI/4623/04.10.07/2017).

4.2 Antibodies (I-III)

Primary antibodies used in the original publications (I-III) are listed in **Table 1**, and the use of those antibodies is described in detail in the original publications.

Table 1. Primary antibodies used in the original publications. WB, western blot; IHC, immunohistochemistry.

Publication	Antigen	Catalog no.	Supplier	Method
I	C1r (H-126)	sc-99195	Santa Cruz Biotechnology, Santa Cruz, CA, USA	IHC
I	C1s (H-69)	sc99196	Santa Cruz Biotechnology, Santa Cruz, CA, USA	IHC
I	MMP-9	AB6001	Millipore, Germany	WB
I	C1r	sc-99195	Santa Cruz Biotechnology, Santa Cruz, CA, USA	WB
I-II	C1r	HPA001551	Sigma-Aldrich, St. Louis, MO, USA	WB
I-II	C1s	HPA018852	Sigma-Aldrich, St. Louis, MO, USA	WB
I-II	Ki-67		Dako Denmark A/S, Denmark	IHC
I-II	CD34	sc-18917	Santa Cruz Biotechnology, Santa Cruz, CA, USA	IHC
I-II	Active caspase-3	#9661S	Cell Signaling Technology Inc., MA, USA	IHC
I-II	p-Akt	9271S	Cell Signaling Technology, Beverly, MA, USA	WB
I-II	Akt	sc-1618	Santa Cruz Biotechnology, Santa Cruz, CA, USA	WB
I-II	p-ERK1/2	9101	Cell Signaling Technology, Beverly, MA, USA	WB
I-II	ERK1/2	9102	Cell Signaling Technology, Beverly, MA, USA	WB
I-II	β-actin	A1978	Sigma-Aldrich, St. Louis, MO, USA	WB
II	MMP-1	MAB3307	Merck Millipore, Temecula, CA, USA	WB
II	MMP-10	MA5-14233	Thermo Fisher Scientific, Waltham, MA, USA	WB
II	MMP-12	MAB919	R&D systems, Minneapolis, MN, USA	WB
II	MMP-13	MAB3321	Merck Millipore, Temecula, CA, USA	WB
II	MMP-13	MAB13424	Merck Millipore, Germany	IHC
II	TIMP-1	MAB3300	Merck Millipore, Temecula, CA, USA	WB
III	C1q	A0136	Dako Denmark A/S, Denmark	IHC
III	CD68	PG-M1	Dako, CA, USA	IHC
III	PDGFRβ	CST3169	Cell Signaling Technology, Danvers, MA, USA	IHC

4.3 Cells and tumor samples

4.3.1 Normal human epidermal keratinocytes (NHEKs) (I)

Primary normal human epidermal keratinocytes (NHEK-PC) were acquired from PromoCell (Heidelberg, Germany). NHEKs were established from normal skin of healthy patients (n = 10) from the upper arm who had obtained mammoplasty at Turku University Hospital (Turku, Finland). NHEKs were cultivated in keratinocyte growth medium-2 with calcium chloride and supplement mix (PromoCell), penicillin–streptomycin mixture and L-glutamine (Gibco, Paisley, Scotland, UK) (Junttila et al, 2007).

4.3.2 Human cSCC cell lines (I-III)

Human cSCC cell lines (n = 8) were established from surgically removed cSCCs. Five cSCC cell lines were from primary cSCCs (UT-SCC-12A, UT-SCC-91, UT-SCC-105, UT-SCC-111 and UT-SCC-118). Three cSCC cell lines were derived from metastatic cSCCs (UT-SCC-7, UT-SCC-59A and UT-SCC-115). These eight cell lines were authenticated by short tandem repeat DNA profiling (DDC Medical,

Fairfield, OH) (Farshchian et al, 2017). cSCC cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Flow Laboratories, Irvine, UK), supplemented with 100 IU penicillin G, 100 µg/ml streptomycin, 10% FCS, 2 mM L-glutamine and Non-Essential Amino Acid (NEAA) (Riihilä et al, 2014).

4.3.3 Tissue samples (I, III)

Tissue samples from normal skin (n = 10) were collected during mammoplasty operations and from the upper arm of healthy volunteers. cSCC tumor tissue samples were collected from surgically removed tumors at the Turku University Hospital (Farshcian et al, 2011). Total RNA was isolated from the tissue samples using RNeasy mini kit (Qiagen, Germantown, MD) and subjected to qRT-PCR analysis (Riddick et al, 2005).

4.3.4 Tissue microarrays (TMAs) (I-III)

Tissue microarrays (TMAs) consisting of formalin fixed and paraffin-embedded samples, together totalling 509, were generated in the Department of Pathology, Turku University Hospital (Riihilä et al, 2015; Farshchian et al, 2015). TMAs consisted of normal human sun-protected skin samples (n = 92), AK (n = 40), cSCCIS (n = 45), UV-induced cSCC (n = 228), RDEBSCC (n = 8), and seborrheic keratosis (n = 17).

4.4 RNA detection

4.4.1 Quantitative real-time PCR (I-III)

RNeasy Mini Kit (Qiagen) was used to extract total RNA from cultured cells using the manufacturer's instructions. cDNA was synthetised from total RNA. To design specific probes and primers, the RealTimeDesign Software (https://www.biosearchtech.com) was used. The mRNA levels of *C1QA, C1QB, C1QC* variant 1*, C1QC* variant 2, *C1R, C1S,* matrix metalloproteinase-1 (*MMP1*), *MMP10, MMP12, MMP13*, and β-actin were analysed by qRT-PCR. **Table 2** lists all primers and probes used in the original publications. All experiments were performed twice and the range of the threshold cycle values was less than 5% of the mean in each measurement. QuantStudio 12K Flex (Thermo Fisher Scientific) system was used for all qRT-PCR reactions at the Finnish Functional Genomics Centre (FFGC) in Turku, Finland. mRNA levels of *C1QA, C1QB, C1QC* variant 1 and *C1QC* variant 2, *C1R, C1S*, and MMPs were corrected against levels of β-actin mRNA. To analyse the results, the standard curve method was used. (Larionov et al, 2005)

Table 2. Primer and probe sequences used in the original publications in qRT-PCR.

Gene	Primer/Probe	Sequence
Human *C1QA*	Forward Primer	5'-TCG ACA CGG TCA TCA CCA A-3'
	Reverse Primer	5'-CGG GTA CAG TGC AGA CGA ATC-3'
	Probe	5'-CCA GGA AGA ACC GTA CCA GAA CCA C-3'
Human *C1QB*	Forward Primer	5'-AAG GTG CCC GGT CTC TAC TA-3'
	Reverse Primer	5'-GCC ACG CAT GAG GTT CAC-3'
	Probe	5'-TTC ACC TAC CAC GCC AGC TCT C-3'
Human *C1QC*	Forward Primer	5'-CAC GTC CAT CCC GGA GAA G-3'
trancscript variant 1	Reverse Primer	5'-CGG AGA AGG AAC TGG GCA GG-3'
	Probe	5'-AAG CAG ATC TGA GGA CAT CTC TGT GCC-3'
Human *C1QC*	Forward Primer	5'-CAC GTC CAT CCC GGA GAA G-3'
trancscript variant 2	Reverse Primer	5'-ATC CCG GAG AAG GAA CTG CA-3'
	Probe	5'-AAG CAG ATC TGA GGA CAT CTC TGT GCC-3'
Human *C1R*	Forward Primer	5'-GCT GCC CAC ACC CTG TAT C-3'
	Reverse Primer	5'-GCC CAG GAA CAC ATC CAA AGA G-3'
	Probe	5´-CAA GGA ACA CGA AGC GCA AAG CA-3'
Human *C1S*	Forward Primer	5'-CTC CTG AGC ATG TGT TTA-3'
	Reverse Primer	5'-CAG TGC AAT GTC ATT ATC AA-3'
	Probe	5'-TCA TCC GGG ATG GAA GCT GCT-3'
Human *ACTB*	Forward Primer	5'-TCA CCC ACA CTG TGC CCA TCT ACG C-3'
	Reverse Primer	5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3'
	Probe	5'-ATG CCC TCC CCC ATG CCA TCC TGC GT-3'
Human *MMP1*	Forward Primer	5'-AAG ATG AAA CGT GGA CCA ACA ATT-3'
	Reverse Primer	5'-CCA AGA GAA TGG AAG AGT TC-3'
	Probe	5'-CAG AGA GTA CAA CTT ACA TCG TGT TGC GGC TC-3'
Human *MMP10*	Forward Primer	5'-GGA CCT GGG CTT TAT GGA GAT AT-3'
	Reverse Primer	5'-CCC AGG GAG TGG CCA AGT-3'
	Probe	5'-CAT CAG GCA CCA ATT TAT TCC TCG TTG CT-3'
Human *MMP12*	Forward Primer	5′-CGC CTC TCT GCT GAT GAC ATA C-3'
	Reverse Primer	5′-GGT AGT GAC AGC ATC AAA ACT CAA A-3′
	Probe	5′-TCC CTG TAT GGA GAC CCA AAA GAG AAC CA-3′
Human *MMP13*	Forward Primer	5'-AAA TTA TGG AGG AGA TGC CCA TT-3'
	Reverse Primer	5'-TCC TTG GAG TGG TCA AGA CCT AA-3'
	Probe	5'-CTA CAA CTT GTT TCT TGT TGC TGC TGC GCA TGA-3'

4.4.2 Nanostring (II-III)

RNA (100 ng) was hybridised at 65°C overnight with the Human Fibrosis Panel (NanoString Technologies, Seattle, WA). The nCounter Prep Station was utilised for purification and binding of the hybridised probes to the cartridge, followed by scanning of the cartridge on the nCounter Digital Analyzer (Nanostring Technologies). nSolver 4.0 (NanoString Technologies) was used to analyse the data. Default quality control settings were used for the normalisation and conformation for quality of the data.

4.4.3 RNA sequencing and bioinformatics analyses (I-II)

miRNAeasy Mini kit (Qiagen, Chatsworth, CA) was used for isolating RNA for mRNA sequencing from C1r, C1s and control small interfering RNA (siRNA) (120 nM) transfected human cSCC cell lines (UT-SCC-12A, UT-SCC-59A, and UT-SCC-91). Illumina TruSeq Stranded mRNA Sample Preparation Kit was used for preparing the sequencing library and Illumina HiSeq3000 (Illumina, San Diego, CA) was used for performing sequencing at the Finnish Functional Genomics Centre, Turku. The reads were aligned against the human reference genome (hg38) and data normalisation used TMM normalisation (R/Bioconductor package edgeR). The Limma package was used for statistical analysis. RNA seq data is available online at the public database Gene Expression Omnibus (GEO; accession number GSE174626 for C1r knockdown samples, and GSE121017 for C1s knockdown samples; NCBI; http://www.ncbi.nlm.nih.gov/geo/).

Morpheus software was used to generate heatmaps (https://software.broadinstitute.org/morpheus). Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA; FC log2>0.5, P<0.05), the Reactome Pathway Knowledgebase (https://reactome.org/), Kyoto Encyclopedia of Genes and Genomes Pathway Analysis (http://www.genome.jp/kegg/), and the gene ontology (GO) Enrichment Analysis (http://geneontology.org/) were used for RNA-seq data bioinformatics analyzes.

4.5 Western blot analysis (I-II)

Western blotting analysis was used to determine the production of C1r and C1s by NHEKs and cSCC cells from aliquots of conditioned media under non-reducing conditions using specific polyclonal rabbit anti-C1r and anti-C1s antibodies. Same anti-C1s antibody was used for detection of the cleaved form of C1s under reducing conditions. (**Table 1**)

Cell lysates were analysed with specific antibodies for phosphorylated Akt (p-Akt), phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2 and total Akt. Equal protein loading was confirmed with β-actin antibody. (**Table 1**)

Production of MMPs (MMP-1, MMP-9, MMP-10, MMP-12 and MMP-13) was determined by western blotting analysis from aliquots of conditioned media under reducing conditions using the specific antibodies listed in **Table 1**. Equal protein loading was confirmed with TIMP-1 antibody. The LI-COR Odyssey® CLx imaging system was used for the quantification of protein expression with Infrared Dye labeled secondary antibodies (LI-COR Biosciences, Lincoln, NE).

4.6 Immunohistochemistry (IHC) (I-III)

The human TMA sections were stained with rabbit polyclonal anti-C1r or anti-C1s antibodies (**Table 1**). Adjacent human TMA sections were stained with rabbit polyclonal anti-C1q antibody. Anti-CD68 antibody was used for staining macrophages. Rabbit anti-platelet-derived growth factor receptor-β (PDGFRβ) antibody was used for staining platelet-derived growth factor receptor β. (**Table 1**) All tissue samples where stained at the same time. For negative controls, the primary antibody was replaced with PBS. C1r, C1s and C1q immunostainings were scored as negative (-), weak (+), moderate (++), or strong (+++) based on cytoplasmic staining intensity. As a strong positive control (+++) liver tissue was used. Panoramic 1000 Slide Scanner (3DHistech, Budapest, Hungary) was used to digitally scan slides of human TMA samples.

4.7 Functional assays

4.7.1 Cell transfections

4.7.1.1 Gene knockdown using small interfering RNAs (I-II)

cSCC cells were grown to 50% confluence and transfected with commercially available siRNAs targetting C1r, C1s, or a negative control siRNA (**Table 3**) in serum-free medium using siLentFect™ Lipid Reagent (Bio-Rad Laboratories, Hercules, CA, USA). Transfection of the cells was done according to the guidelines of the manufacturer (Invitrogen Corporation, Carlsbad, CA, USA). Western blotting was used to confirm the function of C1r and C1s siRNAs. β-actin and TIMP-1 was used as a loading control and ImageJ software version 1.47v (NIM, Bethesda, MD; http://imagej.nih.gov/ij) was used for quantification of western blot band intensities (Schneider et al, 2012).

Table 3. Commercially available siRNAs and CRISPR/Cas9 guide sequences used in original publications.

Publication	Target	Concentration	Catalog no.	Sequence	Supplier
I-II	Hs_C1r_5	120 nM	SI02777068	5´-TCGGGAGAGCCCAGGATTCAA-3´	Qiagen, New Delhi, India
I-II	Hs_C1r_7	75 nM	SI03071586	5´-CAGGGTGAAGCTCGTCTTCCA-3´	Qiagen, New Delhi, India
I-II	Hs_C1r_11	75 nM	SI05075623	5´-CCAGTTGTTGATSSCCACTAA-3´	Qiagen, New Delhi, India
I	Hs_C1s_1	120 nM	SI00027356	5´-GAGGTAGAGTTTGATCATAGA-3´	Qiagen, New Delhi, India
I	Hs_C1s_2	120 nM	SI00027363	5´-AGGATCCGATGCAGATATTAA-3´	Qiagen, New Delhi, India
I	Hs_C1s_5	120 nM	SI03022873	5´-TTCGCTATCATGGAGATCCAA-3´	Qiagen, New Delhi, India
I-II	negative control siRNA	75 nM and 120 nM	SI03650318	proprietary	Qiagen, New Delhi, India
II	CRISPR/Cas9 target: C1r		HS0000245574	GCTTCACCCTGTATCCCGTGGG	Sigma-Aldrich, St. Louis, MO, USA
II	CRISPR/Cas9 target: C1r		HS0000245577	ACTTCTCCAACGAGGAGAATGG	Sigma-Aldrich, St. Louis, MO, USA
II	CRISPR/Cas9 target: C1s		HS0000245615	CCTACCATGTATGGGGAGATCC	Sigma-Aldrich, St. Louis, MO, USA
II	CRISPR/Cas9 target: C1s		HS0000245618	CAATGAAGAGCGTTTTACGGGG	Sigma-Aldrich, St. Louis, MO, USA

4.7.1.2 Gene knockout using CRISPR/Cas9 technology (II)

A genome editing method was used to generate C1r or C1s negative cell lines by transfecting UT-SCC-7 cells with all-in-one CRISPR/Cas9 vector (**Table 3**). Based on the expression of GFP transfected cells were chosen, allowed to grow and examined for C1r and C1s production in cell culture medium. To generate C1r knockout (C1r KO) cell pool four C1r knockout cSCC single cell clones (C1r CRISPR 74_1, 74_2, 74_3, and 77_3) were pooled.

4.7.1.3 Overexpression of C1r (II)

The C1r sequence was acquired from NCBI. C1r cDNA fragment in pEX-A128 was obtained from Eurofins Genomics (Ebersberg, Germany) and cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) including a neomycin resistance gene. The construct was resequenced to confirm integrity of the cloned C1r segment. Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) was used to perform stable transfections of UT-SCC-7 cells with the recombinant C1r expression construct (pcDNA3.1_C1r) or control vector (pcDNA3.1) according to instructions. Cells were grown in the presence of 0.5 mg/ml geneticin (Gibco). cSCC cells were transfected with C1r siRNAs or control siRNA in rescue experiments. Conditioned media with C1r overexpressing (C1r_pcDNA3.1) or vector control (pcDNA3.1) cells was added after 48 hours and incubation continued for 24 hours.

4.7.2 Analysis of cell viability and proliferation (I-II)

cSCC cell cultures and NHEKs were transfected with commercially available C1r siRNAs, C1s siRNAs or the negative control siRNA. UT-SCC-7 and -59A cells (5.0 x 10^3 cells/well), UT-SCC-12A cells (3.5 x 10^3 cells/well), and UT-SCC-91, -105 and -111 cells and NHEKs (1.0 x 10^4 cells/well) were seeded into 96-well plates 24 hours after transfection. UT-SCC-7 C1r KO and wild type (WT) cells (5.0 x 10^3 cells/well) were also seeded into 96-well plates. NHEKs were transfected with C1r, C1s or control siRNAs.

To study the growth of cSCC cells the IncuCyte ZOOM real-time cell imaging system was used (Essen Bio-Science, Ann Arbor, MI). The IncuCyte ZOOM system took images every 2 h and the relative confluence was analysed by the instrument. The confluency of the NHEK cells was measured at the 100 h time point using IncuCyte ZOOM. Experiments were performed with 8 parallel wells at each time point.

Viability of C1r siRNAs, C1s siRNAs or negative control siRNA treated cSCC cells was determined using WST-1 cell proliferation reagent (Roche Diagnostics, Mannheim, Germany). cSCC cells (1.0 x 10^4 cells/well) from UT-SCC-12A, -7 and -105 cell lines were seeded into 96-well plates 24 hours after transfection. The number of viable cells was measured at 0, 24, 48 and 72 hours using WST reagent. Experiments were performed with 5-8 parallel wells at each time point.

For cell viability assays, C1r KO and WT cSCC cells (1.0 x 10^4 cells/well) were seeded into 96-well plates. The number of viable cells was measured at 0, 24, 48 and 72 hours with Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Experiments were performed with 8 parallel wells at each time point.

4.7.3 Cell migration assays (I-II)

C1r siRNAs, C1s siRNAs or control siRNA were used to transfect cSCC cell cultures, and cells were plated (3.5 x 10^4 cells/well) onto an ImageLock 96-well plate (Essen Bioscience, Ann Arbor, MI). UT-SCC-7 C1r KO and WT cells (3.5 x 10^4 cells/well) were also plated onto an ImageLock 96-well plate. cSCC cells were incubated in complete growth medium for 24 hours to reach confluency. 1 mM hydroxyurea (Sigma Aldrich, St. Louis, MO, USA) treatment in DMEM with 10% FCS (fetal calf serum) was used for inhibition of cell division for 6 hours. IncuCyte ZOOM (Essen Bio-Science, Ann Arbor, MI) wound scratcher was used to scratch the cell monolayer and incubation of the cells was continued in DMEM with 0.5 mM hydroxyurea and 1% FCS. IncuCyte ZOOM system took images every 2 h and the relative wound density was analysed by IncuCyte ZOOM software. Experiments were performed with 8 parallel wells at each time point.

4.7.4 Cell invasion assays (II)

24 hours after transfection with C1r siRNAs or negative control siRNA, cSCC cells (5×10^4 cells/well) were plated on a collagen type I (5 µg/cm^2, PureCol; Advanced BioMatrix, San Diego, CA, USA) or Matrigel (100 µg/mL) (Corning, Corning, NY, USA) coated ImageLock 96-well plate (Essen Bioscience, Ann Arbor, MI). C1r KO and WT cells, and siRNA transfected cells were allowed to adhere overnight. The cell monolayer was scratched using an IncuCyte ZOOM (Essen Bio-Science, Ann Arbor, MI, USA) wound scratcher, and Matrigel (4 mg/ml) (UT-SCC-7 and UT-SCC-59A) or collagen type I (UT-SCC-7 and UT-SCC-91) solution was added by mixing type I collagen (PureCol) with 5×DMEM and 0.2 mol/L HEPES buffer (pH 7.4) at a ratio of 7:2:1, respectively. To obtain a final pH of 7.4, NaOH (1M) was added. After allowing the collagen type I solution (2.2 mg/ml) and Matrigel to polymerise for 2 or 4 hours, respectively, cell culture medium with 0.5% FCS at 37°C was added. MMP-13 inhibitor (444283, Merck, Darmstadt, Germany), in 10 µM concentration, was added to the gel and medium. Incucyte S3 real-time cell imaging or IncuCyte ZOOM system (Essen Bioscience) took images every 2 h and Incucyte S3 or the IncuCyte ZOOM software was used to quantitate relative cell invasion. Experiments were performed with 6-7 parallel wells at each time point.

4.7.5 Cell apoptosis assays (I)

Control siRNA, C1r and C1s siRNAs were used to transfect cSCC cells (UT-SCC-7, -12A, -91, -105, -111 and -115). In Situ Cell Death Detection Kit (Roche) was used to detect apoptotic cells 48 hours after transfection. From 6-14 parallel image fields using 20x objective the number of TUNEL positive cells was counted and compared to total cell numbers visualised by Hoechst 33342 (Invitrogen).

4.8 Human cSCC xenograft model (I-II)

Female severe combined immunodeficiency (SCID/SCID) mice (CB17/Icr-*Prkdc*^{scid}/IcrIcoCrl) (Charles River Laboratorie, Wilmington, MA), 6–8 weeks old, were used in all experiments.

UT-SCC-91 cells were transfected with specific siRNAs and after 72 hours control siRNA (n = 8), C1r siRNA (n = 7) and C1s siRNA (n = 8) transfected cSCC cells (7 x 10^6) were injected subcutaneously into the backs of mice. UT-SCC-7 C1r KO or WT cells (5 x 10^6) were injected subcutaneously into the backs of mice (n = 6 in both groups).

In *in vivo* experiments, tumor size was measured twice a week and tumor volume calculated with the formula V = (length × width^2)/2 (Euhus et al, 1986). Mice were sacrificed 16 or 17 days after tumor implantation and tumors were

harvested, measured and fixed in fresh phosphate-buffered 10% formaldehyde. Fixed tumors were embedded in paraffin and 5 µm thick slices sectioned for histological analysis and IHC (Junttila et al, 2007).

Formalin fixed cSCC xenograft tumor sections embedded in paraffin were stained with Mayer's haematoxylin (Sigma-Aldrich, Steinheim, Germany) and eosin to evaluate tumor histology samples (Junttila et al, 2007). Proliferating cells were identified with monoclonal human Ki-67 antibody (**Table 1**) with Mayer's haematoxylin as the counterstain. The relative number of Ki-67 positive cells was determined using ImageJ Software (Schneider et al, 2012). Anti-CD34 antibody (**Table 1**) was used to assess vascularisation of the xenograft tumors by IHC. Blood vessel density was calculated in each sample by counting the number of CD34-positive blood vessels with a 10x objective in three defined microscopic fields. Active caspase-3 antibody (**Table 1**) was used to determine apoptosis of the xenograft tumor cells by IHC. The relative number of active caspase-3 positive cells was determined using ImageJ Software. Mouse monoclonal MMP-13 antibody (**Table 1**) was used to stain MMP-13. QuPath bioimage analysis software version 0.2.3 was used to evaluate the MMP-13 staining intensity in xenograft tumour tissue sections (Bankhead et al, 2017). Van Gieson (VG; BIO300; 3Helix Inc, Salt Lake City, Utah, USA) and Collagen hybridising peptide-biotin (CHP; BIO300; 3Helix Inc, Salt Lake City, Utah, USA) conjugate (**Table 1**) stainings were used according to manufacturer's instructions to visualise collagen (Hwang et al, 2017). All xenograft tissue samples where stained at the same time. The stainings were prepared by the Core Facilities of the Institute of Biomedicine, University of Turku. Labvision Autostainer (Santa Clara, CA, USA) was used for IHC stainings to standardise staining quality and intensity.

Xenograft tumors were stained with rabbit polyclonal anti-C1r and anti-C1s antibodies (**Table 1**). Negative controls replaced the primary staining antibody with PBS. C1r, C1s and C1q immunostainings were semiquantitatively scored as negative (-), weak (+), moderate (++), or strong (+++) based on cytoplasmic staining intensity. Strong positive control staining (+++) used liver tissue. The Panoramic 1000 Slide Scanner (3DHistech, Budapest, Hungary) digitally scanned slides of xenograft samples.

4.9 Statistical analysis (I-III)

SPSS software (IBM, Armonk, NY, USA) was used to determine the significance of differences between two sample groups. The Two-tailed Mann–Whitney U-test was used for qRT-PCR and staining analyses for active caspase-3, CD34 and Ki-67. To analyse variations in xenograft tumor volume and assays of cell numbers, viability, migration and apoptosis Student's t-test was used. For contrast of intensities of IHC staining the $\chi 2$ –test was used.

5 Results

5.1 Expression of C1q, C1r and C1s in cutaneous squamous cell carcinoma cells and tumors (I, III)

First, the mRNA levels of *C1QA, C1QB, C1QC* variants 1 and 2, *C1R* and *C1S* in NHEKs and cSCC cell lines were confirmed by qRT-PCR. Expression levels of *C1QA, C1QB* and *C1QC* variants 1 and 2 were almost negative in cSCC cell lines (n = 8) and NHEKs (n = 7) (I, Figure S1). The mRNA levels of *C1R* and *C1S* were significantly higher (p-value <0.001 and p-value <0.01, respectively) in cSCC cell lines (n = 8) than in NHEKs (n = 10) (I, Figure 1a).

The expression of complement category genes *in vivo* in RNA samples of cSCC tumors and normal skin was verified using Nanostring Cancer Immune Panel. The *in vivo* expression of *C1QA* and *C1QB* mRNAs was higher in cSCC tumor samples, and downstream of the complement classical pathway, *C2* was upregulated, compared to normal skin (III, Figure 1A). Elevated mRNA levels of *C1R* and *C1S* were also noted in cSCC tumor tissue (n = 6) compared with normal skin tissue samples (n = 10) (I, Figure 1c).The mRNA levels of *C1QA*, *C1QB*, *C1QC* variants 1 and 2 in cSCC tumors (n = 6) and normal skin (n = 7) were determined by qRT-PCR *in vivo*. mRNA levels of *C1Q* subunits were significantly higher (p-value <0.05 for *C1QA*, p-value <0.01 for *C1QB, C1QC* variant 1 and 2) in cSCC tumors than in normal skin (III, Figure 1B).

Western blot analysis showed C1r and C1s proteins in conditioned media of cSCC cell lines but the production of C1r and C1s in NHEKs was very low (I, Figure 1b). At the same time there was no correlation between C1r and C1s protein levels produced by cSCC cells (I, Figure 1b). C1r activity was studied by detecting the cleaved form of C1s (B chain) in conditioned media of cSCC cells under reducing conditions with western blotting (I, Figure 1b).

5.2 Expression of C1q, C1r and C1s in cutaneous squamous cell carcinomas *in vivo* (I, III)

The TMA sections were analysed by IHC to study the expression of C1q, C1r and C1s in cSCC tumor tissue *in vivo*. TMAs contained tissue samples from sporadic

UV-induced cSCC (n = 228), RDEBSCC (n = 8), cSCCIS (n = 45), AK (n = 40), normal skin (n = 92) and seborrheic keratosis (n = 17) (I, III).

Notable tumor-cell-specific cytoplasmic expression of C1r was noted in cSCC (I, Figure 2a–b) and RDEBSCC samples (I, Figure 2c). Intensity of the staining for C1r was stronger in cSCC tissue sections than in normal skin, AK, or cSCCIS tissue samples (I, Figure 2d–f). Semiquantitative analysis revealed significantly stronger cytoplasmic staining of C1r in tissue sections in cSCC and RDEBSCC than in cSCCIS and AK (p-value <0.001 for cSCC and p-value <0.01 for RDEBSCC), or normal skin tissue samples (p-value <0.001) (I, Figure 2g).

Marked expression of C1s was noted also in tumor cells in cSCC tissue samples (I, Figure 3a–b) and in RDEBSCC tissue sections (I, Figure 3c). Staining intensity of C1s was weak in normal skin (I, Figure 3d) and staining for C1s in AK (I, Figure 3e) and cSCCIS (I, Figure 3f) was significantly lower than in cSCC samples. Semiquantitative analysis showed stronger cytoplasmic staining for C1s in the majority of tissue sections in the cSCC and RDEBSCC groups (I, Figure 3g) than in AK and cSCCIS. The expression of C1s was negative or weak in normal skin tissue samples (I, Figure 3g).

Staining of C1q was noted on cell membrane and intercellular space in cSCC tumors and in RDEBSCC cells (III, Figure 2A–D). The intensity of C1q staining was weaker in cSCCIS, AK, normal skin, and seborrheic keratosis tissue samples than in cSCC tissue samples on epidermal or tumor cell membrane and intercellular space (III, Figure 2E–H). Semiquantitative analysis revealed significantly stronger staining of C1q on cell membrane and intercellular space in tissue sections in cSCC than in cSCCIS, AK, normal skin, and seborrheic keratosis tissue samples (III, Figure 2I).

5.3 Co-localisation of C1q with stromal macrophages and activated fibroblasts in cSCC microenvironment (III)

Significant staining of C1q was noted in the microenvironment of human cSCC tumors (III, Figure 2A–D). Semiquantitative analysis confirmed stronger staining of C1q in the tumor microenvironment in cSCC and RDEBSCC tissue sections than in the papillary dermis of cSCCIS, AK, normal skin, and seborrheic keratosis (III, Figure 2J). Since strong positive C1q staining was mainly in the tumour microenvironment it was important to study in which cells C1q was expressed. Specific co-localisation of macrophage marker CD68 (III, Figure 4A) and C1q (III, Figure 4B) was noted in cSCC. Semiquantitative analysis showed that there were significantly more CD68 positive cells in cSCC (99%) and in RDEBSCC (100%) than in normal skin (59%), AK (65%), or cSCCIS (65%) (III, Figure 4C). Also in

AK and cSCCIS samples there were more CD68 positive cells than in normal skin. In cSCC (93%) and RDEBSCC (80%) most of the CD68 positive cells were co-localised to C1q (III, Figure 4D). In cSCCIS only 30%, 30% of AK and 23% of normal skin samples scored more than 50% of CD68 positive cells being co-localised, as most CD68 cells co-localised with C1Q (III, Figure 4D).

Co-localization of a marker for activated fibroblasts, PDGFRβ (III, Figure 3A) and C1q (III, Figure 3B) was also seen in cSCC samples. Semiquantitative analysis showed that there are significantly more PDGFRβ positive cells in cSCC tissue samples (90%) than in normal skin (35%), AK (26%), cSCCIS (26%) and in RDEBSCC (50%) (III, Figure 3C). Semiquantitative analysis revealed that most of the cells of C1q and positive PDGFRβ cells are co-localized in cSCC (63%) and RDEBSCC (67%) (III, Figure 3D) but in cSCCIS (87%), AK (87%) and normal skin (100%) of the samples scored no co-location or only some of the cells were co-localized with C1q (III, Figure 3D).

5.4 Regulation of gene expression profile in cutaneous squamous cell carcinoma by C1r or C1s knockdown (I-II)

5.4.1 Alteration of the gene expression profile by C1r (II)

SiRNA was used to knock down C1r expression in three cSCC cell lines (II, Figure S2). Following this, gene expression profiling was done with mRNA sequencing. The mRNA levels of C1R, MAC-inhibitory protein (CD59) and C1RL (complement C1r subcomponent like) decreased significantly after knockdown of C1r, while mRNA levels for other complement components, e.g. C1s, were unchanged (II, Figure S3). After C1r knockdown the most significantly regulated genes ($p<0.05$, fold change log2 1.0) were associated with GO terms *Metallopeptidase activity, Cell-matrix adhesion, Basement membrane* and *Extracellular matrix component*, and with KEGG pathway *Extracellular matrix-receptor interaction.* (II, Figure 3a). The expression of genes that code several integrins and MMPs was significantly increased in GO term *Metalloendopeptidase activity* and KEGG pathway *Extracellular matrix-receptor interaction* (II, Figure 3b). GO term *Extracellular matrix component* included remarkably downregulated genes, e.g. *COL1A1, COL4A6* and *LAMC2* (II, Figure 3b). The study continued to investigate the expression of MMP genes, which are associated with cSCC invasion in more detail. After C1r knockdown *MMP1, MMP10, MMP12* and *MMP13* were among the most significantly downregulated MMP genes (II, Figure 3c). After C1r knockdown the down-regulation of *MMP1, MMP10, MMP12* and *MMP13* expression was confirmed by qRT-PCR (II, Figure 3d–g, S4a–d).

5.4.2 Alteration of the gene expression profile by C1s (I)

Transfection of siRNA was used to knock down C1s expression in three cSCC cell lines and gene expression profiling was done by mRNA sequencing to gain mechanistic insights into the functional role of C1s. Significant downregulation of metastasis-related Ingenuity Pathway Analysis biofunctions after C1s knockdown was noted (I, Figure 7a). After C1s knockdown, significantly regulated genes were associated with GO terms related to signalling pathways regulating cell viability (*Phosphatidylinositol 3-kinase activity and phosphatidylinositol-4,5-bisphosphate 3-kinase activity*) and cell proliferation (*negative regulation of MAPK cascade and negative regulation of MAP kinase activity*) (I, Figure 7). The expression of several dual-specificity phosphatases was also upregulated in GO term *negative regulation of the MAPK cascade* (I, Figure 8a). Furthermore, the term *Phosphatidylinositol 3-kinase activity* included significantly downregulated genes in GO, including *PIK3IP1*, which codes for phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit β, which positively regulates the PI3K signalling pathway, and *PIK3R3*, which codes for PI3K regulatory subunit 3 (I, Figure 8b). After C1s knockdown analysis of cell lysates revealed that the levels of phosphorylated ERK1/2 and phosphorylated Akt were decreased, which indicates inhibition of the activation of the ERK1/2 and PI3K signalling pathways, respectively (I, Figure 9).

5.5 Functional role of C1r and C1s in cutaneous squamous cell carcinoma progression (I-II)

5.5.1 C1r and C1s regulate proliferation of cutaneous squamous cell carcinoma cells by inducing apoptosis (I-II)

The expression of C1r and C1r was silenced by specific siRNAs to study their functional role (I, Figure 4a–b; Figure S2–4). Significant decreases (p-value 0.001-0.01) in the growth of cSCC cells was noted after knockdown of C1r (I, Figure 4c; Figure S2c–g) or C1s (I, Figure 4d; Figure S2a, 2c–g). 72 h after transfection, a significant reduction (p-value <0.001) in the viability of cSCC cells was confirmed with C1r (I, Figure S4c) or C1s siRNAs (I, Figure S4d). 48 h after transfection with C1r (I, Figure 4e, g; Figure S5) or C1s siRNA (I, Figure 4f–g; Figure S5a–b, d) significant (p-value 0.001-0.05) increased number of TUNEL-positive apoptotic cells was noted.

The function of C1r was further studied in more detail and C1r negative cSCC cells were generated from a metastatic human cSCC cell line (UT-SCC-7) using CRISPR/Cas9 technology. The loss of C1r in four cSCC single cell clones was confirmed by western blotting (II, Figure 1a) and sequencing the PCR product

obtained by amplification of genomic DNA from single cell clones in the corresponding area (II, Figure S1). A significant decrease in viability (II, Figure 1b) and growth (II, Figure 1c) of cSCC cell clones was noted after knockout of C1r. Four C1r knockout cSCC single cell clones (C1r CRISPR 74_1, 74_2, 74_3, and 77_3) were pooled to study the functional role after C1r knockout in more detail. The loss of C1r in C1r KO cells was detected by western blotting (II, Figure 1d). Analysis of cell lysates of C1r KO and WT cSCC cells revealed decreased protein levels of Akt and ERK1/2 in C1r KO cells and this was the reason for decreased levels of phosphorylated Akt and ERK1/2 (II, Figure 1e). Additionally, a significant decrease (p-value <0.001) in the growth of C1r KO cSCC cells was noted compared to WT cSCC cells (II, Figure 1f).

C1s negative cSCC cells were also generated from metastatic human cSCC cell line (UT-SCC-7) using CRISPR/Cas9 technology. The loss of C1s was confirmed in four cSCC single cell clones using western blotting (**Figure 12a**). A significant decrease (p-value <0.001) in viability (**Figure 12b**) and growth (**Figure 12c**) of cSCC cell clones was detected after knockout of C1s.

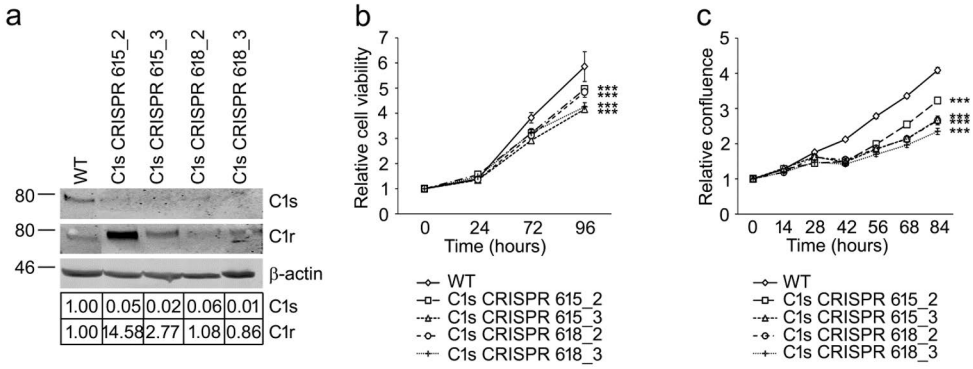

Figure 12. Knockout of C1s suppresses the proliferation of cutaneous squamous cell carcinoma (cSCC) cells. (a) Conditioned media of CRISPR/Cas9-treated UT-SCC-7 single-cell clones and wild type (WT) UT-SCC-7 cells were analysed by western blotting for C1s and C1r levels. β-actin was used as a sample control. (b) The number of viable UT-SCC-7 single-cell clones and WT UT-SCC-7 cells was determined at the time points indicated using WST-1 assay (n = 8). (c) The confluency of UT-SCC-7 single-cell clones and WT cells was determined using IncuCyte ZOOM (n = 8). ***P < 0.001 by Student's t-test.

5.5.2 C1r and C1s regulate migration of cutaneous squamous cell carcinoma cells (I-II)

The migration rate of cSCC cells was studied using 96-well WoundMaker to continue the functional studies and role of C1r and C1s in cSCC cells. At first,

specific siRNAs were used to knockdown C1r or C1s. Then a monolayer culture was scratched. Migration was significantly reduced after knockdown of C1r (I, Figure 5a–b) or C1s (I, Figure 5c–d). The same result was confirmed using CRISPR/Cas9 technology with migration significantly reduced after C1r knockout compared to control cSCC cells (II, Figure 4e).

5.5.3 C1r and C1s promote the growth of the cutaneous squamous cell carcinoma xenograft tumors *in vivo* (I-II)

A xenograft model was used to investigate the role of C1r and C1s on cSCC growth *in vivo*. Human metastatic cSCC cells were transfected with specific C1r, C1s or control siRNA. Transfected cells were incubated for 72 h and then injected subcutaneously into the backs of severe combined immunodeficient (SCID) mice. The same experiment was made with C1r KO and WT cSCC cells. After C1r or C1s knockdown (I, Figure 6a) or C1r KO (II, Figure 2a–b) the growth of cSCC xenograft tumors was decreased (p-value 0.05-0.01) compared with control siRNA tumors or WT tumors. Tumors were harvested 16 or 17 days after implantation. Hematoxylin-eosin (HE) staining of xenografts indicated that the C1r and C1s knockdown tumors contained less tumor tissue compared to control tumors (I, Figure 6b). Xenograft tumors had tumor-cell-specific expression of C1r and C1s (I, Figure 1d). Additionally, the relative number of proliferating Ki-67-positive cells (I, Figure 6b–c, II, Figure 2b–2c) and the number of CD34 positive blood vessels (I, Figure 6b, d; II, Figure 2b, d) were significantly lower in C1r or C1s knockdown or in C1r KO tumors. At the same time the percentage of active caspase-3-positive apoptotic cells was higher in C1r or C1s knockdown or in C1r KO tumors than in control or WT tumors (I, Figure 6b, e; II, Figure 2b, 2e).

5.5.4 C1r regulates invasion of cutaneous squamous cell carcinoma cells and increases the production of MMPs

5.5.4.1 Expression of MMPs in cutaneous squamous cell carcinoma *in vivo* (II)

The expression of MMPs *in vivo* in RNA samples of normal skin and cSCC tumors was verified using a Nanostring Fibrosis Panel. The expression of studied mRNAs for MMPs was higher in cSCCs than in normal skin, except for MMP2 and MMP14. In cSCC the mRNA levels of *MMP1*, *-10*, *-12* and *-13* were upregulated compared to normal skin (II, Figure 4a).

5.5.4.2 C1r regulates production of MMPs by cutaneous squamous cell carcinoma cells (II)

Conditioned media of C1r knockdown (II, Figure 4b) and C1r KO (II, Figure 4c) cSCC cells were studied using western blotting to analyze the production of MMPs. C1r knockdown and C1r KO cSCC cells showed lower production of MMP-1, -10, -12, and -13.

To rescue C1r expression in C1r silenced cells by siRNAs C1r overexpressing cSCC cells were produced. C1r and C1s expression was determined from conditioned culture medium of C1r overexpressing (C1r_pcDNA3.1) or vector control (pcDNA3.1) cSCC cells (II, Figure S6). The production of MMP-13 and MMP-10 increased remarkably after adding conditioned medium from C1r-overexpressing cSCC cells to cultures of cells in which C1r was silenced (II, Figure 4d).

5.5.4.3 C1r affects invasion of cutaneous squamous cell carcinoma cells (II)

A significant decrease of invasion through collagen type I was noted in C1r KO cells compared to WT cSCC cells in culture (II, Figure 4f). Knockdown of C1r also caused significant inhibition of invasion of cSCC cells through collagen type I (II, Figure 4g, Figure S7, S8a) and Matrigel (II, Figure 4h, Figure S8b). The invasion of control siRNA transfected cSCC cells through collagen I (II, Figure 4g, Figure S8a) and Matrigel (II, Figure 4h, Figure S8b) was inhibited by specific MMP-13 inhibitor. Treatment of cSCC cells with specific MMP-13 inhibitor after C1r knockdown had no effect on cell invasion (II, Figure 4g–h).

5.5.4.4 C1r regulates production of MMP-13 *in vivo* (II)

HE and MMP-13 antibody was used to stain C1r KO and WT xenograft tumors. C1r KO tumors had different growth and invasion pattern compared to WT tumors. In 83% of C1r KO xenograft tumor cells were grouped in larger tumor patches and in 67% of WT xenograft tumor cells were scattered into several smaller islets (II, Figure 5a). Positive MMP-13 staining was seen in the invasive edge of xenografts in tumor cells by IHC analysis (II, Figure 5a). In C1r KO xenografts the number of tumor cells, where was strong staining for MMP-13 at the tumor edge, was decreased compared to WT tumors (II, Figure 5a). 33% of WT tumors were classified as strongly stained, 33% as moderate and 33% as weakly stained (II, Figure 5b). 17% of C1r KO tumors were scored as strong, 33% as moderate, and 50% as weakly stained for MMP-13 (II, Figure 5b).

5.5.4.5 C1r affects collagen degradation *in vivo* (II)

Next, the effect of MMP-13 downregulation was investigated to find out the quantity of degraded and total collagen in C1r KO and WT xenografts. VG staining was used to analyse the total amount of collagen in xenografts. C1r KO xenografts had increased numbers of straight, thick, short and coarse collagen bundles at tumor margins between tumour cells (II, Figure 6a). In 83% of C1r knockout tumors and only 17% of WT tumors, the straight, thick, short and coarse collagen bundles surrounded cSCC tumor cells and more than half on tumor edges (II, Figure 6a–b). Xenografts were also stained with CHP, which is known to bind particularly to unfolded triple-helical, degraded collagen. In adjacent sections co-staining of VG and CHP was analysed and found to be weaker in the C1r KO tumor group than in WT tumor group (II, Figure 6c). In C1r KO tumor samples more areas were negative for CHP (67%), even though the staining of VG was positive, which indicates decreased number of degraded collagen. However, 83% of WT tumors showed staining for CHP at the tumor edge connected to VG staining, which indicates triple-helical collagen molecule degradation (II, Figure 6c–d).

6 Discussion

6.1 Molecular markers of cSCC carcinogenesis

Primary cSCC is usually treatable by surgery, but some tumors recur and metastasize with a high risk of mortality. High mortality risk is the reason why early discovery and surgery of cSCC is very important and life-saving for patients. cSCCs can be more aggressive and metastasise when growing on sites of chronic ulcers or infections compared to UV-induced cSCCs. (Tufaro et al, 2011) Usually small lesions of cSCC recur in 8% of cases and large lesions 15% of cases within 5 years. The estimated metastasis rate of primary cSCC is 3-5% and recurrent lesions metastasise in 25-45% of cases. The prognosis of metastatic cSCC is poor, with more than 70% dying of the disease within 3 years. (Alam and Ratner, 2001; Knuutila et al, 2020) Only some specific biomarkers for cSCC have been elucidated – MMP-13 (Airola et al, 1997), MMP-12 (Kerkelä et al, 2000), MMP-7 (Impola et al, 2005; Kivisaari and Kähäri, 2013), E-cadherin (Koseki et al, 1999), signal transducer and activator of transcription 3 (STAT3) (Suiqing et al, 2005), SerpinA1 (Farshchian et al, 2011), complement components of the alternative pathway C3 and FB, and complement inhibitors FH, FI and FD (Rahmati Nezhad et al, 2021; Riihilä et al, 2014; 2015; 2017). In clinical use, however, there are no biological markers to predict the aggressiveness of cSCC and its precancerous forms. In this study, focus was on the complement components of the classical pathway, C1q, C1r, and C1s. Here, the aim was to recognise and characterise new biomarkers and molecular mechanisms for progression of cSCC.

6.2 Expression of C1q, C1r and C1s in normal tissues

The CS consists of more than 50 protein components. These are present in human plasma, cell membranes, and in lower concentrations in other body fluids. Complement components are mainly synthesised in the liver, but other cells and tissues can also produce them, e.g. epidermal keratinocytes. (Bulla et al, 2016; Morgan and Gasque, 1997; Riihilä et al, 2019; Vignesh et al, 2017) C1q is a part of the C1 complex of the classical pathway of the CS. C1q is activated by at least two surface-bound Fc regions of antigen-bound IgGs or IgMs. An antibody-

independent manner can also activate the classical pathway of the CS by binding C1q to other ligands, e.g. CRP, membrane blebs of apoptotic cells, or serum amyloid P component. Activated C1q causes autoactivation of C1r, which then activates C1s. (Deban et al, 2008; Gaboriaud et al, 2004; Kojouharova et al, 2010; Mamidi et al, 2017) Several complement proteins act as serine proteinases, e.g. C1r and C1s. (Forneris et al, 2012; Riihilä et al, 2019). In previous studies, macrophages, immature dendritic cells, many tissue myeloid cells (e.g. mast cells and chondrocytes) and fibroblasts have been shown to express C1q. (Bradley et al, 1996; Ghebrehiwet et al, 2012; van Schaarenburg et al, 2016) It is also known that bone marrow myeloid cells can rescue C1q deficiency (Petry et al, 2001). Other cells in specific organs and tissues, such as glomerular and tubular cells, osteoclasts, microglial cells, and trophoblasts, help to locally produce C1q (Fonseca et al, 2017; Xavier et al, 2017). Recently, it was shown that the local presence of C1q should not automatically be considered as a marker of CS activation (Kouser et al, 2015). Serine proteinases C1r and C1s are produced primarily in liver and macrophages, but also in dendritic cells and monocytes (Lubbers et al, 2017). In this thesis project it has been shown that the expression of C1r and C1s were significantly higher in cSCC cell lines than in NHEKs. There was no expression of C1q in NHEKs.

6.3 C1q, C1r and C1s in cancer

Recently, the role of CS in the progression of cancer has been investigated in more detail. Previously it has been shown that the tumor cell–derived complement components have an autocrine role in the progression of cancer. (Afshar-Kharghan, 2017; Cho et al, 2014; Hajishengallis et al, 2017; Kourtzelis and Rafail, 2016; Nissinen et al, 2016; Reis et al, 2018; Roumenina et al, 2019b) CS is activated in plasma of cancer patients, but also in the microenvironment of tumors. Activation of CS has been considered a host defense mechanism against cancer. (Gorter and Meri, 1999; Pio et al, 2014) It is known that components of CS may also have other functions than being a part of complement pathways. Components of CS can initiate and promote the growth of tumors in areas surrounding chronic inflammation. Chronic inflammation promotes the survival and proliferation of cancer cells, promotes angiogenesis, increases metastasis, and reduces response to chemotherapeutic agents. (Mantovani et al, 2008) In complement expression profiles, different cancer types have an individual role for distinct complement components (Roumenina et al, 2019b). For example, high expression levels of complement component C3 correlates with metastasis rate in primary tumors across the blood–brain barrier (Boire et al, 2017) The gene encoding C3 is present in all cancer types with genes from the classical pathway: *C1QA, C1QB, C1QC,*

C1R, C1S, C4A and *C2* (Hoadley et al, 2018; Roumenina et al, 2019b; Uhlen et al, 2017).

The expression of C1q, C1r and C1s has been detected in clear-cell renal cell carcinoma, mesothelioma, diffuse large B cell lymphoma, glioblastoma, sarcoma, skin cutaneous melanoma, thymoma, lung squamous carcinoma, testicular germ cell tumors, and lung adenocarcinoma. The expression of C1r and C1s has been detected in cholangiocarcinoma, pancreatic adenocarcinoma, cervical squamous carcinoma, invasive breast carcinoma, ovarian serous cystadenocarcinoma, stomach adenocarcinoma, bladder carcinoma, head and neck SCC, prostate adenocarcinoma, adrenocortical carcinoma, and kidney chromophobe. (Hoadley et al, 2018; Roumenina et al, 2019b; Uhlen et al, 2017)

Previous evidence shows that locally produced C1q can act alone without complement activation as a cancer-promoting factor in the tumor microenvironment (Bulla et al, 2016). C1q has an important role in cancer neoangiogenesis via cascade-independent and non-canonical mechanisms. Injury of tumor vessel endothelial cells produces C1q in tumors. (Bulla et al, 2016; Roumenina et al, 2019b) Some studies have shown that C1q can exert antitumoural effects in culture by induction of apoptosis through stimulation of TNF pathway in an ovarian cancer cell line (Kaur et al, 2016). C1q can also activate the tumor suppressor WW (two conserved tryptophans (W) residues) domain-containing oxidoreductase to stimulate apoptosis in prostate and breast cancer cell lines (Bandini et al, 2016; Hong et al, 2009). C1q is often expressed in the microenvironment of human cancers. C1q has been found in colon adenocarcinoma, lung adenocarcinoma, pancreatic adenocarcinoma, breast adenocarcinoma, and melanoma, while the expression of C1s was low. This suggests that the deposition of C1q was not the result of activation of the classical pathway. C1q was mostly expressed by mesenchymal cells, e.g. spindle-shaped fibroblasts, vascular endothelial cells, and by monocytoid cells, which are suggestive of tumor-infiltrating myeloid cells. (Bulla et al, 2016) In this thesis project the expression of C1r and C1s was upregulated in tumor cells in cSCC. C1q was found in the microenvironment in cSCC in macrophages and activated fibroblasts.

6.4 C1q, C1r and C1s in cSCC cells in culture

In this study the expression of C1q, C1r and C1s in cSCC cells was examined. It was noticed that mRNAs for C1q subunits (*C1QA, C1QB, C1QC variant 1* and *2*) are not expressed by cSCC cells in culture. The expression of C1r and C1s were significantly higher in cSCC cell lines than in NHEKs. First, this result was determined by oligonucleotide array–based expression profiling and then confirmed by next–generation–sequencing–based whole transcriptome expression profiling. (Riihilä et al, 2015; 2014) It was determined by western blotting that C1r

and C1s were secreted into the medium of cSCC cells in culture. The expression of C1r and C1s was upregulated in tumor cells in cSCC compared to NHEKs, and C1s was specifically cleaved and activated in the absence of C1q in culture. Previous studies show that the mRNA level of C2, a downstream component of the classical pathway, was increased in cSCC tumors. On the other hand, there was no expression of C2 and C4 in cSCC cells at the mRNA level. (Riihilä et al, 2014; 2015) In this study it was noted that C1s is activated in cSCC cells by western blotting and also C1r is present (I, Figure 1b) but C1q was not expressed by cSCC cells. This suggests that C1r and C1s can promote progression of cSCC tumors independently without activation of the classical pathway of CS. It is possible that C1r and C1s can act without C1q, C4 or C2. C1r is activated autocatalytically without C1q and C1r in turn subsequently activates C1s.

6.5 C1q, C1r and C1s in cSCC cells and cSCC tumor *in vivo*

Tissue material was used to extend the findings *in vivo*. A large panel of tissue sections from cSCC, AK, cSCCIS, and RDEBSCC were collected to generate TMA blocks to examine progression of cSCC. IHC was used to examine the expression of C1q, C1r and C1s in cSCC tumor tissue *in vivo*. It was demonstrated that C1q was not expressed in cSCC cells but is expressed in cSCC tumors, with expression of C1q significantly higher in cSCC tumors compared to normal skin at the RNA level. IHC analysis for C1q staining showed significantly stronger staining in the cSCC tumor microenvironment and stronger staining closer to the tumor edge in the microenvironment. Intensity of the staining was much stronger in cSCC than in normal skin, AK, cSCCIS, or SK stroma. This suggests that C1q expression is mostly derived from the microenvironment of the cSCC tumor. In addition, positive C1q staining was detected in the intercellular space or on cSCC cell membranes in 55% of cSCC samples.

In this study strong tumor-cell-specific expression of C1r and C1s was noted in tumor cells in cSCC tissue samples. The staining intensity for C1r and C1s was significantly stronger in cSCC than in normal skin, AK, or cSCCIS. These results confirm that all components from the C1 complex of the classical pathway are expressed in cSCC tumors or in the microenvironment of cSCC. Also, the expression of C1q, C1r and C1s increases during the progression of cSCC from AK to cSCC. It is possible that macrophages serve C1q to cSCC cells. Then macrophage and fibroblast derived C1q together with cSCC derived C1r and C1s might launch the activation of the classical pathway. It is temting to speculate that the activation of the classical pathway can increase inflammation in the tumor microenvironment and thus stimulate the progression of cSCC. (**Figure 13**)

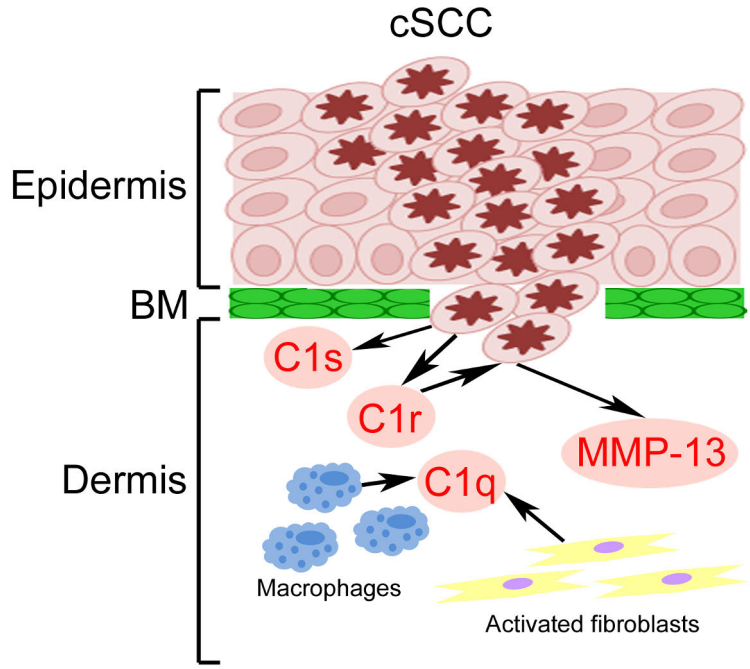

Figure 13. The production of C1q, C1r and C1s in cutaneous squamous cell carcinoma (cSCC) cells and microenvironment. C1r and C1s are produced by cutaneous squamous cell carcinoma (cSCC) cells. C1r increases the production of MMP-13 by cSCC cells and this way promotes degradation of stromal collagen. C1q is mainly produced by macrophages and activated fibroblasts in microenvironment of cSCC. All components from the C1 complex of the classical pathway are present in tumor microenvironment of cSCC. It is possible that C1r and C1s can promote progression of cSCC tumors independently without C1q activation. It is also possible that macrophages and activated fibroblasts serve C1q to cSCC cells and so launch the activation of the classical pathway. BM, basement membrane

6.6 C1q in cSCC tumor microenvironment *in vivo*

The microenvironment of cSCC tumors was further studied to identify the main source of C1q. Previous studies have shown that C1q is produced by macrophages and dendritic cells in the tumor microenvironment (Bulla et al, 2016; Mangogna et al, 2019). In this thesis project the results showed macrophages at tumor cell edges of cSCC located adjacent to tumor cells. The number of CD68 positive macrophages was significantly higher in cSCC tumors than in normal skin, AK and cSCCIS. The number of macrophages increased during the progression of cSCC from normal skin to AK, and from AK to cSCCIS and finally to invasive cSCC, which indicates that macrophages in tumor microenvironments have a role in progression of cSCC. Also, tumor associated macrophages were highly C1q positive in cSCC samples. Marked expression of C1q was seen in macrophages in the peritumoral microenvironment and in intratumoral macrophages in cSCC

tumors. These results show that C1q is mostly derived from macrophages in the microenvironment of cSCC.

In this thesis project the results show that the expression of C1q is derived from the cSCC tumor microenvironment and expression is increased compared to normal skin homeostasis. In the tumor microenvironment several types of cells (e.g. fibroblasts, macrophages, and endothelial cells) can produce C1q subunits *in vivo* (Bossi et al, 2014; Bulla et al, 2016). So, it is possible that C1q from stromal cells allow the formation of the C1 complex in the tumor microenvironment of cSCC, and it is possible that activation of the CS occurs in tumor microenvironment, which also promotes inflammation (Riihilä et al, 2014; 2015). These results suggest that C1q is associated with the progression of cSCC to invasive stage. It has also been shown, that locally produced C1q can be a tumor progression–stimulating factor in a cancer microenvironment independently of complement activation (Bulla et al, 2016). (**Figure 13**)

Activated fibroblast marker PDGFRβ was also used to study for co-localization with C1q. Analysis showed strong co-localization of C1q and PDGFRβ positive fibroblasts compared to normal skin, AK, or cSCCIS. Co-localization was only seen in some cells or was absent in normal skin and precancerous forms of cSCC. These findings confirm that activated PDGFRβ positive fibroblasts are found adjacent to cSCC tumor cells and express C1q. Recent studies have shown the role of the tumor microenvironment in the progression of cancer cells. It has also been shown that functionally active cancer-associated fibroblasts, e.g. PDGFRβ positive fibroblasts, have a major role in the tumor microenvironment by influencing progression, maturation, and metastasis of tumors. (Chen et al, 2021; Kalluri, 2016; Sasaki et al, 2018; Van Hove and Hoste, 2022) The quantity of PDGFRβ-positive stromal tumor associated fibroblasts correlates with poor prognosis of prostate and breast cancers (Frings et al, 2013; Hägglöf et al, 2010; Paulsson et al, 2009).

6.7 Functional roles of C1r and C1s in cSCC

Recently, the role of the CS in cancer has been studied in more detail. Many complement components regulate the growth of tumors. For example, C3a, C5a, and MAC enhance angiogenesis, increase production of tumor-associated growth factors, induce proliferation, prevent apoptosis, and increase the migration and invasion of cancer cells. (Hanahan and Weinberg, 2000; Rutkowski et al, 2010b) Previously it was shown that C1q, C1s, C3, C5, FB, and C9 increase the invasion and migration of cancer. C4 prevents apoptosis and promotes proliferation of tumor cells. (Hanahan and Weinberg, 2000; Rutkowski et al, 2010b)

Previous studies have demonstrated that complement inhibitors FI and FH, and complement components FB, FD, and C3 are significantly upregulated during the

progression of cSCC *in vivo* and have an effect on the proliferation and migration of cSCC cells (Riihilä et al, 2014; 2015; 2017; Rahmati Nezhad et al, 2022). To study the functional role of C1r and C1s siRNAs were used to knock down their expression. Results showed a significant decrease in the growth of cSCC cells, cell viability, proliferation, and migration after silencing the expression of C1r or C1s in cSCC cells in culture. Knockdown of C1r or C1s also promoted apoptosis of cSCC cells. Similar results were noted in xenograft model in SCID mice, where tumor growth and vascularisation was decreased and the number of apoptotic cells was increased *in vivo* after silencing of C1r or C1s in cSCC cells. To specifically study the mechanistic role of C1r in cSCC progression CRISPR/Cas9 technology was used to generate C1r-negative cSCC cells. C1r KO cells showed a significant decrease in proliferation, viability and invasion in culture. Also the growth and vascularisation of xenograft tumors established with C1r KO cSCC cells was suppressed and the number of apoptotic cells was increased. These findings provide new proof for the important roles of C1r and C1s in cSCC tumor growth *in vivo*. (**Figure 14**)

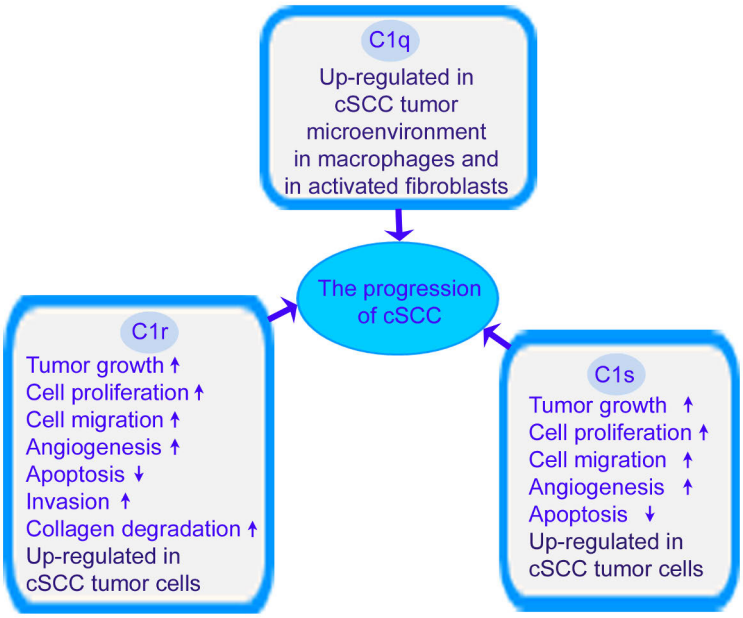

Figure 14. C1q, C1r and C1s in the progression of cutaneous squamous cell carcinoma (cSCC). The viability, proliferation and migration of cSCC cells are increased by C1r and C1s. C1r stimulate the invasion of cSCC cells. Apoptosis of cSCC cells is inhibited by C1r and C1s. C1r and C1s promote the growth of cSCC tumor *in vivo*. Angiogenesis in cSCC tumors is increased by C1r and C1s *in vivo.* C1r promotes collagen degradation of cSCC tumor *in vivo.* C1r and C1s are up-regulated in cSCC tumor cells. C1q is up-regulated in cSCC tumor microenvironment in macrophages and in activated fibroblasts. ↑ indicates stimulation, ↓ indicates inhibition.

In this study the results showed that a decrease in total Akt and ERK1/2 levels resulted in reduced levels of phosphorylated Akt and phosphorylated ERK1/2 in C1r KO cells and after knockdown of C1s. This confirms previous observations that showed that the ERK1/2 cascade components can go through ubiquitination. This causes the degradation of the substrate proteins, which adjust their location and activity. (Laine and Ronai, 2005; Lu et al, 2002; Nguyen et al, 2013)

The molecular mechanisms of the effect of C1r was investigates in more detail. After C1r knockdown, mRNA sequencing was performed on cSCC cells. It was noted that knockdown of C1r regulated the genes, which belong to GO terms, which are associated with invasion of cSCC cells, e.g. genes coding for invasion-associated MMPs – *MMP13, MMP1, MMP10* and *MMP12*. Those MMPs are located in MMP gene cluster locus 11q22.3 (Ujfaludi et al, 2018). Interestingly, STAT3 regulates MMP13, MMP1, MMP10, and MMP12 expression in cSCC cells (Piipponen et al, 2020). It is possible that coordinated upregulation of the production of MMP13, -1, -10, and -12 by cSCC cells promotes invasion of cSCC by generating a strong proteolytic network in the tumor microenvironment (Riihilä et al, 2021). These MMPs also contribute to changes in the structure of basement membranes in premalignant lesions (AK and cSCCIS) and thus promote AK and cSCCIS progression to invasive cSCC (Karppinen et al, 2016).

The molecular mechanisms of the effect of C1s were also studied in more detail. After C1s knockdown with siRNAs mRNA sequencing was made on cSCC cells. Knockdown of C1s caused significant downregulation of genes in biofunctions connected to metastasis in cSCC cells. C1s is a serine proteinase and can activate latent MMP-9 (Sakiyama et al, 1994), which may be an assumed mechanism for C1s to promote the growth, metastasis and angiogenesis of the cancer. Previously it was demonstrated that knockdown of MMP-9 increases the migration and invasion of oral cSCC cells (Väyrynen et al, 2019). After knockdown of C1r or C1s, increased expression of MMP-9 was noted. This recommends that the stimulatory effect of C1r and C1s on cSCC cell migration may include MMP-9.

Knockout of C1r inhibited the invasion of cSCC cells through the collagen type I matrix and also downregulated the production of MMP-13, MMP-1, MMP-10, and MMP-12. Previously it was shown that MMP-13 (collagenase-3) promotes the progression of cSCC (Ala-aho et al, 2004). Fibrillar collagens, basement membrane and many other extracellular matrix components are cleaved by MMP-13. (Knäuper et al, 1996a, 1996b; Nissinen and Kähäri, 2014) The expression of MMP-13 has been noted in stromal fibroblasts and in tumor cells in cSCC, but it is not expressed in NHEKs, AK or cSCCIS (Airola et al, 1997; Impola et al, 2005; Johansson et al, 1997; Kivisaari et al, 2008; Vaalamo et al, 1997). MMP-13 induces the growth and invasion of cSCC tumors *in vivo* and survival of fibroblasts

and cSCC cells. Expression of MMP-13 is also decreased by p53 in cSCC cells. (Ala-aho et al, 2004; 2002; Toriseva et al, 2007) In our study MMP-13 expression by cSCC cells at the invasive edges of cSCC and the number of degraded unfolded triple-helical collagen was reduced in C1r KO xenografts. Tumor cells in WT tumors were widely scattered and in smaller islets, whereas in C1r KO xenografts they were grouped in bigger nests. This shows that C1r has a role in progression cSCC tumors and in the invasion pattern *in vivo*. Those findings prove that knockout of C1r in cSCC tumor cells suppresses the production of MMP-13 and invasion *in vivo*. (**Figure 14**) The effect of C1r on MMP-13 may be direct effect of C1r or it can be mediated by the essential role of C1r in classical pathway activation. This need to be more studied.

Previously it has been shown that the classical pathway of the CS is activated in lung cancer, papillary thyroid carcinoma, and a mouse model of cervical cancer (Ajona et al, 2013; Lucas et al, 1996; Markiewski et al, 2008). The levels of classical pathway components are much higher in follicular and mucosa-associated lymphoid tissue lymphomas and astrocytomas, oral and oropharyngeal SCCs (Ajona et al, 2015; Bu et al, 2007; Mäkelä et al, 2012). The results of this study confirm previous results and provide new evidence for the role of the classical pathway of the CS in cSCC progression.

7 Summary/Conclusions

In this thesis the role and expression of CS components of the classical pathway in the progression of cSCC was studied. The expression of C1r and C1s was up-regulated in cSCC cells compared to NHEKs at the mRNA and protein level. The staining intensity of C1q was stronger in cSCC than its precancerous forms, AK or cSCCIS. IHC analysis revealed that C1q was specifically expressed in stromal activated fibroblasts and in macrophages in tumor microenvironment. These results provide new evidence for the role of fibroblast and macrophage-derived C1q in progression of cSCC to the invasive stage. IHC analysis showed that C1r and C1s were specifically expressed by tumor cells in cSCC *in vivo* and the expression was stronger in cSCC than in AK or cSCCIS.

Knockdown of C1r or C1s was performed with specific siRNAs to study the functional role of C1r and C1s. The knockdown of C1r or C1s inhibited cSCC cell proliferation and migration. KO of C1r was done using CRISPR/Cas9 technology. KO of C1r in cSCC cells caused significantly decreased proliferation, migration, and invasion through collagen type I and Matrigel. Furthermore, C1r KO decreased the expression of invasion proteinases MMP-13, MMP-1, MMP-10, and MMP-12. These findings provide proof for CS independent role of C1r in regulating cSCC cell invasion by increasing the production of invasion-associated MMPs.

For deeper insight into the role of C1r and C1s in tumor growth, cSCC xenograft tumors were generated in SCID mice. The results show that C1r and C1s promote the growth of cSCC xenografts *in vivo* by increasing cell proliferation and vascularisation of tumors. Knockout of C1r also significantly suppressed the growth, MMP-13 production, degradation of collagen and invasion in cSCC xenografts. Knockdown of C1r or C1s and knockout of C1r also decreased the number of proliferating cells in cSCC xenograft tumors.

Altogether, the findings of this study provide new evidence for the roles of C1q, C1r and C1s in cSCC tumor progression. These results suggest cell-derived C1r and C1s, and tumor-microenvironment-derived as putative molecular biomarkers and therapeutic targets for locally advanced and metastatic cSCC. More IHC analysis has to be done for multiple tumor samples and those results need to correlate with clinical parameters.

Acknowledgements

This work was carried out in the Department of Dermatology and Venereology, MediCity Research Laboratory, and the Cancer Research Laboratory of the Western Cancer Centre of the Cancer Center Finland (FICANWest) in the University of Turku and Turku University Hospital, during years 2017-2022. My greatest gratitude goes to the head of the Department of Dermatology and Venereology, Professor Veli-Matti Kähäri for providing very good working facilities and performing high-quality research. I owe gratitude to the Turku Doctoral Programme of Clinical Investigation for conducting a graduate school for allowing to combine the thesis project and clinical work.

I express my deepest gratitude to my supervisor Professor Veli-Matti Kähäri for his invaluable guidance, support and believing in me. I am very lucky to have Docent Liisa Nissinen as my second supervisor and my friend. She is so optimistic and has always time to listen and help. I sincerely thank my third supervisor Pilvi Riihilä for the infinite patience. I am very grateful for your helpful guidance.

I want to thank the members of my doctoral thesis committee, Professor Jyrki Heino and Professor Klaus Elenius for their expert advice and valuable comments to my work.

I sincerely thank Docent Sakari Jokiranta and Professor Kaisa Tasanen-Määttä for reviewing my thesis manuscript and providing valuable feedback.

I express my gratitude to Professor Külli Kingo. I am honoured to have you as my opponent for this dissertation.

I would also like to extend my appreciation to all co-authors: Seppo Meri, Markku Kallajoki, Sirkku Peltonen, Marjaana Ojalill, Mehdi Farshchian, Atte Kivisaari, Juha Peltonen and Jyrki Heino.

I thank personnel of the Histology Core of the Institute of Bioscience, Sinikka Collanus and Erica Nyman for their expert technical assistance. Special thanks to Johanna Markola for your skillful technical assistance in the lab. Johanna, you are irreplaceable!

My warmest thanks go to present and past members of the Skin cancer and proteinase research group including: Johanna Markola, Jaakko Knuutila, Minna Piipponen, Lauri Heiskanen, Maria Salmela and Pegah Rahmati Nezhad. You have

made my days more cheerful and you have inspired me to work harder. I want to warmly thank also the members of the Professor Heino's group for their help and time together during these years. My thanks go also to Emma Rousi for being so positive and friendly.

Doctoral Programme in Clinical Research (DPCR) of the University of Turku, The State Research Funding from the Turku University Hospital, Turku University Foundation, Nordic Dermatology Association, Finnish Cancer Research Foundation, Cancer Foundation of the Southwest Finland, Finnish Cultural Foundation, Finnish Dermatological Society, The Maud Kuistila Memorial Foundation, Ida Montin Foundation, Magnus Ehrnrooth Foundation, K. Albin Johanssons stiftelse sr, The Finnish Medical Foundation, Jane and Aatos Erkko Foundation, and Sigrid Jusélius Foundation are gratefully acknowledged for the financial support of this work.

In addition, I want to thank Eloise Mikkonen for revising the language in my thesis manuscript.

I express my warm thanks to Docent Leena Koulu, Docent Jussi Liippo, and my clinical colleagues at the Department of Dermatology in Turku University Hospital for their support and encouragement during the study.

I am incredibly grateful to Matti Lahti, who has supported me in small and big daily things. It has been a safe feeling knowing I can count on you. I thank my family for supporting me, especially my mother Maire, my sister Katrin, my brother Andres, my grandmother Linda, and my grandfather Garri. The last but not least thanks go to my lovely children Karolina (*alias* Pärlike, *alias* Täpsu) and Kristjan (*alias* Krisu, *alias* Kakuke) for being supportive during these years. You are my sunshine and my "prääniku̇d". Your hugs and kisses have given me strength to continue even when it has been hard.

December, 2022
Kristina Viiklepp

References

Aboul-Fettouh N, Nijhawan RI, 2018. Aggressive squamous cell carcinoma in a patient on the Janus kinase inhibitor ruxolitinib. *JAAD Case Rep*, 4:455–457.

Afshar-Kharghan V, 2017. The role of the complement system in cancer. *J Clin Invest*, 127:780-789.

Airola K, Johansson N, Kariniemi AL, Kähäri VM, Saarialho-Kere UK, 1997. Human collagenase-3 is expressed in malignant squamous epithelium of the skin. *J Invest Dermatol*, 109:225-231.

Ajona D, Pajares MJ, Chiara MD, Rodrigo JP, Jantus-Lewintre E, Camps C, Suarez C, Bagán JV, Montuenga LM, Pio R, 2015. Complement activation product C4d in oral and oropharyngeal squamous cell carcinoma. *Oral Dis*, 2:899-904.

Ajona D, Pajares MJ, Corrales L, Perez-Gracia JL, Agorreta J, Lozano MD, Torre W, Massion PP, de-Torres JP, Jantus-Lewintre E, Camps C, Zulueta JJ, Montuenga LM, Pio R, 2013. Investigation of complement activation product c4d as a diagnostic and prognostic biomarker for lung cancer. *J Natl Cancer Inst*, 105:1385-1393.

Ala-aho R, Ahonen M, George SJ, Heikkilä J, Grénman R, Kallajoki M, Kähäri VM, 2004. Targeted inhibition of human collagenase-3 (MMP-13) expression inhibits squamous cell carcinoma growth in vivo. *Oncogene*, 23:5111-5123.

Ala-aho R, Grénman R, Seth P, Kähäri VM, 2002. Adenoviral delivery of p53 gene suppresses expression of collagenase-3 (MMP-13) in squamous carcinoma cells. *Oncogene*, 21:1187-1195.

Alam M, Ratner D, 2001. Cutaneous squamous-cell carcinoma. *N Engl J Med*, 344:975-983.

Albert MR, Weinstock MA, 2003. Keratinocyte carcinoma. *CA Cancer J Clin*, 53:292-302.

Aleisa AI, Plante JG, Hsia L-LB, 2020. A case of aggressive squamous cell carcinoma with lymphovascular invasion during treatment with the Janus kinase inhibitor tofacitinib. *JAAD Case Rep*, 6:727–730.

Anforth R, Fernandez-Peñas P, Long GV, 2013. Cutaneous toxicities of RAF inhibitors. *Lancet Oncol*, 14:e11-18.

Anforth R, Menzies A, Byth K, Carlos G, Chou S, Sharma R, Scolyer RA, Kefford R, Long GV, Fernandez-Peñas P, 2015. Factors influencing the development of cutaneous squamous cell carcinoma in patients on BRAF inhibitor therapy. *J Am Acad Dermatol*, 72:809-815.e1.

Antonsson A, Forslund O, Ekberg H, Sterner G, Hansson BG, 2000. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. *J Virol*, 74:11636–11641.

Anwar J, Wrone DA, Kimyai-Asadi A, Alam M, 2004. The development of actinic keratosis into invasive squamous cell carcinoma: evidence and evolving classification schemes. *Clin Dermatol*, 22:189-196.

Arlaud GJ, Gaboriaud C, Thielens NM, Budayova-Spano M, Rossi V, Fontecilla-Camps JC, 2002. Structural biology of the C1 complex of complement unveils the mechanisms of its activation and proteolytic activity. *Mol Immuno*, 39:383-394.

Armstrong BK, Kricker A, 2001. The epidemiology of UV induced skin cancer. *J Photochem Photobiol B*, 63:8-18.

Artavanis-Tsakonas S, Rand MD, Lake RJ, 1999. Notch Signaling: Cell Fate Control and Signal Integration in Development. *Science*, 284:770–776.

Arvanitidou IE, Nikitakis NG, Georgaki M, Papadogeorgakis N, Tzioufas A, Sklavounou A, 2018. Multiple primary squamous cell carcinomas of the lower lip and tongue arising in discoid lupus erythematosus: a case report. *Oral Surg Oral Med Oral Pathol Oral Radiol*, 125:e22-e30.

Ascierto PA, Minor D, Ribas A, Lebbe C, O'Hagan A, Arya N, Guckert M, Schadendorf D, Kefford RF, Grob JJ, Hamid O, Amaravadi R, Simeone E, Wilhelm T, Kim KB, Long GV, Martin AM, Mazumdar J, Goodman VL, Trefzer U, 2013. Phase II trial (BREAK-2) of the BRAF inhibitor dabrafenib (GSK2118436) in patients with metastatic melanoma. *J Clin Oncol*, 31:3205-3211.

Attard NR, Karran P, 2011. UVA photosensitization of thiopurines and skin cancer in organ transplant recipients. *Photochem Photobiol Sci*, 11:62-68.

Baibergenova AT, Weinstock MA, Group VT, 2012. Oral prednisone use and risk of keratinocyte carcinoma in non-transplant population. The VATTC trial. *J Eur Acad Dermatol Venereol*, 26:1109–1115.

Balkwill FR, Mantovani A, 2012. Cancer-related inflammation: common themes and therapeutic opportunities. *Semin Cancer Biol*, 22:33-40.

Bandini S, Macagno M, Hysi A, Lanzardo S, Conti L, Bello A, Riccardo F, Ruiu R, Merighi IF, Forni G, Iezzi M, Quaglino E, Cavallo F, 2016. The non-inflammatory role of C1q during Her2/neu-driven mammary carcinogenesis. *Oncoimmunology*, 5:e1253653.

Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, McQuaid S, Gray RT, Murray LJ, Coleman HG, James JA, Salto-Tellez M, Hamilton PW, 2017. QuPath: Open source software for digital pathology image analysis. *Sci Rep*, 7:16878.

Baum CL, Wright AC, Martinez JC, Arpey CJ, Brewer JD, Roenigk RK, Otley CC, 2018. A new evidence-based risk stratification system for cutaneous squamous cell carcinoma into low, intermediate, and high risk groups with implications for management. *J Am Acad Derm*, 78:141–147.

Benjamin CL, Ananthaswamy HN, 2007. p53 and the pathogenesis of skin cancer. *Toxicol Appl Pharmacol*, 224:241-248.

Benjamin CL, Melnikova VO, Ananthaswamy HN, 2008. P53 protein and pathogenesis of melanoma and nonmelanoma skin cancer. *Adv Exp Med Biol*, 624:265-282.

Bergón-Sendín M, Pulido-Pérez A, Nieto-Benito LM, Barchino-Ortiz L, Díez-Sebastián J, Suárez-Fernández R, 2021. Effectiveness of neoadjuvant intralesional methotrexate in cutaneous squamous cell carcinoma: a prospective cohorts study. *Dermatol Ther*, e15233.

Biao T, Cai-Feng H, Xiao-Hong L, Xiao-Li C, Wen-Bei L, Jun W, Chao C, Tao Y, 2022. From Bowen disease to cutaneous squamous cell carcinoma: eight markers were verified from transcriptomic and proteomic analyses. *J Transl Med*, 20:416.

Blakely KM, Drucker AM, Rosen CF, 2019. Drug-Induced Photosensitivity-An Update: Culprit Drugs, Prevention and Management. *Drug Saf*, 42:827-847.

Bock SC, Skriver K, Nielsen E, Thøgersen HC, Wiman B, Donaldson VH, Eddy RL, Marrinan J, Radziejewska E, Huber R, Shows TB, Magnusson S, 1986. Human C1 inhibitor: primary structure, cDNA cloning, and chromosomal localization. *Biochemistry*, 25:4292-4301.

Boire A, Zou Y, Shieh J, Macalinao DG, Pentsova E, Massagué J, 2017. Complement Component 3 Adapts the Cerebrospinal Fluid for Leptomeningeal Metastasis. *Cell*, 168:1101-1113.

Bossi F, Tripodo C, Rizzi L, Bulla R, Agostinis C, Guarnotta C, Munaut C, Baldassarre G, Papa G, Zorzet S, Ghebrehiwet B, Ling GS, Botto M, Tedesco F, 2014. C1q as a unique player in angiogenesis with therapeutic implication in wound healing. *Proc Natl Acad Sci U S A*, 111:4209-4214.

Bouwes Bavinck JN, Feltkamp MCW, Green AC, Fiocco M, Euvrard S, Harwood CA, Nasir S, Thomson J, Proby CM, Naldi L, Diphoorn JCD, Venturuzzo A, Tessari G, Nindl I, Sampogna F, Abeni D, Neale RE, Goeman JJ, Quint KD, Halk AB, Sneek C, Genders RE, de Koning MNC, Quint WGV, Wieland U, Weissenborn S, Waterboer T, Pawlita M, Pfister H; EPI-HPV-UV-CA group, 2018. Human papillomavirus and posttransplantation cutaneous squamous cell carcinoma: A multicenter, prospective cohort study. *Am J Transplant*, 18:1220–1230.

Bradley K, North J, Saunders D, Schwaeble W, Jeziorska M, Woolley DE, Whaley K, 1996. Synthesis of classical pathway complement components by chondrocytes. *Immunology*, 88:648-656.

Brantsch KD, Meisner C, Schönfisch B, Trilling B, Wehner-Caroli J, Röcken M, Breuninger H, 2008. Analysis of risk factors determining prognosis of cutaneous squamous cell carcinoma: A prospective study. *Lancet Oncol*, 9:713–720.

Bretaud S, Guillon E, Karppinen S-M, Pihlajaniemi T, Ruggiero F, 2020. Collagen XV, a multifaceted multiplexin present across tissues and species. *Matrix Biol*, 6-7:100023.

Brewer JD, Shanafelt TD, Khezri F, Seda IMS, Zubair AS, Baum CL, Arpey CJ, Cerhan JR, Call TG, Roenigk RK, 2015. Increased incidence and recurrence rates of nonmelanoma skin cancer in patients with non-Hodgkin lymphoma: A Rochester Epidemiology Project population-based study in Minnesota. *J Am Acad Dermatol*, 72:302–309.

Brougham ND, Dennett ER, Cameron R, Tan ST, 2012. The incidence of metastasis from cutaneous squamous cell carcinoma and the impact of its risk factors. *Journal of surgical oncology*, 106:811-815.

Brougham ND, Tan ST, 2014. The incidence and risk factors of metastasis for cutaneous squamous cell carcinoma--implications on the T-classification system. *J Surg Oncol*, 110:876-82.

Brown VL, Harwood CA, Crook T, Cronin JG, Kelsell DP, Proby CM, 2004. p16INK4a and p14ARF Tumor Suppressor Genes Are Commonly Inactivated in Cutaneous Squamous Cell Carcinoma. *J Investig Dermatol,* 122:1284–1292.

Bu X, Zheng Z, Wang C, Yu Y, 2007. Significance of C4d deposition in the follicular lymphoma and MALT lymphoma and their relationship with follicular dendritic cells. *Pathol Res Pract*, 203:163-167.

Bulla R, Tripodo C, Rami D, Ling GS, Agostinis C, Guarnotta C, Zorzet S, Durigutto P, Botto M, Tedesco F, 2016. C1q acts in the tumour microenvironment as a cancer-promoting factor independently of complement activation. *Nat Commun*, 7:10346.

Cantwell M, Hua T, Pappas J, Kipps TJ, 1997. Acquired CD40-ligand deficiency in chronic lymphocytic leukemia. *Nat Med*, 3:984–989.

Chang AY, Doiron P, Maurer T, 2017. Cutaneous malignancies in HIV. *Curr Opin HIV AIDS*, 12:57-62.

Chen P, Chen F, Zhou B, 2019. Systematic review and meta-analysis of prevalence of dermatological toxicities associated with vemurafenib treatment in patients with melanoma. *Clin Exp Dermatol*, 44:243–251.

Chen Y, McAndrews KM, Kalluri R, 2021. Clinical and therapeutic relevance of cancer-associated fibroblasts. *Nat Rev Clin Oncol*, 8:792-804.

Cho RJ, Alexandrov LB, den Breems NY, Atanasova VS, Farshchian M, Purdom E, Nguyen TN, Coarfa C, Rajapakshe K, Prisco M, Sahu J, Tassone P, Greenawalt EJ, Collisson EA, Wu W, Yao H, Su X, Guttmann-Gruber C, Hofbauer JP, Hashmi R, Fuentes I, Benz SC, Golovato J, Ehli EA, Davis CM, Davies GE, Covington KR, Murrell DF, Salas-Alanis JC, Palisson F, Bruckner AL, Robinson W, Has C, Bruckner-Tuderman L, Titeux M, Jonkman MF, Rashidghamat E, Lwin SM, Mellerio JE, McGrath JA, Bauer JW, Hovnanian A, Tsai KY, South AP, 2018. APOBEC mutation drives early-onset squamous cell carcinomas in recessive dystrophic epidermolysis bullosa. *Sci Transl Med*, 10:eaas9668.

Cho MS, Vasquez HG, Rupaimoole R, Pradeep S, Wu S, Zand B, Han HD, Rodriguez-Aguayo C, Bottsford-Miller J, Huang J, Miyake T, Choi HJ, Dalton HJ, Ivan C, Baggerly K, Lopez-Berestein G, Sood AK, Afshar-Kharghan V, 2014. Autocrine effects of tumor-derived complement. *Cell Rep*, 6:1085-1095.

Chung HJ, Uitto J, 2010. Type VII collagen: the anchoring fibril protein at fault in dystrophic epidermolysis bullosa. *Dermatol Clin*, 28:93-105.

Cockerell CJ, 2000. Histopathology of incipient intraepidermal squamous cell carcinoma ("actinic keratosis"). *J Am Acad Dermatol*, 42:11-17.

Collins L, Quinn A, Stasko T, 2019. Skin Cancer and Immunosuppression. *Dermatol Clin*, 37:83-94.

Condorelli AG, Dellambra E, Logli E, Zambruno G, Castiglia D, 2019. Epidermolysis Bullosa-Associated Squamous Cell Carcinoma: From Pathogenesis to Therapeutic Perspectives. *Int J Mol Sci*, 20:5707.

Conforti C, Paolini F, Venuti A, Dianzani C, Zalaudek I, 2019. The detection rate of human papillomavirus in well-differentiated squamous cell carcinoma and keratoacanthoma: Is there new evidence for a viral pathogenesis of keratoacanthoma? *Br J Dermatol*, 181:1309–1311.

Corchado-Cobos R, García-Sancha N, González-Sarmiento R, Pérez-Losada J, Cañueto J, 2020. Cutaneous Squamous Cell Carcinoma: From Biology to Therapy. *Int J Mol Sci*, 21:2956.

Coulthard LG, Woodruff TM, 2015. Is the complement activation product C3a a proinflammatory molecule? Re-evaluating the evidence and the myth. *J Immunol*, 194:3542-3548.

Dajee M, Lazarov M, Zhang JY, Cai T, Green CL, Russell AJ, Marinkovich MP, Tao S, Lin Q, Kubo Y, Khavari PA, 2003. NF-κB blockade and oncogenic Ras trigger invasive human epidermal neoplasia. *Nature*, 421:639–643.

Dalal AJ, Ingham J, Collard B, Merrick G, 2018. Review of outcomes of 500 consecutive cases of non-melanoma skin cancer of the head and neck managed in an oral and maxillofacial surgical unit in a District General Hospital. *Br J Oral Maxillofac Surg*, 56:805-809.

D'Arcy ME, Pfeiffer RM, Rivera DR, Hess GP, Cahoon EK, Arron ST, Brownell I, Cowen EW, Israni AK, Triplette MA, Yanik EL, Engels EA, 2020. Voriconazole and the Risk of Keratinocyte Carcinomas among Lung Transplant Recipients in the United States. *JAMA Dermatol*, 156:772–779.

de Koning MNC, Weissenborn SJ, Abeni D, Bouwes Bavinck JN, Euvrard S, Green AC, Harwood CA, Naldi L, Neale R, Nindl I, Proby CM, Quint WGV, Sampogna F, Ter Schegget J, Struijk L, Wieland U, Pfister HJ, Feltkamp MCW, The Epi-Hpv-Uv-Ca Group, 2009. Prevalence and associated factors of betapapillomavirus infections in individuals without cutaneous squamous cell carcinoma. *J Gen Virol*, 90:1611–1621.

de Vries E, Trakatelli M, Kalabalikis D, Ferrandiz L, Ruiz-de-Casas A, Moreno-Ramirez D, Sotiriadis D, Ioannides D, Aquilina S, Apap C, Micallef R, Scerri L, Ulrich M, Pitkänen S, Saksela O, Altsitsiadis E, Hinrichs B, Magnoni C, Fiorentini C, Majewski S, Ranki A, Stockfleth E, Proby C; EPIDERM Group, 2012. Known and potential new risk factors for skin cancer in European populations: a multicentre case-control study. *Br J Dermatol*, 167 Suppl 2:1-13.

Deban L, Jarva H, Lehtinen MJ, Bottazzi B, Bastone A, Doni A, Jokiranta TS, Mantovani A, Meri S, 2008. Binding of the long pentraxin PTX3 to factor H: interacting domains and function in the regulation of complement activation. *J Immunol*, 181:8433-8440.

Demehri S, Turkoz A, Kopan R, 2009. Epidermal Notch1 Loss Promotes Skin Tumorigenesis by Impacting the Stromal Microenvironment. *Cancer Cell*, 16:55–66.

Di Nardo L, Pellegrini C, Di Stefani A, Del Regno L, Sollena P, Piccerillo A, Longo C, Garbe C, Fargnoli MC, Peris K, 2020. Molecular genetics of cutaneous squamous cell carcinoma: perspective for treatment strategies. *J Eur Acad Dermatol Venereol*, 34:932-941.

Dovezenski N, Billetta R, Gigli I, 1992. Expression and localization of proteins of the complement system in human skin. *J Clin Invest*, 90:2000-2012.

Drouet C, Ponard D, Ghannam A, 2018. Chapter 23 - C1 Inhibitor. *The Complement FactsBook (Second Edition)*, 241-249.

Euhus DM, Hudd C, LaRegina MC, Johnson FE, 1986. Tumor measurement in the nude mouse. *J Surg Oncol*, 31:229-234.

Farshchian M, Kivisaari A, Ala-Aho R, Riihilä P, Kallajoki M, Grénman R, Peltonen J, Pihlajaniemi T, Heljasvaara R, Kähäri VM, 2011. Serpin peptidase inhibitor clade A member 1 (SerpinA1) is a novel biomarker for progression of cutaneous squamous cell carcinoma. *Am J Pathol*, 179:1110-1119.

Farshchian M, Nissinen L, Grénman R, Kähäri VM, 2017. Dasatinib promotes apoptosis of cutaneous squamous carcinoma cells by regulating activation of ERK1/2. *Exp Dermatol*, 26:89-92.

Farshchian M, Nissinen L, Siljamäki E, Riihilä P, Toriseva M, Kivisaari A, Ala-Aho R, Kallajoki M, Veräjänkorva E, Honkanen HK, Heljasvaara R, Pihlajaniemi T, Grénman R, Peltonen J, Peltonen S, Kähäri VM, 2015. EphB2 Promotes Progression of Cutaneous Squamous Cell Carcinoma. *J Invest Dermatol*, 135:1882-1892.

Fearon DT, 1979. Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. *Proc Natl Acad Sci U S A*, 76:5867-71.

Fearon ER, Vogelstein B, 1990. A genetic model for colorectal tumorigenesis. *Cell*, 61:759–767.

Fernandes MS, Girisha BS, Viswanathan N, Sripathi H, Noronha TM, 2015. Discoid lupus erythematosus with squamous cell carcinoma: a case report and review of the literature in Indian patients. *Lupus*, 24:1562-1566.

Fernandez Figueras MT, 2017. From actinic keratosis to squamous cell carcinoma: pathophysiology revisited. *J Eur Acad Dermatol Venereol*, 31Suppl2:5-7.

Ferrandiz L, Ruiz-de-Casas A, Trakatelli M, de Vries E, Ulrich M, Aquilina S, Saksela O, Majewski S, Ranki A, Proby C, Magnoni C, Pitkänen S, Kalokasidis K, Siskou S, Hinrichs B, Altsitsiadis E, Stockfleth E, Moreno-Ramirez D; EPIDERM Group, 2012. Assessing physicians' preferences on skin cancer treatment in Europe. *Br J Dermatol*, 167 Suppl 2:29-35.

Finnish Cancer Registry, accessed 3.12.2021. https://syoparekisteri.fi/tilastot/syopa-suomessa/tarkeimpia-tilastoja/.

Fitzpatrick TB, 1988. The validity and practicality of sun-reactive skin types I through VI. *Arch Dermatol*, 124:869-871.

Fonseca MI, Chu SH, Hernandez MX, Fang MJ, Modarresi L, Selvan P, MacGregor GR, Tenner AJ, 2017. Cell-specific deletion of C1qa identifies microglia as the dominant source of C1q in mouse brain. *J Neuroinflammation*, 14:48.

Foote JA, Harris RB, Giuliano AR, Roe DJ, Moon TE, Cartmel B, Alberts DS, 2001. Predictors for cutaneous basal- and squamous-cell carcinoma among actinically damaged adults. *Int J Cancer*, 95:7-11.

Forneris F, Wu J, Gros P, 2012. The modular serine proteases of the complement cascade. *Curr Opin Struct Biol*, 22:333-341.

Fortina AB, Piaserico S, Caforio AL, Abeni D, Alaibac M, Angelini A, Iliceto S, Peserico A, 2004. Immunosuppressive level and other risk factors for basal cell carcinoma and squamous cell carcinoma in heart transplant recipients. *Arch Dermatol*, 140:1079-1085.

Fridman WH, Zitvogel L, Sautès-Fridman C, Kroemer G, 2017. The immune contexture in cancer prognosis and treatment. *Nat Rev Clin Oncol*, 14:717-734.

Frings O, Augsten M, Tobin NP, Carlson J, Paulsson J, Pena C, Olsson E, Veerla S, Bergh J, Ostman A, Sonnhammer EL, 2013. Prognostic significance in breast cancer of a gene signature capturing stromal PDGF signaling. *Am J Pathol*, 182:2037-2047.

Fruman DA, Chiu H, Hopkins BD, Bagrodia S, Cantley LC, Abraham RT, 2017. The PI3K pathway in human disease. *Cell*, 170:605–635.

Gaboriaud C, Thielens NM, Gregory LA, Rossi V, Fontecilla-Camps JC, Arlaud GJ, 2004. Structure and activation of the C1 complex of complement: unraveling the puzzle. *Trends Immunol*, 25:368-373.

Garred P, Honoré C, Ma YJ, Munthe-Fog L, Hummelshøj T, 2009. MBL2, FCN1, FCN2 and FCN3-The genes behind the initiation of the lectin pathway of complement. *Mol Immunol*, 46:2737-2744.

Garrett GL, Lowenstein SE, Singer JP, He SY, Arron ST, 2016. Trends of skin cancer mortality after transplantation in the United States: 1987 to 2013. *J Am Acad Dermatol*, 75:106-112.

Ghebrehiwet B, Hosszu KK, Valentino A, Peerschke EI, 2012. The C1q family of proteins: insights into the emerging non-traditional functions. *Front Immunol*, 3:52.

Gibney GT, Messina JL, Fedorenko IV, Sondak VK, Smalley KS, 2013. Paradoxical oncogenesis--the long-term effects of BRAF inhibition in melanoma. *Nat Rev Clin Oncol*, 10:390-399.

Glogau RG, 2000. The risk of progression to invasive disease. *J Am Acad Dermatol*, 42:23-4.
Gorter A, Meri S, 1999. Immune evasion of tumor cells using membrane-bound complement regulatory proteins. *Immunol Today*, 20:576-582.
Grivennikov SI, Greten FR, Karin M, 2010. Immunity, inflammation, and cancer. *Cell*, 140:883-899.
Gros P, Milder FJ, Janssen BJ, 2008. Complement driven by conformational changes. *Nat Rev Immunol*, 8:48-58.
Hägglöf C, Hammarsten P, Josefsson A, Stattin P, Paulsson J, Bergh A, Ostman A, 2010. Stromal PDGFRbeta expression in prostate tumors and non-malignant prostate tissue predicts prostate cancer survival. *PLoS One*, 5:e10747.
Haisma MS, Plaat BEC, Bijl HP, Roodenburg JLN, Diercks GFH, Romeijn TR, Terra JB, 2016. Multivariate analysis of potential risk factors for lymph node metastasis in patients with cutaneous squamous cell carcinoma of the head and neck. *J Am Acad Dermatol*, 75:722–730.
Hajela K, Kojima M, Ambrus G, Wong KH, Moffatt BE, Ferluga J, Hajela S, Gal P, Sim RB, 2002. The biological functions of MBL-associated serine proteases (MASPs). *Immunobiology*, 205: 467-475.
Hajishengallis G, Reis ES, Mastellos DC, Ricklin D, Lambris JD, 2017. Novel mechanisms and functions of complement. *Nat Immunol*, 18:1288-1298.
Hanahan D, Weinberg RA, 2000. The hallmarks of cancer. *Cell*, 100:57-70.
Harwood CA, Mesher D, McGregor JM, Mitchell L, Leedham-Green M, Raftery M, Cerio R, Leigh IM, Sasieni P, Proby CM, 2013. A Surveillance Model for Skin Cancer in Organ Transplant Recipients: A 22-Year Prospective Study in an Ethnically Diverse Population. *Am J Transplant*, 13:119–129.
Heaphy MR Jr, Ackerman AB, 2000. The nature of solar keratosis: a critical review in historical perspective. *J Am Acad Dermatol*, 43:138-150.
Heidorn SJ, Milagre C, Whittaker S, Nourry A, Niculescu-Duvas I, Dhomen N, Hussain J, Reis-Filho JS, Springer CJ, Pritchard C, Marais R, 2010. Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell*, 140:209-221.
Heise R, Vetter-Kauczok CS, Skazik C, Czaja K, Marquardt Y, Lue H, Merk HF, Bernhagen J, Baron JM, 2012. Expression and function of macrophage migration inhibitory factor in the pathogenesis of UV-induced cutaneous nonmelanoma skin cancer. *Photochem Photobiol*, 88:1157-1164.
Heljasvaara R, Aikio M, Ruotsalainen H, Pihlajaniemi T, 2017. Collagen XVIII in tissue homeostasis and dysregulation - Lessons learned from model organisms and human patients. *Matrix Biol*, 57-58:55-75.
Higgins S, Nazemi A, Chow M, Wysong A, 2018. Review of Nonmelanoma Skin Cancer in African Americans, Hispanics, and Asians. *Dermatol Surg*, 44:903-910.
Hoadley KA, Yau C, Hinoue T, Wolf DM, Lazar AJ, Drill E, Shen R, Taylor AM, Cherniack AD, Thorsson V, Akbani R, Bowlby R, Wong CK, Wiznerowicz M, Sanchez-Vega F, Robertson AG, Schneider BG, Lawrence MS, Noushmehr H, Malta TM; Cancer Genome Atlas Network, Stuart JM, Benz CC, Laird PW, 2018. Cell-of-Origin Patterns Dominate the Molecular Classification of 10,000 Tumors from 33 Types of Cancer. *Cell*, 173:291-304.e6.
Hong Q, Sze CI, Lin SR, Lee MH, He RY, Schultz L, Chang JY, Chen SJ, Boackle RJ, Hsu LJ, Chang NS, 2009. Complement C1q activates tumor suppressor WWOX to induce apoptosis in prostate cancer cells. *PLoS One*, 4:e5755.
Howell JY, Ramsey ML, 2021. Squamous Cell Skin Cancer. *StatPearls Publishing*.
Howley PM, Pfister HJ, 2015. Beta genus papillomaviruses and skin cancer. *Virology*, 479–480:290–296.
Hwang J, Huang Y, Burwell TJ, Peterson NC, Connor J, Weiss SJ, Yu SM, Li Y, 2017. In Situ Imaging of Tissue Remodeling with Collagen Hybridizing Peptides. *ACS Nano*, 11:9825-9835.
Impola U, Jeskanen L, Ravanti L, Syrjänen S, Baldursson B, Kähäri VM, Saarialho-Kere U, 2005. Expression of matrix metalloproteinase (MMP)-7 and MMP-13 and loss of MMP-19 and p16 are associated with malignant progression in chronic wounds. *Br J Dermatol*, 152:720-726.

Inman GJ, Wang J, Nagano A, Alexandrov LB, Purdie KJ, Taylor RG, Sherwood V, Thomson J, Hogan S, Spender LC, South AP, Stratton M, Chelala C, Harwood CA, Proby CM, Leigh IM, 2018. The genomic landscape of cutaneous SCC reveals drivers and a novel azathioprine associated mutational signature. *Nat Commun*, 9:3667.

Janssen BJ, Gros P, 2006. Conformational complexity of complement component C3. *Adv Exp Med Biol*, 586:291-312.

Jensen A, Thomsen HF, Engebjerg MC, Olesen AB, Friis S, Karagas MR, Sørensen HT, 2009. Use of oral glucocorticoids and risk of skin cancer and non-Hodgkin's lymphoma: A population-based case–control study. *Br J Cancer*, 100:200–205.

Johansson N, Airola K, Grénman R, Kariniemi AL, Saarialho-Kere U, Kähäri VM, 1997. Expression of collagenase-3 (matrix metalloproteinase-13) in squamous cell carcinomas of the head and neck. *Am J Pathol*, 151:499-508.

Johnson TM, Rowe DE, Nelson BR, Swanson NA, 1992. Squamous cell carcinoma of the skin (excluding lip and oral mucosa). *J Am Acad Derm*, 26:467–484.

Jonason AS, Kunala S, Price GJ, Restifo RJ, Spinelli HM, Persing JA, Leffell DJ, Tarone RE, Brash DE, 1996. Frequent clones of p53-mutated keratinocytes in normal human skin. *Proc Natl Acad Sci USA*, 93:14025–14029.

Junttila MR, Ala-Aho R, Jokilehto T, Peltonen J, Kallajoki M, Grenman R, Jaakkola P, Westermarck J, Kähäri VM, 2007. p38alpha and p38delta mitogen-activated protein kinase isoforms regulate invasion and growth of head and neck squamous carcinoma cells. *Oncogene*, 26:5267-79.

Kadir AR, 2007. Burn scar neoplasm. *Ann Burn Fire Disasters*, 20:185–188.

Kalluri R, 2016. The biology and function of fibroblasts in cancer. *Nat Rev Cancer*, 16:582–598.

Karppinen SM, Honkanen HK, Heljasvaara R, Riihilä P, Autio-Harmainen H, Sormunen R, Harjunen V, Väisänen MR, Väisänen T, Hurskainen T, Tasanen K, Kähäri VM, Pihlajaniemi T, 2016. Collagens XV and XVIII show different expression and localisation in cutaneous squamous cell carcinoma: type XV appears in tumor stroma, while XVIII becomes upregulated in tumor cells and lost from microvessels. *Exp Dermatol*, 25:348-354.

Kaur A, Sultan SH, Murugaiah V, Pathan AA, Alhamlan FS, Karteris E, Kishore U, 2016. Human C1q Induces Apoptosis in an Ovarian Cancer Cell Line via Tumor Necrosis Factor Pathway. *Front Immunol*, 7:599.

Kemper C, Atkinson JP, Hourcade DE, 2010. Properdin: emerging roles of a pattern-recognition molecule. *Annu Rev Immunol*, 28:131-155.

Kerkelä E, Ala-Aho R, Jeskanen L, Rechardt O, Grénman R, Shapiro SD, Kähäri VM, Saarialho-Kere U, 2000. Expression of human macrophage metalloelastase (MMP-12) by tumor cells in skin cancer. *J Invest Dermatol*, 114:1113-1119.

Kerr-Valentic MA, Samimi K, Rohlen BH, Agarwal JP, Rockwell WB, 2009. Marjolin's Ulcer: Modern Analysis of an Ancient Problem. *Plast Reconstr Surg*, 123:184–191.

Khatri ML, 2021. Xeroderma pigmentosum in Yemen. *Int J Dermatol*, 60:314-320.

Khavari PA, 2006. Modelling cancer in human skin tissue. *Nat Rev Cancer*, 6:270–280.

Kim MS, In SG, Park OJ, Won CH, Lee MW, Choi JH, Kim CW, Kim SE, Moon KC, Chang S, 2011. Increased expression of activating transcription factor 3 is related to the biologic behavior of cutaneous squamous cell carcinomas. *Hum Pathol*, 42:954-959.

Kishikawa M, Koyama K, Iseki M, Kobuke T, Yonehara S, Soda M, Ron E, Tokunaga M, Preston DL, Mabuchi K, Tokuoka S, 2005. Histologic characteristics of skin cancer in Hiroshima and Nagasaki: Background incidence and radiation effects. *Int J Cancer*, 117:363–369.

Kishore U, Reid KB, 2000. C1q: structure, function, and receptors. *Immunopharmacology*, 49:159-170.

Kivisaari A, Kähäri VM, 2013. Squamous cell carcinoma of the skin: Emerging need for novel biomarkers. *World J Clin Oncol*, 4:85-90.

Kivisaari AK, Kallajoki M, Mirtti T, McGrath JA, Bauer JW, Weber F, Königová R, Sawamura D, Sato-Matsumura KC, Shimizu H, Csikós M, Sinemus K, Beckert W, Kähäri VM, 2008.

Transformation-specific matrix metalloproteinases (MMP)-7 and MMP-13 are expressed by tumour cells in epidermolysis bullosa-associated squamous cell carcinomas. *Br J Dermatol*, 158:778-785.

Knäuper V, López-Otin C, Smith B, Knight G, Murphy G, 1996a. Biochemical characterization of human collagenase-3. *J Biol Chem*, 271:1544-1550.

Knäuper V, Will H, López-Otin C, Smith B, Atkinson SJ, Stanton H, Hembry RM, Murphy G, 1996b. Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. *J Biol Chem*, 271:17124-17131.

Knuutila JS, Riihilä P, Kurki S, Nissinen L, Kähäri VM, 2020. Risk Factors and Prognosis for Metastatic Cutaneous Squamous Cell Carcinoma: A Cohort Study. *Acta Derm Venereol*, 100:adv00266.

Kojima A, Iwata K, Seya T, Matsumoto M, Ariga H, Atkinson JP, Nagasawa S, 1993. Membrane cofactor protein (CD46) protects cells predominantly from alternative complement pathway-mediated C3-fragment deposition and cytolysis. *J Immunol,* 151:1519-1527.

Kojouharova M, Reid K, Gadjeva M, 2010. New insights into the molecular mechanisms of classical complement activation. *Mol Immunol*, 47:2154–2160.

Kolev V, Mandinova A, Guinea-Viniegra J, Hu B, Lefort K, Lambertini C, Neel V, Dummer R, Wagner EF, Dotto GP, 2008. EGFR signalling as a negative regulator of Notch1 gene transcription and function in proliferating keratinocytes and cancer. *Nat Cell Biol*, 10:902-911.

Koseki S, Aoki T, Ansai S, Hozumi Y, Mitsuhashi Y, Kondo S, 1999. An immunohistochemical study of Ecadherin expression in human squamous cell carcinoma of the skin: relationship between decreased expression of Ecadherin in the primary lesion and regional lymph node metastasis. *J Dermatol*, 26:416-422.

Kourtzelis I, Rafail S, 2016. The dual role of complement in cancer and its implication in anti-tumor therapy. *Ann Transl Med*, 4:265.

Kouser L, Madhukaran SP, Shastri A, Saraon A, Ferluga J, Al-Mozaini M, Kishore U, 2015. Emerging and Novel Functions of Complement Protein C1q. *Front Immunol*, 6:317.

Kramb F, Doerfer C, Meiwes A, Ramakrishnan K, Eigentler T, Garbe C, Keim U, Leiter U, 2022. Real-world Treatment Patterns and Outcomes with Systemic Therapies in Unresectable Locally Advanced and Metastatic Cutaneous Squamous Cell Carcinoma in Germany. *Acta Derm Venereol*, 102:adv00637.

Kubo Y, Urano Y, Yoshimoto K, Iwahana H, Fukuhara K, Arase S, Itakura M, 1994. p53 gene mutations in human skin cancers and precancerous lesions: comparison with immunohistochemical analysis. *J Invest Dermatol*, 102:440-444.

Kueder-Pajares T, Descalzo MA, García-Doval I, Ríos-Buceta L, Moreno-Ramírez D, 2018. Evaluation of Structure Indicators for Assessing Skin Cancer Quality of Care in Dermatology Departments. *Actas Dermosifiliogr (Engl Ed)*, 109:807-812.

Laine A, Ronai Z, 2005. Ubiquitin chains in the ladder of MAPK signaling. *Sci STKE*, 2005:re5.

Larionov A, Krause A, Miller W, 2005. A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics*, 6:62.

Lee DA, Miller SJ, 2009. Nonmelanoma skin cancer. *Facial Plast Surg Clin North Am*, 17:309-324.

Lefort K, Mandinova A, Ostano P, Kolev V, Calpini V, Kolfschoten I, Devgan V, Lieb J, Raffoul W, Hohl D, Neel V, Garlick J, Chiorino G, Dotto GP, 2007. Notch1 is a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1/2 and MRCKalpha kinases. *Genes Dev*, 21:562–577.

Lennard L, 1992. The clinical pharmacology of 6-mercaptopurine. *Eur J Clin Pharmacol*, 43:329-39.

Levi F, Randimbison L, Te VC, La Vecchia C, 1996. Non-Hodgkin's lymphomas, chronic lymphocytic leukaemias and skin cancers. *Br J Cancer*, 74:1847–1850.

Lewis KG, Weinstock MA, 2004. Nonmelanoma skin cancer mortality (1988-2000): the Rhode Island follow-back study. *Arch Dermatol*, 140:837-42.

Li YY, Hanna GJ, Laga AC, Haddad RI, Lorch JH, Hammerman PS, 2015. Genomic analysis of metastatic cutaneous squamous cell carcinoma. *Clin Cancer Res*, 21:1447–1456.

Li Y, Wang J, Xiao W, Liu J, Zha X, 2022. Risk Factors for Actinic Keratoses: A Systematic Review and Meta-Analysis. *Indian J Dermatol*, 67:92.

Lichter MD, Karagas MR, Mott LA, Spencer SK, Stukel TA, Greenberg ER, 2000. Therapeutic ionizing radiation and the incidence of basal cell carcinoma and squamous cell carcinoma. The New Hampshire Skin Cancer Study Group. *Arch Dermatol*, 136:1007–1011.

Lindqvist CA, Christiansson LH, Simonsson B, Enblad G, Olsson-Strömberg U, Loskog ASI, 2010. T regulatory cells control T-cell proliferation partly by the release of soluble CD25 in patients with B-cell malignancies. *Immunology*, 131:371–376.

Ling M, Murali M, 2019. Analysis of the Complement System in the Clinical Immunology Laboratory. *Clin Lab Med*, 39:579-590.

Liszewski MK, Farries TC, Lublin DM, Rooney IA, Atkinson JP, 1996. Control of the complement system. *Adv Immunol*, 61:201-283.

Liszewski MK, Leung M, Cui W, Subramanian VB, Parkinson J, Barlow PN, Manchester M, Atkinson JP, 2000. Dissecting sites important for complement regulatory activity in membrane cofactor protein (MCP; CD46). *J Biol Chem*, 275:37692-37701.

Lober BA, Lober CW, 2000. Actinic keratosis is squamous cell carcinoma. *South Med J*, 93:650-5.

Lomas A, Leonardi-Bee J, Bath-Hextall F, 2012. A systematic review of worldwide incidence of nonmelanoma skin cancer. *Br J Dermatol*, 166:1069-1080.

Lu Z, Xu S, Joazeiro C, Cobb MH, Hunter T, 2002. The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. *Mol Cell*, 9:945-956.

Lubbers R, van Essen MF, van Kooten C, Trouw LA, 2017. Production of complement components by cells of the immune system. *Clin Exp Immunol*, 188:183-194.

Lucas R, McMichael T, Smith W, Armstrong B, 2006. Solar Ultraviolet Radiation: Global Burden of Disease from Solar Ultraviolet Radiation. Environmental Burden of Disease Series, No. 13. Geneva: World Health Organization.

Lucas SD, Karlsson-Parra A, Nilsson B, Grimelius L, Akerström G, Rastad J, Juhlin C, 1996. Tumor-specific deposition of immunoglobulin G and complement in papillary thyroid carcinoma. *Hum Pathol,* 27:1329-1335.

Lujambio A, Akkari L, Simon J, Grace D, Tschaharganeh DF, Bolden JE, Zhao Z, Thapar V, Joyce JA, Krizhanovsky V, Lowe SW, 2013. Non-cell-autonomous tumor suppression by p53. *Cell*, 153:449-460.

Madan V, Lear JT, Szeimies RM, 2010. Non-melanoma skin cancer. *Lancet*, 375:673-685.

Mahé E, Beauchet A, de Paula Corrêa M, Godin-Beekmann S, Haeffelin M, Bruant S, Fay-Chatelard F, Jégou F, Saiag P, Aegerter P, 2011. Outdoor sports and risk of ultraviolet radiation-related skin lesions in children: evaluation of risks and prevention. *Br J Dermatol*, 165:360-367.

Mäkelä K, Helén P, Haapasalo H, Paavonen T, 2012. Complement activation in astrocytomas: deposition of C4d and patient outcome. *BMC Cancer*, 12:565.

Mamidi S, Höne S, Kirschfink M, 2017. The complement system in cancer: Ambivalence between tumour destruction and promotion. *Immunobiology*, 222:45-54.

Mangogna A, Belmonte B, Agostinis C, Zacchi P, Iacopino DG, Martorana A, Rodolico V, Bonazza D, Zanconati F, Kishore U, Bulla R, 2019. Prognostic Implications of the Complement Protein C1q in Gliomas. *Front Immunol*, 10:2366.

Mantovani A, Allavena P, Sica A, Balkwill F, 2008. Cancer-related inflammation. *Nature*, 454:436-444.

March-Rodriguez Á, Bellosillo B, Álvarez-Larrán A, Besses C, Pujol RM, Toll A, 2019. Rapidly Growing and Aggressive Cutaneous Squamous Cell Carcinomas in a Patient Treated with Ruxolitinib. *Ann Derm*, 31:204–208.

Markiewski MM, DeAngelis RA, Benencia F, Ricklin-Lichtsteiner SK, Koutoulaki A, Gerard C, Coukos G, Lambris JD, 2008. Modulation of the antitumor immune response by complement. *Nat Immunol*, 9:1225-1235.

Marks R, Rennie G, Selwood TS, 1988. Malignant transformation of solar keratoses to squamous cell carcinoma. *Lancet*, 1:795-797.

Marks R, 1995. An overview of skin cancers. Incidence and causation. *Cancer*, 75:607-612.

Martincorena I, Roshan A, Gerstung M, Ellis P, Van Loo P, McLaren S, Wedge DC, Fullam A, Alexandrov LB, Tubio JM, Stebbings L, Menzies A, Widaa S, Stratton MR, Jones PH, Campbell PJ, 2015. Tumor evolution. High burden and pervasive positive selection of somatic mutations in normal human skin. *Science*, 348:880-886.

Martins VL, Caley MP, Moore K, Szentpetery Z, Marsh ST, Murrell DF, Kim MH, Avari M, McGrath JA, Cerio R, Kivisaari A, Kähäri VM, Hodivala-Dilke K, Brennan CH, Chen M, Marshall JF, O'Toole EA, 2015. Suppression of TGFβ and Angiogenesis by Type VII Collagen in Cutaneous SCC. *J Natl Cancer Inst*, 108:djv293.

McCarthy EM, Ethridge KP, Wagner RF Jr, 1999. Beach holiday sunburn: the sunscreen paradox and gender differences. *Cutis*, 64:37-42.

Medicus RG, Melamed J, Arnaout MA, 1983. Role of human factor I and C3b receptor in the cleavage of surface-bound C3bi molecules. *Eur J Immunol*, 13:465-470

Medof ME, Iida K, Mold C, Nussenzweig V, 1982. Unique role of the complement receptor CR1 in the degradation of C3b associated with immune complexes. *J Exp Med*, 156:1739-1754.

Meri S, Morgan BP, Davies A, Daniels RH, Olavesen MG, Waldmann H, Lachmann PJ, 1990. Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. *Immunology*, 71:1-9.

Migden MR, Rischin D, Schmults CD, Guminski A, Hauschild A, Lewis KD, Chung CH, Hernandez-Aya L, Lim AM, Chang ALS, Rabinowits G, Thai AA, Dunn LA, Hughes BGM, Khushalani NI, Modi B, Schadendorf D, Gao B, Seebach F, Li S, Li J, Mathias M, Booth J, Mohan K, Stankevich E, Babiker HM, Brana I, Gil-Martin M, Homsi J, Johnson ML, Moreno V, Niu J, Owonikoko TK, Papadopoulos KP, Yancopoulos GD, Lowy I, Fury MG, 2018. PD-1 Blockade with cemiplimab in advanced cutaneous squamous-cell carcinoma. *N Engl J Med*, 379:341–351.

Mittelbronn MA, Mullins DL, Ramos-Caro FA, Flowers FP, 1998. Frequency of pre-existing actinic keratosis in cutaneous squamous cell carcinoma. *Int J Dermatol*, 37:677-681.

Montaudié H, Chiaverini C, Sbidian E, Charlesworth A, Lacour JP, 2016. Inherited epidermolysis bullosa and squamous cell carcinoma: a systematic review of 117 cases. *Orphanet J Rare Dis*, 11:117.

Morgan BP, Gasque P, 1997. Extrahepatic complement biosynthesis: where, when and why? *Clin Exp Immunol*, 107:1-7.

Mortensen SA, Sander B, Jensen RK, Pedersen JS, Golas MM, Jensenius JC, Hansen AG, Thiel S, Andersen GR, 2017. Structure and activation of C1, the complex initiating the classical pathway of the complement cascade. *Proc Natl Acad Sci U S A*, 114:986-991.

Morton CA, Szeimies RM, Basset-Seguin N, Calzavara-Pinton P, Gilaberte Y, Hædersdal M, Hofbauer GFL, Hunger RE, Karrer S, Piaserico S, Ulrich C, Wennberg AM, Braathen LR, 2019. European Dermatology Forum guidelines on topical photodynamic therapy 2019 Part 1: treatment delivery and established indications – actinic keratoses, Bowen's disease and basal cell carcinomas. *JEADV*, 33:2225-2238.

Nakazawa H, English D, Randell PL, Nakazawa K, Martel N, Armstrong BK, Yamasaki H, 1994. UV and skin cancer: specific p53 gene mutation in normal skin as a biologically relevant exposure measurement. *Proc Natl Acad Sci U S A*, 91:360-364.

Neale RE, Weissenborn S, Abeni D, Bavinck JN, Euvrard S, Feltkamp MC, Green AC, Harwood C, de Koning M, Naldi L, Nindl I, Pawlita M, Proby C, Quint WG, Waterboer T, Wieland U, Pfister H, 2013. Human Papillomavirus Load in Eyebrow Hair Follicles and Risk of Cutaneous Squamous Cell Carcinoma. *Cancer Epidemiol Biomark Prev*, 22:719–727.

Nehal KS, Bichakjian CK, 2018. Update on keratinocyte carcinomas. *N Engl J Med*, 379:363–374.

Nelson MA, Einspahr JG, Alberts DS, Balfour CA, Wymer JA, Welch KL, Salasche SJ, Bangert JL, Grogan TM, Bozzo PO, 1994. Analysis of the p53 gene in human precancerous actinic keratosis lesions and squamous cell cancers. *Cancer Lett*, 85:23-29.

Nguyen LK, Kolch W, Kholodenko BN, 2013. When ubiquitination meets phosphorylation: a systems biology perspective of EGFR/MAPK signalling. *Cell Commun Signal*, 11:52.

Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M, Hui C-c, Clevers H, Dotto GP, Radtke F, 2003. Notch1 functions as a tumor suppressor in mouse skin. *Nat Genet*, 33:416–421.

Nissinen L, Farshchian M, Riihilä P, Kähäri VM, 2016. New perspectives on role of tumor microenvironment in progression of cutaneous squamous cell carcinoma. *Cell Tissue Res*, 365:691-702.

Nissinen L, Kähäri VM, 2014. Matrix metalloproteinases in inflammation. *Biochim Biophys Acta*, 1840:2571-2580.

Noris M, Remuzzi G, 2013. Overview of complement activation and regulation. *Semin Nephrol*, 33:479–492.

Nowell CS, Radtke F, 2017. Notch as a tumour suppressor. *Nat Rev Cancer*, 17:145–159.

Olivero C, Lanfredini S, Borgogna C, Gariglio M, Patel GK, 2018. HPV-Induced Field Cancerisation: Transformation of Adult Tissue Stem Cell Into Cancer Stem Cell. *Front Microbiol*, 9:546.

Ortonne JP, 2002. From actinic keratosis to squamous cell carcinoma. *Br J Dermatol*, 146(suppl 61):20–23.

Palaniappan V, Karthikeyan K, 2022. Bowen's Disease. *Indian Dermatol Online J*, 13:177-189.

Palazzo E, Morasso MI, Pincelli C, 2020. Molecular Approach to Cutaneous Squamous Cell Carcinoma: From Pathways to Therapy. *Int J Mol Sci*, 21:1211.

Paulsson J, Sjöblom T, Micke P, Pontén F, Landberg G, Heldin CH, Bergh J, Brennan DJ, Jirström K, Ostman A, 2009. Prognostic significance of stromal platelet-derived growth factor beta-receptor expression in human breast cancer. *Am J Pathol*, 175:334-341.

Perry PK, Silverberg NB, 2001. Cutaneous malignancy in albinism. *Cutis*, 67:427-430.

Petry F, Botto M, Holtappels R, Walport MJ, Loos M, 2001. Reconstitution of the complement function in C1q-deficient (C1qa-/-) mice with wild-type bone marrow cells. *J Immunol*, 167:4033-4037.

Pickering CR, Zhou JH, Lee JJ, Drummond JA, Peng SA, Saade RE, Tsai KY, Curry JL, Tetzlaff MT, Lai SY, 2014. Mutational landscape of aggressive cutaneous squamous cell carcinoma. *Clin Cancer Res,* 20:6582–6592.

Pierceall WE, Goldberg LH, Tainsky MA, Mukhopadhyay T, Ananthaswamy HN, 1991. Ras gene mutation and amplification in human nonmelanoma skin cancers. *Mol Carcinog*, 4:196–202.

Piipponen M, Nissinen L, Riihilä P, Farshchian M, Kallajoki M, Peltonen J, Peltonen S, Kähäri VM, 2020. p53-Regulated Long Noncoding RNA PRECSIT Promotes Progression of Cutaneous Squamous Cell Carcinoma via STAT3 Signaling. *Am J Pathol*, 190:503-517.

Piipponen M, Riihilä P, Nissinen L, Kähäri VM, 2021. The Role of p53 in Progression of Cutaneous Squamous Cell Carcinoma. *Cancers (Basel)*, 13:4507.

Pio R, Corrales L, Lambris JD, 2014. The role of complement in tumor growth. *Adv Exp Med Biol, Sringer*, 772:229-262

Poulikakos PI, Zhang C, Bollag G, Shokat KM, Rosen N, 2010. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature*, 464:427-430.

Pukkala E, Martinsen JI, Weiderpass E, Kjaerheim K, Lynge E, Tryggvadottir L, Sparén P, Demers PA, 2014. Cancer incidence among firefighters: 45 years of follow-up in five Nordic countries. *Occup Environ Med*, 71:398–404.

Quaedvlieg PJ, Tirsi E, Thissen MR, Krekels GA, 2006. Actinic keratosis: how to differentiate the good from the bad ones? *Eur J Dermatol,* 16:335-339.

Que SKT, Zwald FO, Schmults CD, 2018. Cutaneous squamous cell carcinoma: Incidence, risk factors, diagnosis, and staging. *J Am Acad Dermatol*, 78:237–247.

Rahmati Nezhad P, Riihilä P, Knuutila JS, Viiklepp K, Peltonen S, Kallajoki M, Meri S, Nissinen L, Kähäri VM, 2022. Complement Factor D Is a Novel Biomarker and Putative Therapeutic Target in Cutaneous Squamous Cell Carcinoma. *Cancers*, 14:305.

Rahmati Nezhad P, Riihilä P, Piipponen M, Kallajoki M, Meri S, Nissinen L, Kähäri VM, 2021. Complement factor I upregulates expression of matrix metalloproteinase-13 and -2 and promotes invasion of cutaneous squamous carcinoma cells. *Exp Dermatol*, 30:1631-1641.

Raone B, Patrizi A, Gurioli C, Gazzola A, Ravaioli GM, 2018. Cutaneous carcinogenic risk evaluation in 375 patients treated with narrowband-UVB phototherapy: A 15-year experience from our Institute. *Photodermatol Photoimmunol Photomed*, 34:302-306.

Ratushny V, Gober MD, Hick R, Ridky TW, Seykora JT, 2012. From keratinocyte to cancer: the pathogenesis and modeling of cutaneous squamous cell carcinoma. *J Clin Invest*, 122:464-472.

Reinehr CPH, Bakos RM, 2019. Actinic keratoses: review of clinical, dermoscopic, and therapeutic aspects. *An Bras Dermatol*, 94:637-657.

Reis ES, Mastellos DC, Ricklin D, Mantovani A, Lambris JD, 2018. Complement in cancer: untangling an intricate relationship. *Nat Rev Immunol*, 18:5-18.

Ren ZP, Hedrum A, Pontén F, Nistér M, Ahmadian A, Lundeberg J, Uhlén M, Pontén J, 1996. Human epidermal cancer and accompanying precursors have identical p53 mutations different from p53 mutations in adjacent areas of clonally expanded non-neoplastic keratinocytes. *Oncogene*, 12:765–773.

Reygagne P, Rostain G, 2018. Au-delà des kératoses actiniques, le champ de cancérisation cutané [Beyond actinic keratoses: Field cancerization of the skin]. *Ann Dermatol Venereol*, 145:587-592.

Riches JC, Ramsay AG, Gribben JG, 2012. Immune reconstitution in chronic lymphocytic leukemia. *Curr Hematol Malig Rep*, 7:13–20.

Ricklin D, Hajishengallis G, Yang K, Lambris JD, 2010. Complement: A key system for immune surveillance and homeostasis. *Nat Immunol*, 11:785–797.

Riddick AC, Shukla CJ, Pennington CJ, Bass R, Nuttall RK, Hogan A, Sethia KK, Ellis V, Collins AT, Maitland NJ, Ball RY, Edwards DR, 2005. Identification of degradome components associated with prostate cancer progression by expression analysis of human prostatic tissues. *Br J Cancer,* 92:2171-2180.

Ridky TW, Chow JM, Wong DJ, Khavari PA, 2010. Invasive three-dimensional organotypic neoplasia from multiple normal human epithelia. *Nat Med*, 16:1450–1455.

Riihilä P, Nissinen L, Farshchian M, Kallajoki M, Kivisaari A, Meri S, Grénman R, Peltonen S, Peltonen J, Pihlajaniemi T, Heljasvaara R, Kähäri VM, 2017. Complement Component C3 and Complement Factor B Promote Growth of Cutaneous Squamous Cell Carcinoma. *Am J Pathol*, 187:1186-1197.

Riihilä P, Nissinen L, Farshchian M, Kivisaari A, Ala-Aho R, Kallajoki M, Grénman R, Meri S, Peltonen S, Peltonen J, Kähäri VM, 2015. Complement factor I promotes progression of cutaneous squamous cell carcinoma. *J Invest Dermatol*, 135:579-588.

Riihilä P, Nissinen L, Kähäri VM, 2021. Matrix metalloproteinases in keratinocyte carcinomas. *Exp Dermatol*, 30:50-61.

Riihilä P, Nissinen L, Knuutila J, Rahmati Nezhad P, Viiklepp K, Kähäri VM, 2019. Complement System in Cutaneous Squamous Cell Carcinoma. *Int J Mol Sci*, 20:3550.

Riihilä PM, Nissinen LM, Ala-Aho R, Kallajoki M, Grénman R, Meri S, Peltonen S, Peltonen J, Kähäri VM, 2014. Complement factor H: a biomarker for progression of cutaneous squamous cell carcinoma. *J Invest Dermatol*, 134:498-506.

Robinson SN, Zens MS, Perry AE, Spencer SK, Duell EJ, Karagas MR, 2013. Photosensitizing agents and the risk of non-melanoma skin cancer: a population-based case-control study. *J Invest Dermatol*, 133:1950-1955.

Rollison DE, Messina JL, Cherpelis BS, Fenske NA, Schell MJ, Adeegbe DO, Zhao Y, Amorrortu RP, Akuffo AA, Hesterberg RS, Epling-Burnette PK, 2021. Circulating Immunosuppressive

Regulatory T Cells Predict Risk of Incident Cutaneous Squamous Cell Carcinoma. *Front Med (Lausanne)*, 8:735585.

Rosso S, Zanetti R, Martinez C, Tormo MJ, Schraub S, Sancho-Garnier H, Franceschi S, Gafà L, Perea E, Navarro C, Laurent R, Schrameck C, Talamini R, Tumino R, Wechsler J, 1996. The multicentre south European study 'Helios'. II: Different sun exposure patterns in the aetiology of basal cell and squamous cell carcinomas of the skin. *Br J Cancer*, 73:1447-1454.

Roumenina LT, Daugan MV, Noé R, Petitprez F, Vano YA, Sanchez-Salas R, Becht E, Meilleroux J, Clec'h BL, Giraldo NA, Merle NS, Sun CM, Verkarre V, Validire P, Selves J, Lacroix L, Delfour O, Vandenberghe I, Thuilliez C, Keddani S, Sakhi IB, Barret E, Ferré P, Corvaïa N, Passioukov A, Chetaille E, Botto M, de Reynies A, Oudard SM, Mejean A, Cathelineau X, Sautès-Fridman C, Fridman WH, 2019a. Tumor Cells Hijack Macrophage-Produced Complement C1q to Promote Tumor Growth. *Cancer Immunol Res*,7:1091-1105.

Roumenina LT, Daugan MV, Petitprez F, Sautès-Fridman C, Fridman WH, 2019b. Context-dependent roles of complement in cancer. *Nat Rev Cancer*, 19:698-715.

Rowe DE, Carroll RJ, Day CL, 1992. 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 Derm*, 26:976–990.

Röwert-Huber J, Patel MJ, Forschner T, Ulrich C, Eberle J, Kerl H, Sterry W, Stockfleth E, 2007. Actinic keratosis is an early in situ squamous cell carcinoma: a proposal for reclassification. *Br J Dermatol*, 156 Suppl 3:8-12.

Royle JA, Baade PD, Joske D, Girschik J, Fritschi L, 2011. Second cancer incidence and cancer mortality among chronic lymphocytic leukaemia patients: A population-based study. *Br J Cancer,* 105:1076–1081.

Rutkowski MJ, Sughrue ME, Kane AJ, Ahn BJ, Fang S, Parsa AT, 2010a. The complement cascade as a mediator of tissue growth and regeneration. *Inflamm Res*, 59:897-905.

Rutkowski MJ, Sughrue ME, Kane AJ, Mills SA, Parsa AT, 2010b. Cancer and the complement cascade. *Mol Cancer Res,*8:1453-1465.

Ryan MB, Corcoran RB, 2018. Therapeutic strategies to target RAS-mutant cancers. *Nat Rev Clin Oncol*, 15:709–720.

Sakiyama H, Inaba N, Toyoguchi T, Okada Y, Matsumoto M, Moriya H, Ohtsu H, 1994. Immunolocalization of complement C1s and matrix metalloproteinase 9 (92kDa gelatinase/type IV collagenase) in the primary ossification center of the human femur. *Cell Tissue Res*, 277:239-245.

Salasche SJ, 2000. Epidemiology of actinic keratoses and squamous cell carcinoma. *J Am Acad Dermatol*, 42:4-7.

Sasaki K, Sugai T, Ishida K, Osakabe M, Amano H, Kimura H, Sakuraba M, Kashiwa K, Kobayashi S, 2018. Analysis of cancer-associated fibroblasts and the epithelial-mesenchymal transition in cutaneous basal cell carcinoma, squamous cell carcinoma, and malignant melanoma. *Hum Pathol*, 79:1-8.

Schneider CA, Rasband WS, Eliceiri KW, 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9:671-675.

Sekulic A, Kim SY, Hostetter G, Savage S, Einspahr JG, Prasad A, Sagerman P, Curiel-Lewandrowski C, Krouse R, Bowden GT, Warneke J, Alberts DS, Pittelkow MR, DiCaudo D, Nickoloff BJ, Trent JM, Bittner M, 2010. Loss of inositol polyphosphate 5-phosphatase is an early event in development of cutaneous squamous cell carcinoma. *Cancer Prev Res (Phila)*, 3:1277-1283.

Sengupta S, Harris CC, 2005. p53: traffic cop at the crossroads of DNA repair and recombination. *Nat Rev Mol Cell Biol*, 6:44-55.

Shao SC, Lai CC, Chen YH, Lai EC, Hung MJ, Chi CC, 2022. Associations of thiazide use with skin cancers: a systematic review and meta-analysis. *BMC Med*, 20:228.

Sim RB, Laich A, 2000. Serine proteases of the complement system. *Biochem Soc Trans*, 28:545-550.

Sim RB, Tsiftsoglou SA, 2004. Proteases of the complement system. *Biochem Soc Trans*, 32:21-27.

Sinha RP, Häder DP, 2002. UV-induced DNA damage and repair: a review. *Photochem Photobiol Sci*, 1:225-236.

Sinikumpu SP, Jokelainen J, Keinänen-Kiukaanniemi S, Huilaja L, 2022. Skin cancers and their risk factors in older persons: a population-based study. *BMC Geriatr,* 22:269.

Sørensen HT, Mellemkjaer L, Nielsen GL, Baron JA, Olsen JH, Karagas MR, 2004. Skin cancers and non-hodgkin lymphoma among users of systemic glucocorticoids: a population-based cohort study. *J Natl Cancer Inst*, 96:709-711.

Sorensen R, Thiel S, Jensenius JS, 2005. Mannan-binding-lectin-associated serine proteases, characteristics and disease associations. *Springer Semin Immunopathol*, 27: 299-319.

South AP, Purdie KJ, Watt SA, Haldenby S, den Breems N, Dimon M, Arron ST, Kluk MJ, Aster JC, McHugh A, Xue DJ, Dayal JH, Robinson KS, Rizvi SH, Proby CM, Harwood CA, Leigh IM, 2014. NOTCH1 mutations occur early during cutaneous squamous cell carcinogenesis. *J Invest Dermatol*, 134:2630-2638.

Spencer JM, Kahn SM, Jiang W, DeLeo VA, Weinstein IB, 1995. Activated ras genes occur in human actinic keratoses, premalignant precursors to squamous cell carcinomas. *Arch Dermatol*, 131:796–800.

Stenehjem JS, Robsahm TE, Bråtveit M, Samuelsen SO, Kirkeleit J, Grimsrud TK, 2017. Aromatic hydrocarbons and risk of skin cancer by anatomical site in 25 000 male offshore petroleum workers. *Am J Ind Med*, 60:679–688.

Stratigos AJ, Garbe C, Dessinioti C, Lebbe C, Bataille V, Bastholt L, Dreno B, Concetta Fargnoli M, Forsea AM, Frenard C, Harwood CA, Hauschild A, Hoeller C, Kandolf-Sekulovic L, Kaufmann R, Kelleners-Smeets NWJ, Malvehy J, Del Marmol V, Middleton MR, Moreno-Ramirez D, Pellecani G, Peris K, Saiag P, van den Beuken-van Everdingen MHJ, Vieira R, Zalaudek I, Eggermont AMM, Grob JJ, 2020. European Dermatology Forum (EDF), the European Association of Dermato-Oncology (EADO) and the European Organization for Research and Treatment of Cancer (EORTC). European interdisciplinary guideline on invasive squamous cell carcinoma of the skin: Part 2. Treatment. *Eur J Cancer*, 128:83-102.

Strippoli S, Fanizzi A, Quaresmini D, Nardone A, Armenio A, Figliuolo F, Filotico R, Fucci L, Mele F, Traversa M, De Luca F, Montagna ES, Ruggieri E, Ferraiuolo S, Macina F, Tommasi S, Sciacovelli AM, De Risi I, Albano A, Massafra R, Guida M, 2021. Cemiplimab in an Elderly Frail Population of Patients With Locally Advanced or Metastatic Cutaneous Squamous Cell Carcinoma: A Single-Center Real-Life Experience From Italy. *Front Oncol*, 11:686308.

Su F, Viros A, Milagre C, Trunzer K, Bollag G, Spleiss O, Reis-Filho JS, Kong X, Koya RC, Flaherty KT, Chapman PB, Kim MJ, Hayward R, Martin M, Yang H, Wang Q, Hilton H, Hang JS, Noe J, Lambros M, Geyer F, Dhomen N, Niculescu-Duvaz I, Zambon A, Niculescu-Duvaz D, Preece N, Robert L, Otte NJ, Mok S, Kee D, Ma Y, Zhang C, Habets G, Burton EA, Wong B, Nguyen H, Kockx M, Andries L, Lestini B, Nolop KB, Lee RJ, Joe AK, Troy JL, Gonzalez R, Hutson TE, Puzanov I, Chmielowski B, Springer CJ, McArthur GA, Sosman JA, Lo RS, Ribas A, Marais R, 2012. RAS mutations in cutaneous squamous-cell carcinomas in patients treated with BRAF inhibitors. *N Engl J Med*, 366:207-215.

Suiqing C, Min Z, Lirong C, 2005. Overexpression of phosphorylatedSTAT3 correlated with the invasion and metastasis of cutaneous squamous cell carcinoma. *J Dermatol*, 32:354-360.

Tanvetyanon T, Padhya T, McCaffrey J, Kish JA, Deconti RC, Trotti A, Rao NG, 2015. Postoperative concurrent chemotherapy and radiotherapy for high-risk cutaneous squamous cell carcinoma of the head and neck. *Head Neck*, 37:840–845.

Tejera-Vaquerizo A, Descalzo-Gallego MA, Otero-Rivas MM, Posada-García C, Rodríguez-Pazos L, Pastushenko I, Marcos-Gragera R, García-Doval I, 2016. Skin Cancer Incidence and Mortality in Spain: A Systematic Review and Meta-Analysis. *Actas Dermosifiliogr*, 107:318-328.

Terui T, Ishii K, Ozawa M, Tabata N, Kato T, Tagami H, 1997. C3 production of cultured human epidermal keratinocytes is enhanced by IFNgamma and TNFalpha through different pathways. *J Invest Dermatol*, 108:62-67.

Thai AA, Lim AM, Solomon BJ, Rischin D, 2021. Biology and Treatment Advances in Cutaneous Squamous Cell Carcinoma. *Cancers (Basel)*, 13:5645.

Thomas-Ahner JM, Wulff BC, Tober KL, Kusewitt DF, Riggenbach JA, Oberyszyn TM, 2007. Gender differences in UVB-induced skin carcinogenesis, inflammation, and DNA damage. *Cancer Res*,67:3468-3474.

Thompson AK, Kelley BF, Prokop LJ, Murad MH, Baum CL, 2016. Risk factors for cutaneous squamous cell carcinoma recurrence, metastasis, and disease-specific death: A systematic review and meta-analysis. *JAMA Derm*, 152:419–428.

Timár KK, Dallos A, Kiss M, Husz S, Bos JD, Asghar SS, 2007. Expression of terminal complement components by human keratinocytes. *Mol Immunol*, 44:2578-2586.

Timár KK, Pasch MC, van den Bosch NH, Jarva H, Junnikkala S, Meri S, Bos JD, Asghar SS, 2006. Human keratinocytes produce the complement inhibitor factor H: synthesis is regulated by interferon-gamma. *Mol Immunol*, 43:317-325.

Toll A, Salgado R, Yébenes M, Martín-Ezquerra G, Gilaberte M, Baró T, Solé F, Alameda F, Espinet B, Pujol RM, 2009. MYC gene numerical aberrations in actinic keratosis and cutaneous squamous cell carcinoma. *Br J Dermatol*, 161:1112-1118.

Toll A, Salgado R, Yébenes M, Martín-Ezquerra G, Gilaberte M, Baró T, Solé F, Alameda F, Espinet B, Pujol RM, 2010. Epidermal growth factor receptor gene numerical aberrations are frequent events in actinic keratoses and invasive cutaneous squamous cell carcinomas. *Exp Dermatol*, 19:151-153.

Toriseva MJ, Ala-aho R, Karvinen J, Baker AH, Marjomäki VS, Heino J, Kähäri VM, 2007. Collagenase-3 (MMP-13) enhances remodeling of three-dimensional collagen and promotes survival of human skin fibroblasts. *J Invest Dermatol*, 127:49-59.

Tosi M, Journet A, Duponchel C, Couture-Tosi E, Meo T, 1989. Human complement C1r and C1s proteins and genes: studies with molecular probes. *Behring Inst Mitt*, 65-71.

Traianou A, Ulrich M, Apalla Z, De Vries E, Bakirtzi K, Kalabalikis D, Ferrandiz L, Ruiz-de-Casas A, Moreno-Ramirez D, Sotiriadis D, Ioannides D, Aquilina S, Apap C, Micallef R, Scerri L, Pitkänen S, Saksela O, Altsitsiadis E, Hinrichs B, Magnoni C, Fiorentini C, Majewski S, Ranki A, Proby CM, Stockfleth E, Trakatelli M; EPIDERM Group, 2012. Risk factors for actinic keratosis in eight European centres: a case-control study. *Br J Dermatol*, 167 Suppl 2:36-42.

Trinchieri G, 2012. Cancer and inflammation: an old intuition with rapidly evolving new concepts. *Annu Rev Immunol*, 30:677-706.

Tufaro AP, Chuang JC, Prasad N, Chuang A, Chuang TC, Fischer AC, 2011. Molecular markers in cutaneous squamous cell carcinoma. *Int J Surg Oncol*, 2011:231475.

Uhlen M, Zhang C, Lee S, Sjöstedt E, Fagerberg L, Bidkhori G, Benfeitas R, Arif M, Liu Z, Edfors F, Sanli K, von Feilitzen K, Oksvold P, Lundberg E, Hober S, Nilsson P, Mattsson J, Schwenk JM, Brunnström H, Glimelius B, Sjöblom T, Edqvist PH, Djureinovic D, Micke P, Lindskog C, Mardinoglu A, Ponten F, 2017. A pathology atlas of the human cancer transcriptome. *Science,* 357:eaan2507.

Ujfaludi Z, Tuzesi A, Majoros H, Rothler B, Pankotai T, Boros IM, 2018. Coordinated activation of a cluster of MMP genes in response to UVB radiation. *Sci Rep*, 8:2660.

Vaalamo M, Mattila L, Johansson N, Kariniemi AL, Karjalainen-Lindsberg ML, Kähäri VM, Saarialho-Kere U, 1997. Distinct populations of stromal cells express collagenase-3 (MMP-13) and collagenase-1 (MMP-1) in chronic ulcers but not in normally healing wounds. *J Invest Dermatol*, 109:96-101.

Van Hove L, Hoste E, 2022. Activation of fibroblasts in skin cancer. *J Invest Dermatol,* 142:1026-1031.

van Schaarenburg RA, Suurmond J, Habets KL, Brouwer MC, Wouters D, Kurreeman FA, Huizinga TW, Toes RE, Trouw LA, 2016. The production and secretion of complement component C1q by human mast cells. *Mol Immunol*, 78:164-170.

Väyrynen O, Åström P, Nyberg P, Alahuhta I, Pirilä E, Vilen ST, Aikio M, Heljasvaara R, Risteli M, Sutinen M, Salo T, 2019. Matrix metalloproteinase 9 inhibits the motility of highly aggressive HSC-3 oral squamous cell carcinoma cells. *Exp Cell Res*, 376:18-26.

Vignesh P, Rawat A, Sharma M, Singh S, 2017. Complement in autoimmune diseases. *Clin Chim Acta,* 465:123-130.

Villiers CL, Arlaud GJ, Colomb MG, 1985. Domain structure and associated functions of subcomponents C1r and C1s of the first component of human complement. *Proc Natl Acad Sci U S A*, 82:4477-4481.

Walport MJ, 2001. Complement. First of two parts. *N Engl J Med*, 344:1058-1066.

Wang H, Wang HS, Liu ZP, 2011. Agents that induce pseudo-allergic reaction. *Drug Discov Ther,*5:211–219.

Watt FM, Estrach S, Ambler CA, 2008. Epidermal Notch signalling: Differentiation, cancer and adhesion. *Curr Opin Cell Biol*, 20:171–179.

Weissenborn SJ, Nindl I, Purdie K, Harwood C, Proby C, Breuer J, Majewski S, Pfister H, Wieland U, 2005. Human papillomavirus-DNA loads in actinic keratoses exceed those in non-melanoma skin cancers. *J Investig Dermatol*, 125:93–97.

Wilkins K, Turner R, Dolev JC, LeBoit PE, Berger TG, Maurer TA, 2006. Cutaneous malignancy and human immunodeficiency virus disease. *J Am Acad Dermatol*, 54:189-206.

Wu X, Nguyen B-C, Dziunycz P, Chang S, Brooks Y, Lefort K, Hofbauer GFL, Dotto GP, 2010. Opposing roles for calcineurin and ATF3 in squamous skin cancer. *Nature*, 465:368–372.

Xavier S, Sahu RK, Landes SG, Yu J, Taylor RP, Ayyadevara S, Megyesi J, Stallcup WB, Duffield JS, Reis ES, Lambris JD, Portilla D, 2017. Pericytes and immune cells contribute to complement activation in tubulointerstitial fibrosis. *Am J Physiol Renal Physiol*, 312:F516-F532.

Xiang F, Song HP, Huang YS, 2019. Clinical features and treatment of 140 cases of Marjolin's ulcer at a major burn center in southwest China. *Exp Med*, 17:3403–3410.

Xu C, Jung M, Burkhardt M, Stephan C, Schnorr D, Loening S, Jung K, Dietel M, Kristiansen G, 2005. Increased CD59 protein expression predicts a PSA relapse in patients after radical prostatectomy. *Prostate*, 62:224-232.

Yarosh DB, Pena AV, Nay SL, Canning MT, Brown DA, 2005. Calcineurin Inhibitors Decrease DNA Repair and Apoptosis in Human Keratinocytes Following Ultraviolet B Irradiation. *J Investig Dermatol*, 125:1020–1025.

Yoshinaga S, Hauptmann M, Sigurdson AJ, Doody MM, Freedman DM, Alexander BH, Linet MS, Ron E, Mabuchi K, 2005. Nonmelanoma skin cancer in relation to ionizing radiation exposure among U.S. radiologic technologists. *Int J Cancer*, 115:828–834.

Yu HS, Liao WT, Chai CY, 2006. Arsenic carcinogenesis in the skin. *J Biomed Sci*, 13:657–666.

Zhao L, Li W, Marshall C, Griffin T, Hanson M, Hick R, Dentchev T, Williams E, Werth A, Miller C, Bashir H, Pear W, Seykora JT, 2009. Srcasm inhibits Fyn-induced cutaneous carcinogenesis with modulation of Notch1 and p53. *Cancer Res*, 69:9439-9447.

Zheng Q, Capell BC, Parekh V, O'Day C, Atillasoy C, Bashir HM, Yeh C, Shim EH, Prouty SM, Dentchev T, Lee V, Wushanley L, Kweon Y, Suzuki-Horiuchi Y, Pear W, Grice EA, Seykora JT, 2021. Whole-Exome and Transcriptome Analysis of UV-Exposed Epidermis and Carcinoma In Situ Reveals Early Drivers of Carcinogenesis. *J Invest Dermatol*,141:295-307.

Ziegler A, Jonason AS, Leffell DJ, Simon JA, Sharma HW, Kimmelman J, Remington L, Jacks T, Brash DE, 1994. Sunburn and p53 in the onset of skin cancer. *Nature*, 372:773–776.

Zipfel PF, Heinen S, Józsi M, Skerka C, 2006. Complement and diseases: defective alternative pathway control results in kidney and eye diseases. *Mol Immunol*, 43:97-106.

TURUN
YLIOPISTO
UNIVERSITY
OF TURKU

Painosalama, Turku, Finland 2022

ISBN 978-951-29-9119-8 (PRINT)
ISBN 978-951-29-9120-4 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)