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MARI VALKONEN

HYALURONAN AND ITS METABOLIZING ENZYMES IN MELANOCYTIC TUMORS AND DIFFUSELY INFILTRATING ASTROCYTOMAS

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ABSTRACT

Hyaluronan is an abundant glycosaminoglycan in the extracellular space of many human tissues. It is produced by three hyaluronan synthases (HAS1-3) in the inner leaf of the plasma membrane and catabolized by hyaluronidases (HYALs). Hyaluronan and its metabolizing enzymes participate in many essential processes such as inflammation, wound healing and embryogenesis. Thus, alterations in hyaluronan metabolism have become an interesting topic in cancer research. Hyaluronan concentrations often change in malignant tumors compared to benign tissues. The purpose of this thesis was to investigate hyaluronan metabolism in cutaneous melanocytic tumors and in diffusely infiltrating astrocytomas of the central nervous system.

It is reported that hyaluronan content is low in cutaneous melanomas compared to benign nevi. However, the mechanisms underlying this finding have not been investigated previously. In this thesis, increased staining of hyaluronan and immunostaining of HAS2 were seen in *in situ* melanomas compared to benign melanocytic lesions. Interestingly, declined expression of HAS1 and HAS2 and a subsequent decrease in hyaluronan content were observed in melanoma lesions. Dysplastic nevi, *in situ* melanomas, invasive melanomas and lymph node metastasis of melanoma displayed increased expression of HYAL2 compared to benign nevi. In melanomas, decreased HAS2 immunostaining was associated with several adverse histopathological findings including increased mitotic rate, nodular subtype and reduced number of tumor-infiltrating lymphocytes. Furthermore, decreased HAS1 and HAS2 immunostainings were associated with shorter survival and recurrence-free times of patients.

Diffusely infiltrating astrocytomas are malignant glial cell tumors which include the aggressive grade IV glioblastomas. All astrocytomas demonstrated high hyaluronan content and expression of HAS1, HAS2 and HYAL2 were elevated in the high grade astrocytomas. High level immunopositivity of HAS2 and HYAL2 was associated with increased proliferation of tumor cells, while reduced expression of HAS2 correlated with an increased amount of positive prognostic marker *IDH1* mutations in tumors. High HAS2 staining was associated with shorter overall survival time of patients.

This thesis provides new information on hyaluronan metabolism in two aggressive tumor types. In cutaneous melanoma, hyaluronan content was low due to decreased HAS expression and this correlates with poor prognosis, while in diffusely infiltrating astrocytomas, hyaluronan metabolism shows a different pattern. In astrocytomas, increased HAS2 immunostaining correlated with poor prognosis. Thus, hyaluronan and its metabolizing enzymes are involved in the regulation of cancer progression.

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Medical Subject Heading: Hyaluronic Acid; Hyaluronan Synthases; Melanoma; Skin Neoplasms; Astrocytoma; Enzymes; Nervous System; Staining and Labeling; Prognosis

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TIIVISTELMÄ

Hyaluronaani on yleinen glykosaminoglykaani ihmisen kudoksissa. Sitä tuottavat kolme hyaluronaanisyntaasia (HAS1-3) ja hajottavat hyaluronidaasientsyymit (HYAL:t). Hyaluronaani ja sitä metaboloivat entsyymit osallistuvat useisiin biologisiin prosesseihin kuten tulehdusvasteeseen, haavan paranemiseen ja embryogeneesiin. Tämän vuoksi hyaluronaanista on tullut kiinnostava kohde myös syöpätutkimuksessa. Adenokarsinoomissa hyaluronaanin määrä on yleensä lisääntynyt verrattuna vastaaviin hyvänlaatuisiin kudoksiin. Hyaluronaanimetabolian ennusteellinen ja syöpäbiologinen merkitys on kuitenkin monissa kasvaimissa vielä kiistanalainen. Tämän väitöskirjatyön tarkoituksena oli tutkia hyaluronaanimetaboliaa ihon melanosyyttisissä kasvaimissa ja keskushermoston diffuuseissa astrosyyttisissä glioomissa.

Ihomelanoomissa on aiemmissa tutkimuksissa todettu matalia hyaluronaanipitoisuuksia. Tässä väitöskirjatyössä *in situ*-melanoomissa todettiin vahvaa hyaluronaanivärjäytymistä sekä lisääntynyttä HAS2:n immunovärjäytymistä verrattuna hyvänlaatuisiin melanosyyttisiin (ihonsisäinen-, raja- ja yhdistelmäluomi) luomiin. Vähäinen HAS1- ja HAS2- ilmentyminen ja matala hyaluronaanipitoisuus todettiin pahanlaatuisissa melanoomissa. Korkea HYAL2-ilmentyminen todettiin muissa melanosyyttisissä muutoksissa verrattuna hyvänlaatuisiin ihon pigmenttiluomiin. Vähentynyt HAS2:n immunovärjäytyvyys assosioitui ennusteellisesti epäedullisiin histopatologisiin tekijöihin kuten lisääntyneeseen solujen jakaantumiseen, nodulaariseen melanoomatyyppiin ja vähentyneeseen kasvaimissa esiintyvien lymfosyyttien määrään. Vähäinen HAS1- ja HAS2- immunovärjäytyvyys assosioitui lyhentyneeseen elossaoloaikaan ja lyhentyneeseen taudittomaan aikaan.

Diffuusit astrosyyttiset glioomat ovat pahanlaatuisia neurogliaalisia kasvaimia, joihin kuuluu myös aggressiivinen gradus IV-glioblastooma. Kaikissa astrosytoomissa todettiin korkeita hyaluronaanipitoisuuksia. HAS1-, HAS2- ja HYAL2- ilmentyminen oli vahvaa korkean graduksen astrosytoomissa. Vahva HAS2:n ja HYAL2:n ilmentyminen yhdistyi suurentuneeseen kasvainsolujen jakaantumiseen ja vähentynyt HAS2-ilmentyminen liittyi ennusteellisesti positiiviseen tuumorien *IHD1*-mutaation olemassaoloon. Lisäksi runsas HAS2-ilmentyminen liittyi lyhentyneeseen potilaiden kokonaiselossaoloaikaan.

Tämä väitöskirja tarjoaa uutta tietoa hyaluronaanin metaboliasta sekä hyvän- että pahanlaatuisissa melanosyyttisissä kasvaimissa ja astrosytoomissa. Tulosten perusteella kasvaimissa tapahtuvat hyaluronaanin määrän muutokset johtuvat hyaluronaania tuottavien ja hajottavien entsyymien ilmentymisen muutoksista.

Luokitus: QU 83, WR 500, QZ 360, QZ 380, WB 142
Yleinen suomalainen asiasanasto: hyaluronaani; melanooma; astrosytoomat; entsyymit; histologia; ennusteet

To Tuomas and Albert

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II **Poukka M**, Bykachev A, Siiskonen H, Tyynelä-Korhonen K, Auvinen P, Pasonen-Seppänen S, Sironen R. Decreased expression of hyaluronan synthase 1 and 2 associates with poor prognosis in cutaneous melanoma. *BMC Cancer 16: 313, 2016*

III **Valkonen M**, Haapasalo H, Rilla K, Tyynelä-Korhonen K, Ylermi S, Pasonen-Seppänen. Elevated expression of hyaluronan synthase 2 associates with decreased survival in diffusely infiltrating astrocytomas. *BMC Cancer 18: 644, 2018*

CONTENTS

ABBREVIATIONS

3LL	Lewis Lung Carcinoma
4-MU	4-Methylumbelliferone
AJCC	American Joint Committee on Cancer
Akt	Protein kinase B
ATP	Adenosine triphosphate
ATRX	Alpha-thalassemia/mental retardation syndrome X-linked
bHABC	Biotinylated hyaluronan-binding complex
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BSA	Bovine serum albumin
bFGF	Basic fibroblast growth factor
CAM	Chicken chorioallantoic membrane
CCL5	Chemokine (C-C motif) ligand 5
CD4+	Cluster of differentiation 4
CD44	Cluster of differentiation 44
CEMIP	Cell migration inducing hyaluronan binding protein
CIS	Carcinoma *in situ*
CISH	Chromogenic in situ hybridisation
CHO	Chinese hamster ovary cell
CNS	Central nervous system
COS-1	CV-1 in origin with SV40 genes
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCR7	C-X-C chemokine receptor type 7
Da	Dalton
DAB	3,3-diaminobenzidine
DNA	Deoxyribonucleic acid
DSS	Disease-specific survival
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERBB2	Erythroblastic oncogene B, known also as HER2
ERK	Extracellular signal–regulated kinase
ERM	Ezrin-radixin-moesin
FAK	Focal adhesion kinase
GAG	Glycosaminoglycan
GlcUA	Glucuronic acid
GlcNAc	N-acetyl-D-glucosamine
GPI	Glycosylphosphatidylinositol
HA	Hyaluronan
HABP	Hyaluronan-binding protein
HaCat	Human epidermal keratinocytes
HARE	Hyaluronic acid receptor for endocytosis
HAS	Hyaluronan synthase
HER2 2	Human epidermal growth factor
HIMEC	Human intestinal microvessel endothelial cells
HMW	High molecular weight
HUVEK	Human umbilical vein endothelial cell

HYAL	Hyaluronidase
HYALP1	Hyaluronoglucosaminidase pseudogene 1
HYBID	Hyaluronan-binding protein involved in hyaluronan depolymerization
IαI	Inter-α-inhibitor
IDH	Isocitrate dehydrogenase
IL	Interleukin
iNOS	Nitric oxide synthases
JAK2	Janus kinase 2
LMW	Low molecular weight
LN	Lymph node
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor
MAPK	Mitogen-activated protein kinase
MEKK3	Mitogen activated protein kinase kinase kinase 3
MGMT	O6-methylguanine-DNA methyltransferase
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NF-kappaB	Nuclear factor kappaB
NOS	Not otherwise specified melanoma
NRAS	Neuroblastoma RAS viral oncogene homolog
o-HA	Hyaluronan oligosaccharides
PB	Phosphate buffer
PD-1	Programmed death-1
PDGF-BB	Platelet-derived growth factor, dimer of two beta polypeptides
PEG-PH20	Pegylated PH20 hyaluronidase
PI3-K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and tensin homolog
PolyI:C	Polyinosinic acid:polycytidylic acid
PSA	Prostate specific antigen
RFS	Recurrence-free survival
RHAMM	Hyaluronan-mediated motility receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma
STAT3	Signal transducer and activator of transcription 3
TAM	Tumor-associated macrophages
TGF-β	Transforming growth factor beta
TIL	Tumor-infiltrating lymphocytes
TIMP-1	Tissue metalloproteinase inhibitor 1
TMEM2	Transmembrane protein 2
TNF-α	Tumor necrosis factor alpha
TLR	Toll-like receptor
TSG-6	Tumor necrosis factor-inducible protein 6
UDP	Uridine diphosphate
UV	Ultraviolet
UVB	Ultraviolet B
VALVIRA	Finnish National Supervisory Authority for Welfare and Health
VEGF	Vascular endothelial growth factor

WHO	World Health Organization

1 INTRODUCTION

Hyaluronan is a large glycosaminoglycan that is found abundantly in the extracellular matrixes of human tissues. Since it was first found in the vitreous body of the eye, its involvement in the development of cancer has become clear in recent decades. Tumoral or stromal content of hyaluronan is altered in several epithelial carcinomas such as carcinomas of breast, colon, pancreas and cutaneous and laryngeal squamous cell carcinomas (Ropponen et al., 1998, Hirvikoski et al., 1999, Auvinen et al., 2000, Karvinen et al., 2003, Cheng et al., 2013). Many of these alterations are linked with an adverse patient prognosis (Auvinen et al., 2013, Cheng et al., 2013). In cancers of non-epithelial origin, hyaluronan metabolism is less studied. There is evidence that hyaluronan metabolism may be involved in the pathogenesis of melanomas, gliomas and lymphomas (Karjalainen et al., 2000, Yoshida, T. et al., 2012, Jelicic et al., 2016).

Cutaneous melanoma is an aggressive cancer type arising from melanocytic skin cells. In recent decades, the incidence of cutaneous melanoma has risen (The Finnish Cancer Registry, 2018) and new prognostic markers have emerged alongside new treatment methods. Medications blocking the function of mutated BRAF oncogene and immunotherapies have provided new treatments for many patients with metastatic melanoma. Nevertheless, melanoma causes excess morbidity and mortality which makes it important to continue research into new prognostic and predictive markers.

Diffusely infiltrating astrocytomas are tumors emerging from neuroglial astrocytes or their precursors cells. Their embryological background is similar to melanomas; both of these tumor types originate from neural crest cells thus belonging to the tissues arising from the ectoderm in embryogenesis (Bhatt, Diaz & Trainor, 2013). Diffusely infiltrating astrocytomas consist of tumors with different levels of malignancy but they all share a similar infiltrative growth type in the central nervous system. The most prevalent form of diffusely infiltrating astrocytomas is glioblastoma, which has a notoriously aggressive behavior despite advances in diagnostics and treatment modalities.

Despite advancements, both cancer types can progress quickly and lead to early demise. Therefore, the mechanisms affecting their pathogenesis need to be studied to enable development of better treatments and diagnostic methods. The results of this thesis show that low tumoral hyaluronan content in melanomas is associated with decreased expression of hyaluronan synthases 1 and 2 (HAS1 and HAS2). This expression pattern is linked with adverse histopathological markers and poor overall survival of patients. Instead, high hyaluronan content was detected in diffusely infiltrating astrocytomas. Increased expression of HAS1, HAS2 and hyaluronan catabolizing hyaluronidase 2 (HYAL2) was observed in high grade astrocytomas. As in melanomas, HAS2 emerged as a prognostic factor as its increased expression associated with poor patient survival.

2 REVIEW OF THE LITERATURE

2.1 HYALURONAN AND HYALURONAN BINDING PROTEINS

2.1.1 Structure and localization of hyaluronan

Hyaluronan is a large, linear and nonsulfated glycosaminoglycan (GAG) residing in the extracellular matrixes (ECM) of nearly all human tissues. Hyaluronan is able to bind large amounts of water forming gel-like structures as seen in the vitreous body of an eye or in the synovial fluid. The vitreous body of the cow`s eye was actually the first tissue where hyaluronan was identified (Meyer, Palmer, 1934). Since then, hyaluronan has been found in various other tissues including skin, central nervous system (CNS) and lungs (Tammi et al., 1988, Tammi et al., 1989, Struve et al., 2005, Papakonstantinou et al., 2008, Cargill et al., 2012). Hyaluronan is distributed widely in many tissue types but its amount is especially high in the ECM of connective and stratified epithelial tissues. Stratified epithelia display hyaluronan as seen in the epithelia of the esophagus, skin and oral mucosa (Tammi et al., 1988, Wang, C. et al., 1996, Siponen et al., 2015). Hyaluronan is localized on the bottom layers of stratified epithelium, whereas the uppermost layers are nearly hyaluronan negative (Tammi et al., 1988, Wang et al., 1996, Siponen et al., 2015). Contrary to the stratified epithelia, simple epithelia are generally devoid of hyaluronan as seen in the colon, stomach and breast (Wang et al., 1996, Auvinen et al., 1997). Although the epithelium is negative for hyaluronan, the connective tissue adjacent to the epithelium shows high hyaluronan content (Wang et al., 1996, Auvinen et al., 1997, Ågren et al., 1997).

Skin is one of the major organs where substantial amounts of hyaluronan have been detected. Cutaneous hyaluronan resides in the epidermis and dermis. Epidermal hyaluronan locates in basal and spinous cell layers, whereas the uppermost layer is devoid of it (Tammi et al., 1988, Tammi et al., 1989). In the dermis, fibroblasts are the main cell type producing hyaluronan and other extracellular matrix molecules.

Similarly, to other GAG's, hyaluronan is composed of disaccharide units. Repeating units of D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) form hyaluronan molecules of varying sizes (Fig. 1). Hyaluronan has various functions in the ECM depending on its location and molecular size. The average size of hyaluronan varies from 10^6 to 10^7 Dalton (Da) (Toole, 2004). Furthermore, there are smaller hyaluronan oligosaccharides and low-molecular weight (LMW) hyaluronan (approximately 10^4-0.5 x 10^6 Da) which are formed via cleavage of high molecular weight (HMW) hyaluronan. The molecular size of hyaluronan oligosaccharides is under 10 kDa and they are approximately 24 disaccharide units long. Hyaluronan fragments are hyaluronan molecules which are formed by the cleavage of hyaluronan and can be of varying sizes.

Hyaluronan is synthesized on the plasma membrane and extruded directly into the ECM, contrary to other GAGs which are formed intracellularly in the Golgi apparatus. After its synthesis, hyaluronan can either be discharged into the ECM, where it can reside without binding to the cell surfaces or alternatively it can remain pericellularly attached to its receptors or hyaluronan synthases (Kultti et al., 2006, Pasonen-Seppänen et al., 2012a). Pericellular hyaluronan forms hyaluronan cables or coats which surround the cells (de la Motte, C. A. et al., 2003, Kultti et al., 2006, Jokela et al., 2008). In the ECM, hyaluronan can bind to other molecules and affect surrounding cells.

Hyaluronan is also found intracellularly (Tammi et al., 2001, Evanko, Parks & Wight, 2004). The role of intracellular hyaluronan is not clear, but since both hyaluronan and its intracellular receptor hyaluronan-mediated motility receptor (RHAMM) co-localize in the mitotic spindle, hyaluronan is suggested to bind to RHAMM and affect cell mitosis (Assmann et al., 1999, Maxwell et al., 2003, Evanko, Parks & Wight, 2004, Tolg et al., 2010). Nevertheless, unlike extracellular hyaluronan, the function of intracellular hyaluronan seems to be rather unclear as the majority of intracellularly-detected hyaluronan are intracellular hyaluronan oligosaccharides which remain in vesicles without distributing in the cytosol; this

indicates that it is possibly on the way to be catabolized (Siiskonen et al., 2013). Collectively, the literature suggests hyaluronan is mostly an ECM molecule which can affect cells through different receptors and binding proteins.

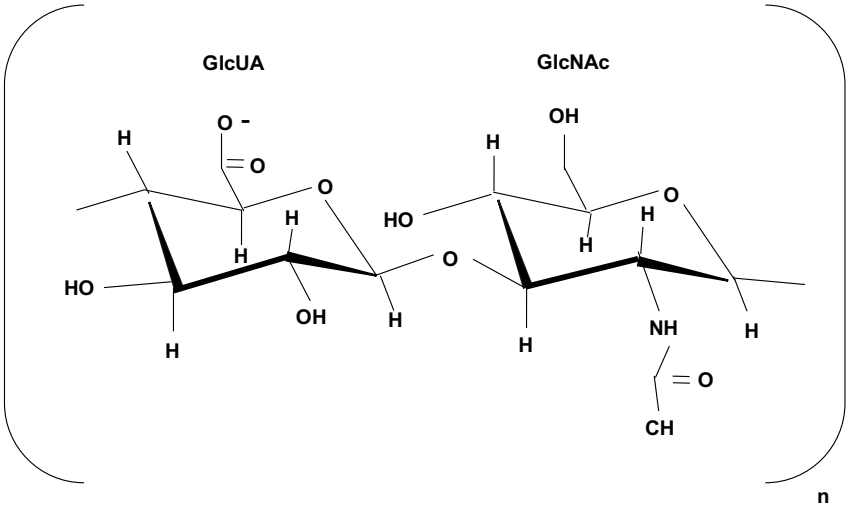

Figure 1. Repeating units of glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) form an unbranched hyaluronan molecule. Modified from Siiskonen, 2013.

2.1.2 Hyaluronan receptors and binding proteins

Once hyaluronan is synthesized it can interact with cells via various receptors and binding proteins. Some of these proteins possess a link domain capable of hyaluronan binding. Hyaluronan binding proteins containing this link domain include CD44, aggrecan, versican, neurocan, brevican, lymphatic vessel endothelial hyaluronan receptor (LYVE-1), stabilin-1/ hyaluronic acid receptor for endocytosis (HARE) and tumor necrosis factor-inducible protein 6 (TSG-6) (Day, Prestwich, 2002). A transmembrane CD44 is probably the best described receptor for hyaluronan (Underhill, Thurn & Lacy, 1985); this is a plasma membrane receptor with a binding site for hyaluronan that resides in its single extracellular domain. Similar to hyaluronan, CD44 is widely expressed in its standard form (CD44s) in multiple different human tissues including hematopoietic tissues, skin, CNS, gastrointestinal tract and endocrine organs (Fox et al., 1994). CD44 binds to standard sized hyaluronan but also small hyaluronan oligosaccharides down to three disaccharide units (Misra et al., 2015). The CD44 gene resides in chromosome 11 and codes for receptors of varying sizes through alternative splicing. The alternative splicing modifies the extracellular part of CD44, but these different splicing variants (CD44v) of CD44 are still able to bind hyaluronan. CD44v are expressed in human tissues although in lesser and slightly different patterns (Fox et al., 1994, Mackay et al., 1994). Multiple CD44vs are expressed in different epithelia whereas hematopoietic tissues express only certain CD44vs (Fox et al., 1994). Hyaluronan-CD44 interactions can lead to the activation of other cell membrane receptors such as numerous receptor tyrosine kinases, including erythroblastic oncogene B/human epidermal growth factor receptor 2 (ERBB2/HER2) and epidermal growth factor receptor (EGFR), which meditate activation of growth factor´ signals (Misra, Toole & Ghatak, 2006). Aside from hyaluronan, CD44 can interact with other extracellular molecules including osteopontin (Dalal et al., 2014), integrins (McFarlane et al., 2015), fibronectin (McFarlane et al., 2015) and matrix metalloproteinases (MMPs) (Chetty et al., 2012).

One of the hyaluronan binding proteins containing link domain (LYVE-1) has a specific role as the major hyaluronan receptor in lymphatic endothelial cells (Banerji et al., 1999). The pattern of LYVE-1 expression

in human tissues is quite restricted. Besides lymphatic endothelial cells, only a few other tissues such as sinusoidal endothelial cells of spleen expresses LYVE-1 (Banerji et al., 1999). Similar to CD44, LYVE-1 is a transmembrane receptor containing a single binding site for hyaluronan. Unlike LYVE-1 and CD44, chondroitin sulfate proteoglycans are extracellular matrix molecules. Chondroitin sulfate proteoglycans that can bind extracellular hyaluronan are aggrecan, versican, neurocan and brevican. These proteoglycans have been identified in several tissues of which cartilage and brain are the best described. In particular, aggrecan has been identified as an important part of cartilage. In cartilage, a hyaluronan-aggrecan interaction is important for the formation of the pericellular matrixes around chondrocytes (Knudson, 1993). The lack of aggrecan has been linked with destruction of cartilage; damaged cartilages express higher levels of catabolized aggrecan (Lark et al., 1997). Besides cartilage, all of the chondroitin sulfate proteoglycans have been detected during brain development and in postnatal brains of mice (Milev et al., 1998). Aggrecan, neurocan and versican are also expressed in neural stem cells (Abaskharoun et al., 2010).

The other classes of hyaluronan binding proteins do not have a link module. These proteins include RHAMM, inter-α-inhibitors (IαI) and plasma hyaluronan-binding protein (HABP2/PHBP) (Day, Prestwich, 2002). IαI are ECM protease inhibitors, which have been linked with inflammation processes together with hyaluronan (Bogdani et al., 2014, Huth et al., 2015).

The best described hyaluronan binding protein without a link module is RHAMM. It was first cloned in 1992 and it is located in chromosome 5 in humans (Hardwick et al., 1992, Spicer et al., 1995). RHAMM locates on the cell surface but it does not have an intracellular domain. Unlike CD44 or LYVE-1, RHAMM can also reside intracellularly (Assmann et al., 1999, Maxwell et al., 2003, Tolg et al., 2010). In particular, RHAMM has been showed to localize with microtubules, which are vital for the successful mitosis of cells (Assmann et al., 1999, Tolg et al., 2010). Thus, the loss of RHAMM causes defects in mitosis (Tolg et al., 2010). Unlike CD44, the plasma membrane expression of RHAMM is not constant but cellular stress generally induces it (Savani et al., 1995, Schwertfeger et al., 2015).

2.2 BIOLOGICAL FUNCTIONS OF HYALURONAN AND ITS RECEPTORS

Originally, it was thought that the primary role of hyaluronan is just to act as a supporting structural molecule. However, it was later discovered that hyaluronan has multiple roles besides being a passive "space filler" molecule.

2.2.1 Proliferation and migration

Hyaluronan is thought to provide hydrated space through which cells can easily migrate. It has been shown to increase migration of several cell types including fibroblasts, vascular smooth muscle cells and glial cells (Kozlova et al., 2012, Kashima et al., 2013, Piao, Wang & Duncan, 2013). Hyaluronan induced cell migration is generally dependent on CD44 as seen in glial and vascular smooth muscle cells (Kashima et al., 2013, Piao, Wang & Duncan, 2013). As these processes need hyaluronan, it is expected that changes in the amount of hyaluronan metabolic enzymes could lead to similar results as is seen in the rat fibroblast 3Y1 cell line (Itano et al., 2002). In this cell line, overexpression of all three hyaluronan synthases (HAS1-3) leads to increased migration of cells and loss of contact inhibition (Itano et al., 2002).

Another important cell process which is partly regulated by hyaluronan is proliferation. Hyaluronan has been shown to induce proliferation of vascular smooth muscle cells (Kashima et al., 2013) and fibroblasts (Kozlova et al., 2012). Overexpression of HASes in confluent fibroblast cell cultures increases the proportion of cells in S and G2/M phase compared to control cells, which indicates that overproduction of hyaluronan can induce proliferation (Itano et al., 2002). Treatment with uridine diphosphate glucose (UDP-Glc) increases hyaluronan synthesis and cellular proliferation and migration in keratinocytes (Jokela et al., 2014). These studies indicate that hyaluronan has a substantial influence on cell motility and growth.

2.2.2 Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a vital process in tissue development. It is a process which allows epithelial type cells to acquire a mesenchymal cell phenotype, which leads to enhanced cell migration and loss of cell adhesion. Knock-down of HAS2 has been showed to disrupt EMT in cardiac development (Camenisch et al., 2000). Interestingly, EMT could be restored by administering exogenous hyaluronan (Camenisch et al., 2000). In murine epicardial cells, increased cell motility and expression of mesenchymal cell markers can be induced with transforming growth factor (TGF-β) 2 (Craig et al., 2010). Craig et al showed that TGF-β2 increases hyaluronan synthesis via mitogen activated protein kinase kinase kinase 3 (MEKK3) - extracellular signal–regulated kinase (ERK) 1/2- ERK5 signaling route (Craig et al., 2010). This caused up-regulation of HAS2 and subsequent EMT, which could be inhibited with hyaluronidase treatment and inhibition of CD44 (Craig et al., 2010). In rat mesothelial cells, EMT can be induced with wounding or epidermal growth factor (EGF) treatment. This causes increased hyaluronan synthesis via up-regulation of HASes and expression of CD44 indicating that hyaluronan and CD44 have roles in EMT (Koistinen et al., 2017).

2.2.3 Inflammation

Hyaluronan and its receptors participate in various inflammatory responses. High hyaluronan staining is observed in basal squamous cells in oral lichen planus, indicating a possible association of hyaluronan with chronic inflammation (Siponen et al., 2015). In another chronical autoimmune disease, multiple sclerosis, hyaluronan accumulation has been detected in demyelinated plaques and HMW hyaluronan prevents remyelination in mice (Back et al., 2005). Hyaluronan also participates in acute inflammation. Treatment with a viral mimic, polyinosinic acid:polycytidylic acid (polyI:C), increases the amount of hyaluronan in cell surfaces and induces formation of hyaluronan cables in mucosal smooth muscle cells (de la Motte, C. A. et al., 2003). These cables facilitate the binding of mononuclear leucocytes into cell surfaces of mucosal smooth muscle cells (de la Motte, C. et al., 2009). Similar hyaluronan cables have also been demonstrated in epidermal keratinocytes, airway smooth muscle cells and renal proximal tubular epithelial cells (Selbi et al., 2006, Jokela et al., 2008, Lauer et al., 2009).

Hyaluronan and CD44 participate in the attraction of inflammatory cells as CD44-hyaluronan interaction is needed in leucocyte rolling and adhesion in cell extravasation from blood vessels (DeGrendele, Estess & Siegelman, 1997, Nandi, Estess & Siegelman, 2004). Removal of hyaluronan and knock-down of CD44 decreases lymphocyte extravasation in an experimental autoimmune encephalomyelitis mice model (Winkler et al., 2012). Similar results have been observed in intestinal inflammation. Hyaluronan initiates inflammation by clustering around blood vessels during the early stages of intestinal inflammation (Kessler et al., 2008). Tumor necrosis factor alpha (TNF-α) induces hyaluronan deposition in human intestinal microvessel endothelial cells (HIMEC) (Kessler et al., 2008). This hyaluronan is able to bind mononuclear leukocytes to HIMECs (Kessler et al., 2008).

2.2.4 Tissue injury

Hyaluronan levels of tissues change after different stimulations. Ultraviolet (UV) radiation causes skin irritation and damage. Exposure to UV radiation leads to hyaluronan accumulation in the epidermis whereas the amount of hyaluronan in the dermis declines after UV exposure; this result suggests a possible function for hyaluronan in normal tissue responses to injuries (Averbeck et al., 2007). Similarly, increased hyaluronan and CD44 levels are seen in the epidermis following a physical injury (Tammi et al., 2005). Inhibition of hyaluronan synthesis with mannose decreases neutrophil infiltration, migration and monocyte binding of dermal fibroblasts in a rat wound model (Jokela et al., 2013). This result suggests hyaluronan participates in normal inflammation processes and the creation of granulation tissue during wound healing in the skin (Jokela et al., 2013).

Similar to the skin, hyaluronan has a role in tissue healing in the CNS (Struve et al., 2005, Lin et al., 2009), but its effects in the CNS differ from the skin. In the CNS, hyaluronan prevents glial scar formation and decreases the amount of glial cells in the area of injury (Lin et al., 2009). In contrast, inhibition of hyaluronan synthesis in the skin reduces migration of dermal fibroblasts (Jokela et al., 2013). Injection of hyaluronidase has been showed to promote proliferation of astrocytes in the spinal cord of mice (Struve et al., 2005). Shortly after a spinal cord injury in mice hyaluronan is degraded and afterwards its amount is increased compared to surrounding tissue; this suggests a possible role for hyaluronan in directing the proliferation of astrocytes at the side of injury (Struve et al., 2005).

2.2.5 Development

Cell migration and proliferation are generally needed when tissues rapidly change, as in embryogenesis. Hyaluronan and HAS2 enable normal embryogenesis and normal cardiovascular development (Camenisch et al., 2000). Knock-down of HAS2 causes a non-vital phenotype in mice (Camenisch et al., 2000). Abundant hyaluronan is also seen in the early stages of skin development (Ågren et al., 1997).

The hyaluronan content of tissues can change during development and ageing as seen in the brain, where hyaluronan accumulates over time (Cargill et al., 2012). The changes in hyaluronan metabolism can impair normal CNS development as CD44 knock-down mice have defects in hippocampal memory retention (Raber et al., 2014). Furthermore, knock-down of hyaluronan synthase 3 (HAS3) causes hyaluronan depletion in the ECM of the hippocampus, which caused increased epileptic seizures in mice (Arranz et al., 2014).

2.3 HYALURONAN SYNTHASES

2.3.1 The structure and localization of hyaluronan synthases

Hyaluronan is produced by enzymes named hyaluronan synthases (Fig. 2). All mammalians have three hyaluronan synthase enzymes (HAS1-3), which produce hyaluronan from its precursor sugars, UDP-GlcNAc and UDP-GlcUA, directly into the ECM (Fig. 3). All vertebrates possess the ability to synthetize hyaluronan but not all animals have this ability; for example, insects and sponges are not able to synthetize hyaluronan (DeAngelis, 2002, Weigel, DeAngelis, 2007). Besides vertebrates, some bacteria can produce hyaluronan (Weigel, DeAngelis, 2007). Thus far, only one virus, a phycodnavirus, has been identified with an active HAS (DeAngelis et al., 1997, Weigel, DeAngelis, 2007). In humans HAS1 and HAS2 were identified in 1996 with high level of homology between mouse and human HASes (Itano, Kimata, 1996, Shyjan et al., 1996, Spicer, Augustine & McDonald, 1996, Watanabe, Yamaguchi, 1996). HAS3 was identified in 1997 and it is also highly homologous (Spicer, Olson & McDonald, 1997). In humans, *HAS1* is located on chromosome 19, *HAS2* on chromosome 8 and *HAS3* on chromosome 16 (Spicer et al., 1997).

Enzymatically, active HASes are located on the plasma membrane and they contain both transmembrane and membrane-associated domains (Fig. 2). HASes have 4-6 transmembrane domains and eukaryotic cells have two more transmembrane domains compared to bacterial HASes (Weigel, Hascall & Tammi, 1997). There are also 1-2 membrane-associated domains which do not go through the whole plasma membrane (Weigel, DeAngelis, 2007). All three HASes produce hyaluronan in the inner leaf of the plasma membrane, where UPD-sugars are added to the reducing end of hyaluronan. Thus, the synthesis of hyaluronan is considerably different from other GAGs which are synthesized in the Golgi apparatus. HASes are active enzymes only when they reside on the plasma membrane, but they can also reside intracellularly where they are produced. HAS1 is localized intracellularly near the Golgi area and both HAS1 and HAS2 are observed in the endoplasmic reticulum (ER) (Törrönen et al., 2014). Instead, low amounts of intracellular HAS3 have been found immunohistochemically compared to HAS1 and HAS2 (Törrönen et al., 2014).

HASes form hyaluronan from its two intracellular substrates, UDP-GlcNAc and UDP-GlcUA with β-1-3 and β-1-4 glycosidic bonds. Enzyme activities are dependent on the concentration of substrates. HAS1

has a higher K_m value for both substrates than HAS2 and HAS3 indicating that high concentrations of substrates are necessary for efficient hyaluronan synthesis (Itano et al., 1999). Similarly, *in vitro* studies suggest that HAS1 requires significantly higher concentrations of both UPD-sugars in order to produce hyaluronan coats in transfected COS-1 cells (Rilla et al., 2013).

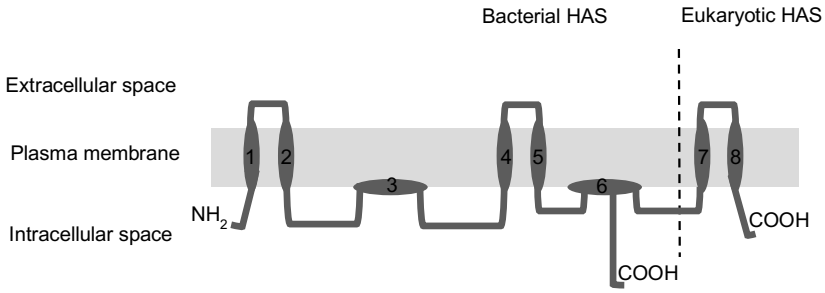

Figure 2. The proposed topology of hyaluronan synthases (HASes). Both N- and C-terminal ends and enzymatically active site reside intracellularly. Modified from Weigel, Hascall & Tammi, 1997.

As enzymatic activities of HASes differ from each other, the molecular forms of produced hyaluronan also varies. The size of the hyaluronan coat produced by different HASes varies: HAS1-transfectants produce smaller pericellular hyaluronan coats compared to HAS2- and HAS3-transfectants on COS-1 and rat 3Y1 fibroblasts cell lines (Itano et al., 1999). Also, the actual size of the hyaluronan molecules produced by HASes differ. HAS2 generally produces hyaluronan with the highest molecular mass, e.g., larger than 3.9×10^6 in Chinese hamster ovary (CHO) cells (Brinck, Heldin, 1999). The molecular sizes depend on the cell type as HAS2 in 3Y1 fibroblasts produces hyaluronan with molecular masses of 2×10^5 to 2×10^6 Da compared to larger than 3.9×10^6 in CHO cells (Brinck, Heldin, 1999, Itano et al., 1999). In 3Y1 fibroblasts, HAS1- and HAS2-transfectants produce larger hyaluronan than HAS3-transfectans with molecular mass of 2×10^5 to $\sim 2 \times 10^6$ Da (Itano et al., 1999). These differences in molecular mass are important as some of the biological effects of hyaluronan depend on its size.

2.3.2 Regulation of hyaluronan synthesis

Hyaluronan synthesis can be altered by regulation of HASes via three mechanisms: by the availability of precursor units (UDP-GlcNAc and UDP-GlcUA) and by transcriptional or posttranslational regulation of HASes.

The substrates of hyaluronan originate from intermediates in glycolysis, thus glucose metabolism can affect the availability of UDP-GlcNAc and UDP-GlcUA. Increased substrate availability due to hyperglycemia and lack of insulin favor cancer progression via increased hyaluronan synthesis in mice models (Twarock et al., 2017). This effect was caused by increased substrate availability of hyaluronan by disrupting glycolysis (Twarock et al., 2017). Extracellular UDP-Glc has also been shown to induce expression of HAS2 in keratinocytes via activation of G_i-coupled receptors, janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) (Jokela et al., 2014). The hyaluronan coat produced by HAS1 is inducible with various cytokines, but increased concentrations of glucose and GlcN can also induce coat formation (Siiskonen et al., 2014). These studies show that hyaluronan synthesis can be affected via extracellular changes in the amount of UDP-sugars.

Many growth factors and cytokines affect hyaluronan metabolism by changing the transcriptional activity of HASes. EGF increases HAS2 messenger RNA (mRNA) levels in rat epidermal keratinocytes causing increased hyaluronan production (Pienimäki et al., 2001) (Pienimäki et al., 2001). Interleukin-1-β

(IL-1β), TGF-β and TNF-α can induce hyaluronan synthesis via activation of HAS1 (Siiskonen et al., 2014). Human skin fibroblasts have showed enhanced hyaluronan production via activation of HAS2 with TGF-β1, basic fibroblast growth factor (bFGF), EGF, and platelet-derived growth factor, dimer of two beta polypeptides (PDGF-BB) (Nagaoka et al., 2015). TGF-β1 also caused increased hyaluronan production by increasing HAS1 up-regulation (Nagaoka et al., 2015). Interestingly, the molecular size of produced hyaluronan was dependent on the growth factor that was used (Nagaoka et al., 2015). This phenomenon may be explained by differential down-regulation of hyaluronan-binding protein involved in hyaluronan depolymerization (HYBID) caused by the same growth factors (Nagaoka et al., 2015). PDGF-BB also stimulates hyaluronan synthesis in cardiomyocytes (Hellman et al., 2010). The influence of growth factors also depends on the cell type. EGF causes increased hyaluronan production and increased mRNA levels of HAS2 and HAS3 in organotypic rat epidermal keratinocyte cell cultures whereas TGF-β decreased hyaluronan synthesis via reduced levels of HAS2 and HAS3 (Pasonen-Seppänen et al., 2003). In contrast, in human skin fibroblasts, TGF-β causes activation of HAS1 and increased hyaluronan synthesis (Nagaoka et al., 2015). Interestingly, TGF-β has been shown to up-regulate expression of HAS1 and at the same time it downregulates HAS3 in fibroblast-like synoviocytes (Stuhlmeier, Pollaschek, 2004). Despite the opposite effects on HASes, TGF-β treatment increased hyaluronan accumulation (Stuhlmeier, Pollaschek, 2004). Extracellular adenosine triphosphate (ATP) induces increased mRNA levels of HAS2 in human epidermal keratinocytes (HaCaT cells) and a subsequent increase in hyaluronan synthesis, despite ATP not being a growth factor or cytokine (Rauhala et al., 2018). The release of ATP into the extracellular space is generally related to cellular stress, such as physical trauma (Yin et al., 2007) or UV radiation (Inoue, Hosoi & Denda, 2007). This is consistent with the notion that hyaluronan content of tissues can change in such situations.

Post-translational modifications for hyaluronan synthesis include ubiquitination, dimerization and phosphorylation. Monoubiquitination of HAS2 is vital for its activity as a mutation in the ubiquitination acceptor site diminishes the activity of HAS2 (Karousou et al., 2010). Dimerization of HAS2 also seems to be needed for its activity (Karousou et al., 2010). Phosphorylation of HAS2 decreases hyaluronan synthesis in human aortic smooth muscle cells (Vigetti et al., 2011). Interestingly, the effect of phosphorylation can be opposite as seen in ovarian tumor cells where phosphorylation of HAS1-3 increases their activity (Bourguignon, L. Y., Gilad & Peyrollier, 2007).

Tissue inflammation and trauma alter the hyaluronan content of tissues, which is often associated with altered expression of HASes. Mechanical trauma in the articular cartilage leads to activation of HAS1 which increases hyaluronan deposition in the injured area (Chan et al., 2015). Epidermal wounding also causes increased mRNA levels of HAS2 and HAS3 (Tammi et al., 2005). Ultraviolet B (UVB) radiation up-regulates all three HASes (Kakizaki et al., 2008, Rauhala et al., 2013). The increased expression of HASes regulates normal tissue remodeling after injury. HAS1-/- mice showed increased fibrosis in the injury site after trauma in the articular cartilage but there were no differences in hyaluronan levels of wild type and HAS1-/- mice (Chan et al., 2015). Instead, HAS1-/- mice showed increased activation of genes associated with inflammation and tissue fibrosis causing increased fibrosis and non-existent regeneration in their cartilage, compared to wild type mice (Chan et al., 2015).

2.4 HYALURONAN CATABOLISM

Hyaluronan degradation is catalyzed by hyaluronidases. In humans, there are five functional hyaluronidase enzymes, HYAL1, HYAL2, HYAL3, HYAL4 and PH-20 (HYAL5) (Fig. 3). Of these, HYAL1 and HYAL2 are mainly responsible for hyaluronan degradation in humans. In addition, there is a sixth enzyme known as hyaluronoglucosaminidase pseudogene 1 (HYALP1/PHYAL1), which is not an active hyaluronidase in humans, but it is transcribed into RNA (Csoka, Scherer & Stern, 1999). In murine sperm HYALP1 is an active enzyme (Miller, Shao & Martin-DeLeon, 2007). *HYAL1*, *HYAL2* and *HYAL3* are located in chromosome 3 and *HYAL4*, *PH-20* and *HYALP1* are located in chromosome 7 (Csoka, Scherer & Stern, 1999, Stern, Jedrzejas, 2006).

Hyaluronidases can cleave β-1-4 glycosidic bonds which degrades hyaluronan into smaller molecules. HYAL2 cleaves hyaluronan into oligosaccharides of ~ 50 disaccharide units, whereas HYAL1 produces smaller oligosaccharides (Stern, Jedrzejas, 2006). These cleavage products are active molecules in tissues, and compared to HMW hyaluronan, they can cause differential effects on cells. For example, small hyaluronan oligosaccharides can induce rat fibroblast migration, whereas HMW hyaluronan (500 kDa) decreases migration of the same cells (Tolg, Telmer & Turley, 2014). HYAL2 overexpression induces shedding of CD44 from the plasma membrane of rat fibroblast cells preventing formation of the hyaluronan coat and decreasing cell motility (Duterme et al., 2009). Shedding of CD44 was caused by separating CD44 from intracellular ezrin-radixin-moesin (ERM) and decreasing activation of the ERM (Duterme et al., 2009).

HYAL2 is a cell membrane-bound enzyme with a glycosylphosphatidylinositol (GPI)-anchor (Andre et al., 2011). After it has created smaller hyaluronan units, this catabolized hyaluronan is endocytosed via receptor-mediated endocytosis (Tammi et al., 2001). The oligosaccharides are further cleaved into smaller oligosaccharides by lysosomal HYAL1 (Stern, 2003). Hyaluronidases are also able to catabolize chondroitin and sulfated chondroitins.

HYAL1 and HYAL2 are widely expressed in most human tissues (Csoka, Scherer & Stern, 1999). The turnover of hyaluronan is high in humans. It has been estimated that there is 15 g hyaluronan in 70 kg human and 5 g of this is turnovered daily (Stern, Jedrzejas, 2006). Hyaluronan is metabolized locally in tissues but also especially in the lymphatic system and liver. Intravenously administrated hyaluronan accumulates in mouse liver and spleen, and less so in the kidneys (Jadin, Bookbinder & Frost, 2012). Subcutaneously administrated hyaluronan was degraded into LMW hyaluronan (under 17 kDa) and in four days most of the hyaluronan signal had disappeared from the superficial skin layers (Jadin, Bookbinder & Frost, 2012). HYAL1 is the main hyaluronidase responsible for the degradation of hyaluronan in liver, whereas knock-down of HYAL2 causes HMW hyaluronan accumulation in the lymph nodes and plasma (Bourguignon, V., Flamion, 2016). Both hyaluronidases metabolize hyaluronan in peripheric tissues as knock-down of HYAL1 or HYAL2 leads to accumulation of hyaluronan in skin and muscles (Bourguignon, Flamion, 2016). Both hyaluronidases are also active in kidneys as their lack causes accumulation of hyaluronan (Colombaro et al., 2015). HYAL1 and HYAL2 null mice had increased hyaluronan concentrations in plasma and medulla and outer cortex of kidneys (Colombaro et al., 2015). Additionally, HYAL2 null mice had increased hyaluronan content in the inner medulla of the kidneys (Colombaro et al., 2015).

Hyaluronidases also contribute to tissue homeostasis. Protein levels of HYALs decrease after trauma in aged mice skin, which may partly contribute to slower healing (Reed et al., 2013). In embryogenesis, HYAL2 affects development of the heart (Chowdhury et al., 2013). HYAL2 knock-out causes thickening of the cardiac valves and some of the HYAL2-/- mice exhibited atrial dilatation, cardiac hypertrophy and thickening of the pulmonary alveolar septa (Chowdhury et al., 2013). All the HYAL2-/- mice had increased hyaluronan accumulation in myocardium and heart valves (Chowdhury et al., 2013); some were viable, but the mortality rate was higher compared to wild type mice (Chowdhury et al., 2013). Interestingly, HAS2 deficiency also impairs cardiovascular development and causes a non-vital phenotype (Camenisch et al., 2000). Currently the only known human disorder caused by changes in hyaluronan metabolism is mucopolysaccharidos IX. Mucopolysaccharidosis IX is a rare disorder with hyaluronidase deficiency caused by mutation in HYAL1 (Natowicz et al., 1996, Triggs-Raine et al., 1999). Unlike HYAL1 and HYAL2, HYAL3 does not have an essential role in hyaluronan turnover as its knock-down does not cause hyaluronan accumulation or any major organ defects in murine model (Atmuri et al., 2008). This finding emphasizes the role HYAL1 and HYAL2 as the main hyaluronidases in humans.

The enzyme activity and expression of hyaluronidases are regulated by growth factors and cytokines. PDGF-BB causes up-regulation of HYAL1 mRNA in dermal human fibroblasts without changes in the protein levels of HYAL1 and HYAL2 (Li, L. et al., 2007). TGF-β does not affect the expression levels of hyaluronidases but it enhances hyaluronidase activity significantly in these cells (Li et al., 2007). The inflammatory cytokines, TNF-α and IL-1 β, can up-regulate together hyaluronidase activity and expression of HYAL1, HYAL2 and HYAL3 in human bronchial epithelial cells (Monzon et al., 2008). Furthermore,

cytokines can up-regulate expression of HYAL1 mRNA in human periodontal ligament fibroblasts and up-regulate expression of HYAL2 and HYAL3 mRNA in cartilage chondrocytes (Flannery et al., 1998, Ohno et al., 2002). Increased hyaluronidase activities have been reported in bronchoalveolar lavages asthmatic people suggesting an involvement of HYALs in inflammatory responses (Monzon et al., 2008). Physical stimulus, e.g. UVB radiation, may increase expression of HYAL1 and HYAL2 (Rauhala et al., 2013). Reactive oxygen species (ROS), which are able to fragment hyaluronan, can also increase expression of HYAL2 and hyaluronidase activity of human bronchial epithelial cells (Monzon et al., 2010).

Hyaluronan can alternatively be catabolized by ROS, cell migration inducing hyaluronan binding protein (CEMIP or KIAA1199) or transmembrane protein 2 (TMEM2). CEMIP is expressed in human skin and synovial fibroblasts (Yoshida, H., Nagaoka, Kusaka-Kikushima et al., 2013). Unlike hyaluronidases, hyaluronan cleavage by CEMIP involves the clathrin-coated pit pathway and cleaves β-endo-N-acetylglucosamine bonds of hyaluronan (Yoshida et al., 2013, Yoshida, H., Nagaoka, Nakamura et al., 2013). Reduction of ROS leads to decline of hyaluronan catabolism in skin organ type culture and in airway epithelial cells suggesting its active role in hyaluronan catabolism (Ågren, Tammi & Tammi, 1997, Manzanares et al., 2007).

HYAL1 and HYAL2 are only able to degrade hyaluronan in low pH, while the optimum pH for a recently found hyaluronan degrading enzyme, TMEM2, is between 6 and 7. TMEM2 is able to degrade extracellular hyaluronan in a Ca2+ -dependent manner into intermediate-sized fragments which are thereafter internalized and completely degraded in the lysosomes (Yamamoto et al., 2017).

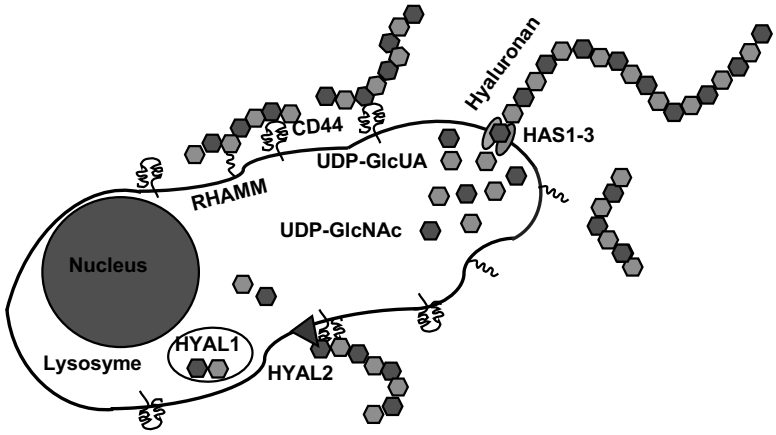

Figure 3. Hyaluronan synthesis and catabolism in a cell. Hyaluronan is formed from intracellular uridine diphosphate glucuronic acid (UPD-GlcUA) and uridine diphosphate N-acetyl-D-glucosamine (UDP-GlcNAC) substrates by three cell membrane hyaluronan synthases (HAS1-3). Hyaluronan is catabolized in the plasma membrane by hyaluronidase 2 (HYAL2) and intracellularly by lysosomal enzyme hyaluronidase 1 (HYAL1). CD44 and hyaluronan-mediated motility receptor (RHAMM) are the main receptors of hyaluronan. CD44 is a plasma membrane receptor, whereas RHAMM can locate either intracellularly or on the plasma membrane.

2.5 HYALURONAN OLIGOSACCHARIDES AND LOW MOLECULAR WEIGHT HYALURONAN INDUCE ANGIOGENESIS AND PARTICIPATE IN INFLAMMATION

The effects of hyaluronan depend on its molecular size. HMW hyaluronan (10^6 to 10^7 Da) is the native form of hyaluronan in human tissues and as previously noted it increases cell migration and proliferation

(Kashima et al., 2013, Piao, Wang & Duncan, 2013). Hyaluronan influences cellular functions via binding to its cell surface receptors. Hyaluronan oligosaccharides (under 10 kDa and 24 disaccharides long) and LMW hyaluronan (approximately 10^4-0.5 x 10^6 Da) have been shown to promote angiogenesis and lymphangiogenesis (Gao et al., 2008, Lennon et al., 2014, Wu, M. et al., 2014). Hyaluronan oligosaccharides can also increase migration, proliferation and wound healing of endothelial cells and thus stimulate angiogenesis (Gao et al., 2008). Reduced vascularization has been observed in tumors originating from the B16 melanoma cell line and in wound healing experiments on CD44-null mice; these results suggest a significant role for CD44 in angiogenesis (Cao et al., 2006). Blocking of CD44 decreases endothelial proliferation and blocking of RHAMM decreases migration of endothelial cells and *in vivo* vascularization in mice (Savani et al., 2001). Inhibition of LYVE-1, the major hyaluronan receptor in lymphatic endothelial cells, decreases the proliferation, migration and tube formation of lymphatic endothelial cells induced by LMW hyaluronan (Wu et al., 2014). Hyaluronan oligosaccharides of 6 (o-HA6), 8 (o-HA8) and 10 (o-HA10) saccharide residues stimulate human umbilical vein endothelial cell (HUVEK) proliferation and angiogenesis in the chicken chorioallantoic membrane (CAM) assay (Cui et al., 2009). Furthermore, these hyaluronan oligomers stimulate the expression of vascular endothelial growth factor (VEGF) in the same cells (Cui et al., 2009). Recently, Wang and co-workers (2016) found that hyaluronan oligosaccharides promote angiogenesis in a diabetic mouse model, which enhanced diabetic wound healing (Wang, Y. et al., 2016). Hyaluronan oligosaccharides increased the phosphorylation of Src and ERK, and expression of TGF-β1, which may be responsible for accelerated proliferation, migration and tube formation of endothelial cells (Wang et al., 2016). Thus, hyaluronan oligosaccharides seem to accelerate angiogenesis which affects endothelial cells directly and indirectly via upregulation of angiogenic growth factor expression.

In addition, hyaluronan oligosaccharides have shown to act both as pro-inflammatory and anti-inflammatory mediators. In many cases they act as pro-inflammatory mediators by activating nuclear factor kappaB (NF-kappaB) via CD44 and toll-like receptor-4 (TLR-4) causing increased expression of inflammatory cytokines TNF-α, IL-6 and IL-1b (Campo et al., 2010). TNF-α increases LMW hyaluronan production in normal murine synovial fibroblasts and fibroblasts exposed to collagen induced arthritis, which mimic rheumatoid arthritis (Campo et al., 2012). Inhibition of HYAL1-3 decreased TNF-α inducible up-regulation of inflammatory cytokines (IL-1β, IL-6), MMP-13 and nitric oxide synthases (iNOS) (Campo et al., 2012). This result suggests that hyaluronan catabolism and production of LMW hyaluronan can induce inflammatory responses in synovial fibroblasts (Campo et al., 2012). Similar results have been obtained in other cell types; hyaluronan fragments (2000 and 50 saccharides) derived from platelets induce IL-6 and IL-8 production in monocytes suggesting that platelets can induce inflammatory processes by hyaluronan cleavage (de la Motte et al., 2009). LMW hyaluronan (approximately 200 kDa) has been shown to induce M1 macrophage formation which are pro-inflammatory macrophages (Sokolowska et al., 2014). Interestingly, hyaluronan fragments (50-200 kDa) have also been shown to induce conversion of monocytes into M2 type immunosuppressive macrophages (Kuang et al., 2007). Although hyaluronan oligosaccharides mediate inflammatory responses, opposite results have been reported as both pegylated PH20 hyaluronidase (PEG-PH20) and hyaluronan oligosaccharides treatment delay the onset of symptoms and decrease demyelination in experimental autoimmune encephalomyelitis in mice (Winkler et al., 2012, Winkler et al., 2013). Hyaluronan oligosaccharides impair lymphocyte rolling which is involved in the development of demyelinating diseases of the CNS, which would indicate that hyaluronan oligosaccharides can function as anti-inflammatory mediators in the CNS (Winkler et al., 2013). Thus, the effects of hyaluronan oligosaccharides on inflammatory processes seem to depend on cell and tissue type.

Hyaluronan oligosaccharides participate also in wound healing. 6 and 8mer hyaluronan oligosaccharides accelerate wound healing both *in vitro* and *in vivo* (Tolg, Telmer & Turley, 2014). 6mer hyaluronan oligosaccharides also increase immunostainings of TGF-β1 and M1 and M2 macrophages in wound healing areas in murine models, indicating healthy wound repair (Tolg, Telmer & Turley, 2014). In addition, in a murine model, oligosaccharide treatment has been shown to enhance recovery from spinal cord injury (Wakao et al., 2011).

2.6 HYALURONAN IN CANCER

2.6.1 Hyaluronan and its receptors in epithelial cancers

Changes in the hyaluronan content of tumors have been reported in several types of cancers (Sironen et al., 2011) (Table 1). Hyaluronan content may be changed either in cancer cells or in the tumor stroma. Tumoral hyaluronan content is associated with the tissue type from which the tumor originated. Hyaluronan content is high in tumors originating from tissues that normally have low amounts or totally devoid of hyaluronan, such as carcinomas of the breast, colon, rectum, pancreas and prostate (Ropponen et al., 1998, Auvinen et al., 2000, Lipponen et al., 2001, Josefsson et al., 2011, Auvinen et al., 2013, Cheng et al., 2013). This increased hyaluronan accumulation can either be seen in stroma (Auvinen et al., 2000, Lipponen et al., 2001, Josefsson et al., 2011, Auvinen et al., 2013) or in tumor cells (Ropponen et al., 1998, Auvinen et al., 2000, Lipponen et al., 2001, Auvinen et al., 2013, Cheng et al., 2013). In colorectal (Ropponen et al., 1998), breast (Auvinen et al., 2000, Auvinen et al., 2013), prostate (Josefsson et al., 2011), ovarian (Anttila et al., 2000) and pancreatic ductal adenocarcinomas (Cheng et al., 2013), high hyaluronan amount in cancer cells is associated with shorter overall survival time. Similarly, increased stromal hyaluronan concentration is associated with shorter survival time in breast (Auvinen et al., 2000, Auvinen et al., 2013) and prostate carcinomas (Lipponen et al., 2001, Josefsson et al., 2011). Furthermore, the role in tumor progression is indicated by high stromal hyaluronan content that is associated with increased postoperative prostate specific antigen (PSA) levels (Rizzardi et al., 2014). High hyaluronan amount in the primary tumor is associated with increased lymph node and distant metastasis in prostate and breast carcinomas (Lipponen et al., 2001, Auvinen et al., 2013). In breast, strong stromal hyaluronan staining is also associated with HER2-positivity, being a negative prognostic factor (Auvinen et al., 2013).

Hyaluronan is an abundant constituent of the normal stratified squamous epithelium. Compared to adenocarcinomas, the metabolism of hyaluronan is different in cancers originating from squamous epithelia. Cutaneous and esophageal carcinoma *in situ* show higher hyaluronan content than the normal squamous epithelia in those organs (Wang et al., 1996, Karvinen et al., 2003). In a murine model, hyaluronan is increased in hyperplastic skin caused by UV radiation indicating that increased hyaluronan accumulation is observed in non-malignant lesions (Siiskonen et al., 2011). Hyaluronan content of tumors change in invasive phenotypes. The poorly differentiated cutaneous squamous cell carcinomas show patchy and low hyaluronan staining, whereas well-differentiated carcinomas have strong and diffuse patterns (Karvinen et al., 2003). Similarly, low hyaluronan content is an adverse prognostic factor in oral squamous cell carcinoma (Kosunen et al., 2004). Patchy hyaluronan accumulation with varied staining intensity is associated with lymph node metastasis in oral and laryngeal squamous cell carcinomas (Hirvikoski et al., 1999, Kosunen et al., 2004). In laryngeal carcinoma, this is also associated with distant metastases (Hirvikoski et al., 1999). Low hyaluronan content in the cancer cells of tumors arising from squamous epithelium seem to be associated with worsened prognosis of patients, contrary to adenocarcinomas. The stromal staining of hyaluronan is generally intense in tumors arising from stratified squamous epithelium (Wang et al., 1996, Karvinen et al., 2003, Kosunen et al., 2004), but does not associate with poor prognosis in oral squamous cell carcinoma (Kosunen et al., 2004).

Alternations of CD44 expression are similar to that of hyaluronan in several cancers. Thus, the high amount of hyaluronan is associated with high levels of CD44. High expression of stromal CD44 is associated with shorter survival time of breast carcinoma patients (Auvinen et al., 2013). Similarly, poorly differentiated squamous cell carcinomas show patchy and weak staining compared to well-differentiated tumors (Karvinen et al., 2003). Irregular staining of CD44 has been shown to correlate with lymph node and distant metastases in laryngeal squamous cell carcinoma (Hirvikoski et al., 1999). High levels of another hyaluronan receptor, RHAMM, is associated with raised PSA in prostate carcinoma after prostatectomy (Rizzardi et al., 2014).

2.6.2 Hyaluronan and its receptors in non-epithelial cancers

In non-epithelial cancers, such as lymphomas, leukemias, mesenchymal tumors and cancers arising from neural crest, the knowledge about hyaluronan metabolism is rather limited. Cells originating from the neural crest include neurons, glial cells, melanocytes, connective tissue cells, cartilage cells and bone cells. High hyaluronan content in cancer cells is known to be associated with shorter overall survival time in malignant peripheral nerve sheath tumors (Ikuta et al., 2014). In contrast, the overall and recurrence-free survival times are shortened in melanomas with low hyaluronan levels (Karjalainen et al., 2000). Low hyaluronan levels in melanoma cells is associated with negative prognostic markers, such as high Breslow thickness and more advanced stage (Karjalainen et al., 2000). In contrast, stromal hyaluronan staining does not correlate with the prognosis in melanoma (Karjalainen et al., 2000).

In lymphomas, high CD44 levels in serum has been linked with poorer survival in pediatric Hodgkin`s and non-Hodgkin`s lymphomas (Taçyildiz et al., 2001). High serum levels of the CD44 splicing variant, CD44v6, is also associated with decreased overall survival of patients with aggressive non-Hodgkin`s lymphomas (Sasaki, Niitsu, 2000). In the follicular lymphoma, high immunoreactivity of CD44 is associated with worsened survival of patients (Jelicic et al., 2016). Thus, CD44 seems to be a negative prognostic marker in aggressive and more indolent lymphomas.

In tumors arising from the neural crest, changes in CD44 expression have been linked with patient survival. High CD44 immunoreactivity in metastatic osteosarcomas implicates poorer prognosis than tumors with weaker CD44 staining (Gvozdenovic et al., 2013). Still, the overall survival of all patients with osteosarcomas, including non-metastatic diseases, did not correlate with CD44 immunostaining significantly (Gvozdenovic et al., 2013). High CD44 expression is also associated with an increased grade of astrocytomas (Yoshida et al., 2012). The prognostic significance is more controversial; one study noted an association with decreased survival of glioblastoma patients (Pietras et al., 2014). In another study, the difference was not significant (Guadagno et al., 2016) and in one study increased CD44 transcription correlated with better prognosis (Wei et al., 2010). In proneural types of glioblastomas, low CD44 expression is linked with improved survival time (Pietras et al., 2014). Interestingly, perivascular areas of high-grade gliomas are rich with CD44 (Yoshida et al., 2012, Guadagno et al., 2016). Contrary to other tumors arising from the neural crest, CD44 expression is linked to the amount of hyaluronan in melanoma (Karjalainen et al., 2000). Thus, decreased CD44 expression is associated with shorter overall and recurrence-free survival (Karjalainen et al., 2000). However, brain metastases have high CD44v6 expression and the expression in the primary melanoma predicts increased risk of brain metastasis (Marzese et al., 2015).

2.7 BIOLOGICAL FUNCTIONS OF HYALURONAN AND ITS RECEPTORS IN TUMOR CELLS

2.7.1 Hyaluronan and its receptors impact on cancer cells proliferation and chemotherapy resistance

The biological role of hyaluronan and CD44 in various cancers is complex. Proliferation of melanoma and breast cancer cells partly depends on hyaluronan as inhibition of hyaluronan synthesis significantly decreases cell proliferation in both cell types (Kultti et al., 2009). Proliferation of melanoma cells has also been shown to increase with exogenous hyaluronan (Ahrens et al., 2001). Similar results have also been obtained in lung cancer cells (Song et al., 2019). However, opposite results have been reported as overexpression of HAS3 decreases MV3 melanoma cells proliferation, which was restored with application of hyaluronidase (Takabe et al., 2015). The overexpression of HAS3 induces cell cycle arrest at G1/G0 (Takabe et al., 2015). Similarly, proliferation of breast cancer cells reduces, and apoptosis increases with application of extremely HMW hyaluronan (Zhao et al., 2019). CD44 also participates in tumor growth as treatment with anti-CD44 antibody induces cellular apoptosis (Wiranowska et al., 2010). In an *in vivo*-pancreatic cancer model, overexpression of HASes and the consequent high tumoral hyaluronan content

results in larger tumors (Kultti et al., 2014). On the other hand, knock-down of CD44 decreases tumor growth of glioblastomas and increases survival in animal models (Xu, Stamenkovic & Yu, 2010). Inhibition of tumor growth was caused by increased apoptosis and decreased proliferation of glioma cells (Xu, Stamenkovic & Yu, 2010). Knock-down of CD44 also sensitized mice to the effects of temozolomide and carmustine thus improving their survival times (Xu, Stamenkovic & Yu, 2010). This sensitization was caused by increased activation of the Hippo signaling pathway, which leads to increased cell apoptosis induced by oxidative stress (Xu, Stamenkovic & Yu, 2010). High expression of CD44 inhibited activation of the Hippo signaling pathway (Xu, Stamenkovic & Yu, 2010).

Hyaluronan and CD44 also seem to participate in chemotherapy resistance in squamous carcinoma cells through EGFR-mediated pathways as inhibition of EGFR reduces hyaluronan-induced resistance to cisplatin (Wang, S. J., Bourguignon, 2006). Silencing CD44 decreases phosphorylation of EGFR in oral carcinoma cells and causes decreased expression of EGFR and phosphorylated EGFR in xenograft tumors on mice; this led to inhibition of tumor growth (Perez et al., 2013). In the same study, overexpression of CD44 led to increased migration and proliferation of cancer cells and resistance to cisplatin (Perez et al., 2013). Antibodies against EGFR are currently being used as a treatment for several metastatic cancers, including head and neck carcinomas and the association of EGFR with CD44 represents a potentially new therapeutic option (Perez et al., 2013).

2.7.2 Hyaluronan and its receptors impact on cancer cells migration and invasion

Some effects of hyaluronan are derived from its ability to change the structure of the ECM surrounding cancer cells. Because hyaluronan is capable of binding to water, it is thought to provide a hydrated space in which tumor cells can easily migrate and invade surrounding tissue. Invasion of cells is an ability that is closely related to cell migration, but invasion of cells includes other mechanisms than just cell movement. During invasion, cancer cells degrade the surrounding ECM, with for example an increased expression of MMPs.

High tumoral hyaluronan content is in many cases favorable for cancer cell migration (Wang, Bourguignon, 2006, Cheng et al., 2016, Shigeeda et al., 2017). Hyaluronan and CD44 facilitate migration of glioma cells (Yoshida et al., 2012, DeSouza et al., 2013) and anti-CD44 antibodies decrease the migration of glioma and squamous cell carcinoma cells, indicating that hyaluronan mediated migration is CD44-dependent (Wang, Bourguignon, 2006, Yoshida et al., 2012). Cleavage of CD44 via MMP-9 also induces migration of glioma cells (Chetty et al., 2012). The extracellular domain region of CD44 seems to be partly needed for secretion and gelatinolytic activity of MMP-9 and thus for migration and invasion of glioma cells (Chetty et al., 2012). Inhibition of hyaluronan synthesis or degradation of hyaluronan with *Streptomyces* hyaluronidase induces the cleavage of focal adhesion kinase (FAK), which is a known cellular adhesion protein also contributing to cell proliferation and migration (Twarock et al., 2010). Decreased hyaluronan levels cause inhibition of migration, proliferation and filopodia formation in esophageal carcinoma cells (Twarock et al., 2010). Inhibition of hyaluronan synthesis or RHAMM induces inactivation of extracellular ERK1/2, a part of the signaling pathway that normally transmits extracellular growth signals (Twarock et al., 2010).

Hyaluronan induces tumor cells invasion (Shigeeda et al., 2017) and the hyaluronan-CD44 interaction participates in the invasion of glioma cells (Kim, Y., Kumar, 2014). As in migration, both CD44 and RHAMM can facilitate invasion. CD44 participates the invasion of glioma cells (Chetty et al., 2012, Kim, Kumar, 2014) and blocking of RHAMM decreases hyaluronan-mediated invasion of malignant pleural mesothelioma cells (Shigeeda et al., 2017). Inhibition of hyaluronan synthesis with 4-MU decreases invasion of melanoma cells in fibroblast-contracted collagen lattices (Edward et al., 2010); these results are contrary to *in vivo* studies on melanoma (Karjalainen et al., 2000).

Although increased hyaluronan content seems to be favorable for the dissemination of some cancer cell types, opposite results have also been published. Migration of MV3 melanoma cells decreases with overexpression of HAS3, despite a large pericellular hyaluronan coat created by this overexpression

(Takabe et al., 2015). These effects are likely transmitted through the inactivation of the ERK1/2 pathway as ERK phosphorylation was reduced with the overexpression of HAS3 (Takabe et al., 2015). Similarly, increased hyaluronan content is not always associated with increased tumor growth. HMW hyaluronan is formed in many tissues including skin and heart, in the long lived rodent species, the naked mole rats (Tian et al., 2013). This HMW hyaluronan prevents tumor formation in these animals (Tian et al., 2013). Degradation of hyaluronan with activation of HYAL2 or prevention of its formation by downregulation of HAS2 in naked mole rats skin fibroblasts leads to tumor promotion in xenograft experiments in immunodeficient mice (Tian et al., 2013). The control mice, which were injected with fibroblasts capable of producing HMW hyaluronan, did not form tumors (Tian et al., 2013). These results indicate that although in many cases high hyaluronan expression seems to be beneficial for cancer cells, in some cases hyaluronan might actually have a significant inhibitory effect on carcinogenesis. The function of hyaluronan in cancer cells might change depending on the situation. High pericellular hyaluronan content surrounding ovarian cancer cells decreases adhesion to peritoneal cells in an *in vitro*-model compared to cells with a low hyaluronan content; this indicates that high hyaluronan content could prevent peritoneal dissemination of ovarian cancer (Tamada et al., 2012). Interestingly, migration of cells that produce abundant hyaluronan was increased, compared to cells with low hyaluronan content (Tamada et al., 2012). Although higher hyaluronan levels increased cell migration and would therefore indicate increased malignancy, the peritoneal dissemination is a common metastatic pathway in ovarian carcinoma which highlights the importance of low hyaluronan amount in the dissemination of ovarian carcinoma (Tamada et al., 2012). The role of hyaluronan in carcinogenesis seems to differ depending on the cell type but also inside the same cancer; this may indicate that it is not yet known which factors affect the content of hyaluronan in tissues. Furthermore, some of the effects of hyaluronan might depend on its size.

2.7.3 LMW hyaluronan in cancer dissemination and angiogenesis

Hyaluronan fragments have been discovered in prostate cancer tissues whereas normal prostate tissues do not exhibit such hyaluronan, thus providing evidence that smaller molecular weight hyaluronan is produced in human cancers (Lokeshwar et al., 2001). Higher serum levels of LMW hyaluronan have been measured in patients with lymph node metastases of breast carcinoma, compared to patients without metastases (Wu, M. et al., 2015). In contrast, total hyaluronan levels were similar in patients with lymph node metastases, compared to patients without metastases; these results indicate that LMW hyaluronan levels predict breast carcinoma metastases better than total hyaluronan levels (Wu et al., 2015). LMW hyaluronan injected into the tumor region increases lymph node metastases of melanoma in a mouse model, whereas HMW hyaluronan did not change the incidence of lymph node metastases, compared to control mice (Du et al., 2016). This was likely caused by increased melanoma cell migration across the disrupted lymphatic endothelial cell layer which was caused by reduced vascular endothelial cadherin-mediated intercellular adhesion (Du et al., 2016).

Lower molecular weight hyaluronan could also facilitate the tumor progression via MMP's. Instead of HMW hyaluronan, expression of MMP-9 and MMP-13 are induced by hyaluronan oligosaccharides in primary murine embryonic fibroblasts and Lewis Lung Carcinoma (3LL) cells (Fieber et al., 2004). LMW hyaluronan promotes growth and migration of thyroid carcinoma cells via cell membrane TLR-4, which causes expression of C-X-C chemokine receptor type 7 (CXCR7) (Dang et al., 2013). Chemokine receptors are able to promote tumor dissemination in multiple ways, such as increased cell migration and angiogenesis (Kakinuma, Hwang, 2006). LMW hyaluronan seems to promote carcinogenesis through the activation of this receptor type. These results were supported by increased immunopositivity of TLR-4 and CXCR7 in papillary thyroid carcinoma tissues (Dang et al., 2013). In a murine model, LMW hyaluronan induced tumor growth was blocked via knock-down of TLR4 or CXCR7 (Dang et al., 2013). The effects of LMW hyaluronan may depend on tumor type. Mice treated with systemic LMW hyaluronan produced smaller tumors than those treated with HMW hyaluronan in a colorectal carcinoma model (Alaniz et al., 2009). Small hyaluronan oligosaccharides can diminish versican inducible ovarian cells migration and

formation of the pericellular hyaluronan coat, indicating a potential role for tumor suppressors in ovarian cancer (Ween et al., 2011).

Angiogenesis is vital for tumor growth and progression. LMW hyaluronan has been shown to increase *in vivo* angiogenesis and the capability of human endothelial cells to form capillary-like structures (Lennon et al., 2014). The effect of LMW hyaluronan can be blocked by silencing CD44 indicating that LMW hyaluronan-mediated angiogenesis is transferred through CD44 (Lennon et al., 2014). Angiogenesis seems to be dependent on lower molecular weight hyaluronan as normal molecular weight hyaluronan is incapable of promoting endothelial cell migration and proliferation (Gao et al., 2008). In endothelial cells, hyaluronan oligosaccharides can also increase VEGF mRNA levels, whose increased expression promotes angiogenesis (Cui et al., 2009). Expression of another angiogenetic growth factor, bFGF, remained unchained after hyaluronan oligosaccharide treatment (Cui et al., 2009).

2.8 HYALURONAN SYNTHASES IN CANCER

The HASes have been shown to affect prognosis of several cancers. High HAS2 immunostaining is associated with decreased survival of patients with pancreatic ductal adenocarcinoma (Cheng et al., 2013). Similar results have been obtained in mice, as overexpression of HAS3 in pancreatic cancer cells causes increased accumulation of hyaluronan and tumor size (Kultti et al., 2014). Increased transcription levels of HAS1 is associated with poorer survival in colon carcinoma patients; this result fits together with increased hyaluronan content in human colorectal carcinomas (Ropponen et al., 1998, Yamada et al., 2004). The immunostaining intensities of HAS1-3 are increased in the endometrial carcinoma, compared to normal endometrium, but there were no changes in the respective mRNA levels, indicating increased turnover times of HASes in carcinomas (Nykopp et al., 2010). Increased HAS1-3 transcription and protein expression levels have also been detected in bladder carcinomas and elevated HAS1 mRNA levels are associated with increased metastases (Kramer et al., 2011). Aligning with these results, silencing of HAS1 leads to decreased growth and increased apoptosis of bladder carcinoma cells indicating a possible anti-apoptotic role for HAS1 (Golshani et al., 2008). Elevated expression of HAS1-3 in the stromal cells of human breast carcinomas is associated with shorter overall survival time and the expression of all three correlates with increased relapse rate (Auvinen et al., 2014). In contrast, some *in vivo* animal models indicate that high expression of HASes can inhibit tumor growth. Thus, overexpression of HAS2 inhibits tumor formation in intracranial and subcutaneous tumors derived from a murine astrocytoma cell line incapable of producing HYALs (Enegd et al., 2002). Similarly, silencing of HAS2 in naked mole rat cells induces increased tumor formation in mice (Tian et al., 2013).

HAS2 has also been suggested to promote carcinogenesis through mechanisms independent of hyaluronan. TGF-β is an inducer of EMT in epithelial cells. Porsch and co-workers showed that silencing of HAS2 prevents TGF-β-dependent EMT and this was independent of extracellular hyaluronan and CD44 (Porsch et al., 2013). Exogenous hyaluronan is also not able to counteract decreased migration and cell proliferation caused by silencing of HAS2 in breast cancer cells, suggesting that HAS2 has routes other than hyaluronan synthesis that influence breast cancer cell motility (Li, Y. et al., 2007). One possible mechanism is the suppression of tissue metalloproteinase inhibitor 1 (TIMP-1) by HAS2 (Bernert, Porsch & Heldin, 2011). HAS2 silencing induces TIMP-1 expression and decreases breast cancer cell invasion and phosphorylation of FAK, which were rescued with inhibition of TIMP-1 (Bernert, Porsch & Heldin, 2011). This result indicates that HAS2 regulates the expression of TIMP-1, which in turn can affect activation of FAK and subsequent signaling routes (Bernert, Porsch & Heldin, 2011). Similar results have been obtained in esophageal squamous carcinoma cells as silencing of HAS3 and HAS2 has been showed to induce cleavage of FAK in them (Twarock et al., 2010). HASes can also affect the expression of MMP's as silencing of HAS2 and HAS3 in colon carcinoma cell line inhibits expression of MMP-7 (Dunn et al., 2009).

2.9 HYALURONIDASES IN CANCER

Altered expression of HYALs is another characteristic mechanism through which tumor cells can modify hyaluronan metabolism in the tumors. Low level immunostaining and low mRNA levels of HYAL1 have been demonstrated in endometrial carcinoma (Nykopp et al., 2010, Nykopp et al., 2015). Decreased immunostaining of HYAL1 is also associated with decreased recurrence-free survival time and adverse clinicopathological factors in endometrial carcinoma (Nykopp et al., 2015). Similarly, mRNA levels of HYAL1 are low in serous ovarian tumors correlating with high hyaluronan content (Nykopp et al., 2009). Weak immunostaining of HYAL1 is also associated with poor survival of patients in pancreatic ductal adenocarcinoma (Cheng et al., 2013). Knowledge about HYALs is rather limited in non-epithelial cancers, but diffuse large B-cell lymphomas contain low mRNA levels of HYAL2 compared to reactive lymph nodes, small lymphocytic lymphoma/chronic lymphocytic leukemias, follicular lymphomas and mantel cell lymphomas (Bertrand et al., 2005). In this setting, increased hyaluronan accumulation was also observed in diffuse large B-cell lymphomas, although there were no significant differences in hyaluronidase activity in respective tumor tissues (Bertrand et al., 2005). This could be explained by changes in the activity of other HYALs (Bertrand et al., 2005).

Although decreased expression of HYALs seems to be associated with adverse factors in many cancers, the role of HYALs in carcinogenesis is quite diverse as increased expression of HYALs has been reported in cancers that have increased hyaluronan accumulation or increased expression of HASes. Increased transcription levels and immunostaining of HYAL1 is associated with high grade bladder cancer, metastatic disease and declined survival (Kramer et al., 2011). High levels of HYAL1 mRNA and high enzymatic activity of hyaluronidases have also been reported in colorectal cancer (Bouga et al., 2010), despite the fact that in this cancer epithelial hyaluronan accumulation is associated with poorer prognosis (Ropponen et al., 1998). This would indicate that there is high hyaluronidase activity in colorectal cancer; naturally it is possible that hyaluronan synthesis activity is simultaneously increased in colorectal cancer thus explaining the high hyaluronan content in them.

In line with results obtained from clinical material, results from *in vitro* studies have been conflicting. Overexpression of HYAL1 increases migration, invasion, proliferation and angiogenesis of breast cancer cells (Tan et al., 2011). Similarly, overexpression of HYAL1 led to increased migration and proliferation of prostate carcinoma cells (Bharadwaj et al., 2009, McAtee et al., 2015). This suggests that HYALs enhance cancer cell dissemination and tumor growth despite the fact that in prostate and breast cancer hyaluronan accumulation is a negative prognostic factor (Josefsson et al., 2011, Auvinen et al., 2013). Nevertheless, opposite results have been reported as overexpression of HYAL1 causes decreased proliferation of rat colon carcinoma cell, compared to HAS2 overexpressive cells (Jacobson et al., 2002).

Fast intracranial tumor growth and rich vascularization is associated with increased expression of HYAL2 in tumors formed by murine astrocytoma cells (Novak et al., 1999). In contrast, colon carcinoma cells overexpressing HYAL1 led to smaller tumor growth and increased necrosis compared to mock-transfected and HAS2 overexpressing tumors (Jacobson et al., 2002). Interestingly, overexpression of HYAL1 with HAS2 or HAS3 caused considerably enhanced prostate cancer cell migration and tumor growth and metastasis in an *in vivo* mouse prostate carcinoma model (Bharadwaj et al., 2009). Such a synergistic effect could be explained by the creation of hyaluronan oligosaccharides as both hyaluronan synthesis and degradation is needed for their formation. HYALs facilitate tumor progression by creating hyaluronan oligosaccharides, which can induce cleavage of CD44 and tumor cell migration (Sugahara et al., 2006). Expression of HYALs seem to be tightly regulated as prostate carcinoma cells which had high hyaluronidase activity of HYAL1 or blocked HYAL1 expression showed decreased tumor formation in an *in vivo* mouse model and decreased cell proliferation (Lokeshwar et al., 2005). In contrast, cancer cells with moderate hyaluronidase activity formed larger tumors (Lokeshwar et al., 2005). These results would suggest that the optimal amount of HYALs for tumor growth and dissemination of prostate carcinoma is somewhere between high and low expression. It might be that synergistic expression with HASes enhances carcinogenesis the most.

There are ongoing studies concerning the role of HYALs in cancer treatment, in cancers with high hyaluronan content. Pancreatic adenocarcinomas are tumors with high hyaluronan content that are associated with poor prognosis (Cheng et al., 2013). PEGPH20 hyaluronidase has been demonstrated to lower hyaluronan content, thus improving chemotherapeutic drug delivery and increasing function of blood vessels in tumor (Jacobetz et al., 2013). The combination of PEGPH20 with gemcitabine increased survival in pancreatic adenocarcinoma murine model (Jacobetz et al., 2013). Currently this same drug is going through phase III trials.

2.10 THE EFFECTS OF TUMOR MICROENVIRONMET ON CANCER DISSEMINATION

As previously stated, the changes in the accumulation of hyaluronan may occur in tumor tissue but also in the peritumoral microenvironment. In human prostate carcinoma, hyaluronan accumulation is observed in the intratumoral stroma and morphologically non-malignant peritumoral prostatic stroma. This accumulation is associated with increased carcinoma-specific death. Increased mRNA levels of HAS1 are also seen in non-malignant stroma from a mouse model indicating that cancer cells could induce hyaluronan synthesis in the surrounding non-malignant cells (Josefsson et al., 2011).

Cancer cells can affect the hyaluronan synthesis of stromal cells. Melanoma cells activate hyaluronan synthesis via up-regulation of HAS2 in fibroblasts (Pasonen-Seppänen et al., 2012b, Willenberg et al., 2012). This causes increased cellular proliferation, cell movement and expression of MMPs in fibroblasts (Pasonen-Seppänen et al., 2012b). Alterations in the hyaluronan metabolism in stromal cells can affect the survival of cancer cells. Knock-down of CD44 in cancer-associated fibroblasts impairs drug resistance of tumor cells (Kinugasa, Matsui & Takakura, 2014). Increased expression of HAS2 is seen in cancer associated fibroblasts cultured with esophageal squamous cell carcinoma cells (Kretschmer et al., 2016). This co-culturing increased the expression of chemokine (C-C motif) ligand 5 (CCL5) in fibroblasts and the increase could be abrogated with inhibition of hyaluronan synthesis via 4-MU (Kretschmer et al., 2016). CCL5 is a proinflammatory chemokine, which takes part in the recruitment of inflammatory cells. Interestingly, hyaluronidase treatment could inhibit binding of CD4+ T-helper cells in xenograft tumor models (Kretschmer et al., 2016). These results indicate that tumor associated fibroblasts can change recruitment of immune system cells in tumors (Kretschmer et al., 2016).

Hyaluronan can affect the recruitment of immune cells by forming hyaluronan cables. These are known to attract mononuclear leukocytes (de la Motte, C. A. et al., 2003). Activation of the immune system can have dramatic effects on tumors. Suppression of HAS2 in tumoral fibroblasts causes diminished angiogenesis and lymphangiogenesis with declined recruitment of tumor-associated macrophages (TAM) in a breast cancer model (Kobayashi et al., 2010). Recruitment of TAMs has an impact on tumor progression as increased number of TAMs has been shown to correlate with high hyaluronan and CD44 content in breast carcinoma and it decreased overall survival of patients (Tiainen et al., 2015). In particular, M2-like TAMs can promote tumor progression via multiple mechanisms including anti-inflammation, angiogenesis and tumor cell proliferation (Hao et al., 2012).

The ECM includes multiple molecules other than hyaluronan, depending on the tissue type. Some of these interact with hyaluronan and can modulate its effects on carcinogenesis. Silencing of a hyaluronan binding ECM molecule, versican, causes decreased cell proliferation, migration and increased adhesion to type I collagen, laminin and fibronectin in melanoma cells indicating its role in dissemination of melanoma (Hernandez et al., 2011). Osteopontin is an ECM protein whose expression correlates with poor prognosis in high grade gliomas (Erpolat et al., 2013). It also participates in glioma cell movement with hyaluronan. Hyaluronan induced migration is partly regulated by osteopontin in glioma cells lacking phosphatase and tensin homolog (PTEN) tumor suppressor gene via the phosphatidylinositol 3-kinase (PI3-K) /Protein kinase B (Akt) pathway (Kim, M. S. et al., 2005). This indicates that PTEN regulates glioma cells migration partly via osteopontin expression as wild type PTEN cell migration was unaffected by hyaluronan treatment (Kim et al., 2005). Glioma cell motility was significantly decreased with anti-osteopontin

antibody treatment in PTEN mutated cells (Kim et al., 2005). It seems that the effects of hyaluronan can be widely modified via other ECM molecules.

Table 1. The association of hyaluronan (HA), CD44, HAS1, HAS2, HAS3, HYAL1 and HYAL2 with poor prognosis (overall or disease-free survival) on cancer patients in human cancers. Location indicates which part of the has used in assessment (cells, stroma, serum or tissue). Arrows indicate the direction of changes in hyaluronan, CD44, HAS1-3 and HYAL1-2. ↑ Increased ↓ Decreased.

Cancer	Location	HA	CD44	HAS1	HAS2	HAS3	HYAL1	HYAL2	Reference
Breast	Cells	↑	ns						Auvinen et al., 2013
Breast	Cells			↑	ns	ns			Auvinen et al., 2014
Breast	Stroma	↑	↑						Auvinen et al., 2013
Breast	Stroma			↑	↑	↑			Auvinen et al., 2014
Breast, triple-negative	Cells							↑	Maierthaler et al., 2015
Colorectal	Cells	↑							Ropponen et al., 1998
Colorectal	Cells		↑						Wu, Q. et al., 2015
Colorectal	Stroma	ns							Ropponen et al., 1998
Colon	Tissue			↑	ns	ns			Yamada et al., 2004
Endometrial	Cells						↓		Nykopp et al., 2015
Follicular lymphoma	Cells		↑						Jelicic et al., 2016
Glioblastoma	Tissue		↓						Wei et al., 2010
Glioblastoma	Tissue		↑						Pietras et al., 2014
Glioblastoma	Cells		ns						Guadagno et al., 2016
Hodgkin`s lymphoma	Serum		↑						Taçyildiz et al., 2001
Kidney	Tissue		↑	ns	↑		ns		Chi et al., 2012
Lung adenoca.	Cells	ns							Pirinen et al., 2001
Lung adenoca.	Stroma	↑							Pirinen et al., 2001
Lung adenoca.	Cells		↓						Sung et al., 2015
NSCLC**	Cells		↓						Pirinen et al., 2000
NSCLC**	Cells	↓							Pirinen et al., 2001

Cancer	Location	HA	CD44	HAS1	HAS2	HAS3	HYAL1	HYAL2	Reference
NSCLC**	Stroma	ns							Pirinen et al., 2001
MPNST*	Cells	↑		ns	ns	ns			Ikuta et al., 2014
Melanoma	Cells	↓	↓						Karjalainen et al., 2000
Melanoma	Stroma	ns							Karjalainen et al., 2000
Myeloma	Cells			ns					Adamia et al., 2005
Myeloma	Cells			↑HAS1Vb					Adamia et al., 2005
Myeloma	Cells			↑HAS1Va					Adamia et al., 2005
Myxo-fibrosarcoma	Tissue		↓ CD44s						Matuschek et al., 2014
Myxo-fibrosarcoma	Tissue		↑ CD44v6						Matuschek et al., 2014
non-Hodgkin`s lymphoma	Serum		↑						Taçyildiz et al., 2001
non-Hodgkin`s lymphoma	Serum		↑						Sasaki, Niitsu, 2000
Oral squamous	Cells	↓							Kosunen et al., 2004
Oral squamous	Stroma	ns							Kosunen et al., 2004
Ovarian	Cells	ns							Anttila et al., 2000
Ovarian	Stroma	↑							Anttila et al., 2000
Prostate	Cells	↑							Josefsson et al., 2011
Prostate	Stroma	↑							Josefsson et al., 2011
Pancreatic	Cells	↑			↑		↓		Cheng et al., 2013
Pancreatic	Tissue		↑						Li, X. et al., 2015
Upper urinary tract	Cells					↓			Chang et al., 2015
Urinary bladder	Cells		↓						Stavropoulos et al., 2001
Urinary bladder	Cells					↓			Chang et al., 2015
Urinary bladder	Tissue		ns	↑	ns	ns	↑		Kramer et al., 2011

Cells = cancer cells, stroma = tumor stroma, tissue = cells and stroma were not separated in analyses. Ns = not-significant, empty not studied. * Malignant peripheral nerve sheath tumors, ** Non-small cell lung carcinoma

2.11 CUTANEUS MELANOMA

2.11.1 Epidemiology

Cutaneous melanoma is an aggressive form of skin cancer. Incidence of cutaneous melanoma has increased: from 1989 to 1991 the incidence was 13.94 per 100 000 person-years and from 2007 to 2009 the incidence was 21.87 per 100 000 person-years in the United States (Shaikh et al., 2015). This increasing incidence is mainly detected among the non-Hispanic whites (Jemal et al., 2011). In Finland, the increasing trend has been similar. Between 2011 and 2015, the incidence of melanoma was 30.42 and 23.4 per 100 000 for men and women, respectively (The Finnish Cancer Registry, 2018). In comparison, between 1991 and 1995, the incidence of melanoma was 15.01 and 10.82 per 100 000 for men and women, respectively (The Finnish Cancer Registry, 2018). Both superficial and deep melanomas have increased incidence (Kruijff et al., 2012, Geller et al., 2013, Shaikh et al., 2015). There is evidence that the superficial (T1-T2) melanomas are thinner than reported previously, whereas the thickness of the deeper (T3-T4) melanomas is increasing (Shaikh et al., 2015). At the same time the prognosis of melanoma has improved significantly (Geller et al., 2013, Shaikh et al., 2015); between 1950 and 1954 one patient died for every 1.5 patient due to melanoma. Since then, the prognosis has improved; between 2003 and 2007 there was one death for every 7.8 melanoma patients (Geller et al., 2013). The reasons behind this improvement could be improved diagnostics of melanoma, increased public awareness and improved treatment rates.

2.11.2 Etiology

Cutaneous melanoma originates from melanin-pigment producing melanocytes of the skin. Normal skin melanocytes can be found in the basal layer of the epidermis. As the melanoma evolves, cancer cells arising from these melanocytes acquire invasive potential, thus breaching the basement membrane and invading the dermis. Macroscopically, patients either develop a new pigmented skin lesion (*de novo*, no previous nevus present) or a preexisting melanocytic nevus starts to change. Estimation of the melanomas arising from previously normal nevi vary a lot (Stolz et al., 1989, Kaddu et al., 2002, Bevona et al., 2003, Haenssle et al., 2015). In a prospective study including high-risk patients, over 54% of melanomas arose from previously normal melanocytic nevi (Haenssle et al., 2015). In retrospective studies, the percentage of nevi associated melanomas varied between 20-30% (Stolz et al., 1989, Kaddu et al., 2002, Bevona et al., 2003). In both cases, a dark, unsymmetrical and unregularly growing pigmented lesion is noted. The lesion may ulcerate and can rapidly change its appearance.

The etiology of melanoma is not yet fully understood but one of the most influential factors in its development is increased exposure to UV radiation (Dennis et al., 2008, Moan et al., 2015). This increased UV radiation exposure has been mainly affecting fair-skinned populations in the recent decades. It is estimated that periodic intense UV exposure (e.g. sunburns) is more important than total (Moan et al., 2015). According to a meta-analysis, increased amount of sunburns at any stage of life elevates the risk for melanoma (Dennis et al., 2008). Other risk factors are a high number of normal melanocytic nevi and the presence of atypical nevi (Grulich et al., 1996, Gandini et al., 2005). In a meta-analysis, people with a high number (101-120) of normal nevi had an approximately 7-fold increased risk for melanoma, compared to people with a low number (0-15) of normal nevi (Gandini et al., 2005). In the same study an increased number of atypical nevi was associated with an increased risk of melanoma (Gandini et al., 2005).

2.11.3 Pathogenesis and classification of melanoma

Currently the most influential and important classification of melanoma is the TNM classification of American Joint Committee on Cancer (AJCC) (Table 2) (Gershenwald et al., 2017). This classification is further divided into pTNM- and cTNM-classifications. The cTNM classification is done according to clinical staging with the help of laboratory tests and radiology. The pTNM-classification is based on pathological staging obtained from surgical samples. According to this classification, primary cutaneous melanomas are divided into a four-level scale based on the tumor thickness (Gershenwald et al., 2017).

Tumor thickness of melanoma is called Breslow thickness. According to the tumor thickness, melanomas are divided into four different categories: from T1 to T4 (Table 2) (Gershenwald et al., 2017). Ulceration is another factor included in the classification of primary melanoma (Gershenwald et al., 2017). Previously, mitotic count has been a part of the classification of T1 melanomas but it was disregarded in the most recent TNM classification. The N category in the TNM classification classifies the number of metastatic lymph nodes and possible in-transit and satellite metastases. M category informs possible distant metastases, lung, liver, brains etc. of melanoma. In localized disease, the tumor thickness has the most influence on the prognosis of patients. According to TNM classification, the ten-year survival rate for TIaN0M0 melanoma is 98 % whereas ten-year survival rate for T4bN0M0 is 75 % (Gershenwald et al., 2017). Ulceration of the tumor worsens the prognosis; for example, the five-year survival rate for T3a melanoma (non-ulcerated) is 94 % whereas the five-year survival rate for T3b (ulcerated) is 86 % (Gershenwald et al., 2017). Previously, melanomas have also been divided according to Clark`s level which is based on the invasion level of the melanoma in the skin layers.

Table 2. The eight edition of the American Joint Committee on Cancer (AJCC) tumor, node, metastasis (TNM) staging system.

T category	Breslow (mm)	Ulceration status
Tis	**(melanoma in situ)**	
T1	≤ 1	
T1a	< 0.8	No
T1b	< 0.8	Yes
	0.8 to 1.0	Yes or No
T2	> 1.0 to 2.0	
T2a		No
T2b		Yes
T3	> 2.0 to 4.0	
T3a		No
T3b		Yes
T4	> 4.00	
T4a		No
T4b		Yes

N category	No. of metastatic nodes	Nodal metastatic burden
N0	0	
N1		
N1a	1	Clinically occult tumor-involved node
N1b	1	Clinically detected tumor-involved node
N1c	0	Any number of in-transit, satellite, and/or microsatellite metastases with no tumor-involved nodes
N2		
N2a	2-3	Clinically occult tumor-involved nodes
N2b	2-3	At least one of which was clinically detected tumor-involved node

N2c	1	Any number of in-transit, satellite, and/or micro- satellite metastases with 1 tumor-involved node
N3		
N3a	≥ 4	Clinically occult tumor-involved nodes
N3b	≥ 4	At least one of which was clinically detected, or the presence of any number of matted nodes tumor-involved node
N3c	≥ 2	Any number of in-transit, satellite, and/or microsatellite metastases with 2 or more tumor-involved nodes and/or presence of any number of matted nodes

M category	**Site**	**Serum lactate dehydrogenase**
M0	No distant metastases	
M1a	Distant skin, soft tissue including muscle, and/or non-regional lymph node	M1a (0): Normal M1a (1): Elevated
M1b	Lung metastases	M1b (0): Normal M1b (1): Elevated
M1c	All other visceral non-CNS metastases	M1c (0): Normal M1c (1): Elevated
M1d	Distant metastasis to CNS	M1d (0): Normal M1d (1): Elevated

Melanoma is regarded as quite a heterogeneous cancer and apart from TNM classification, different melanoma subtypes with different clinical appearances are recognized. The most notable ones are nodular melanoma, superficially spreading melanoma, lentigo maligna melanoma and acral melanoma. The frequency rates of different types of melanomas differ in previous studies. In most studies, not otherwise specified melanomas (NOS) form a large group with frequency rates varying between 20.1 % for women, 22.3% for men and 13-41.4 % (both genders included) (Shaikh, Xiong & Weinstock, 2012, Mar et al., 2013, Bay et al., 2015). Superficially spreading melanoma is the most common type of melanoma (Shaikh, Xiong & Weinstock, 2012, Mar et al., 2013). In an Australian study approximately 56% of melanomas belong to this group (Mar et al., 2013). 14% of melanomas were nodular type and 13% were lentigo maligna melanomas (Mar et al., 2013). Other types of melanoma are far less prevalent (Shaikh, Xiong & Weinstock, 2012, Mar et al., 2013). Nodular type melanomas are generally known for their aggressive behavior and they account for a large number of deaths caused by melanoma (Shaikh, Xiong & Weinstock, 2012, Mar et al., 2013). Nodular melanoma has a distinctive growth type where it spreads in early stages vertically invading the dermis deeply. The more common type, superficially spreading melanoma, grows radially first (a radial growth phase) and later starts to invade deeply into the dermis (a vertical growth phase). After this stage, the first place of metastasis is generally the adjacent lymph nodes. Distant metastases are generally formed later as melanoma progresses hematogenously (Fig. 4).

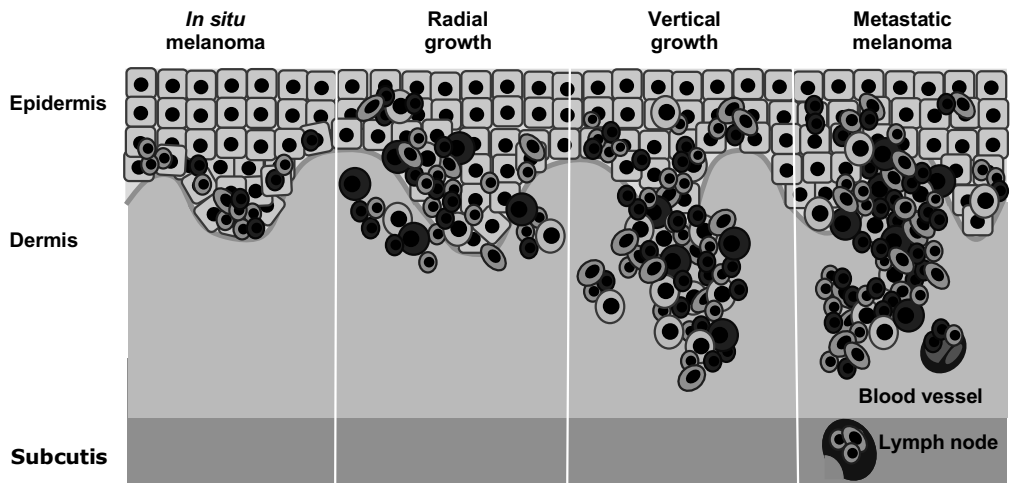

Figure 4. In situ melanomas reside in the epidermis without invading the dermis. Superficially spreading melanomas go through a radial growth phase first, after which they start to invade deeper into the skin (vertical growth phase). Subsequently, tumor cells can spread further either through the lymphatic system or hematogenously.

Approximately 54-61 % of melanomas have activating BRAF mutations (Davies et al., 2002, Libra et al., 2005, Boursault et al., 2013). There are no differences in mutation frequencies between primary melanomas and metastatic melanomas, which suggests that mutation in BRAF occurs early in the melanoma pathogenesis (Libra et al., 2005, Boursault et al., 2013). The most common mutation in the BRAF gene is V600E, but others such as V600E2 and V600K are also observed (Boursault et al., 2013). These mutations cause increased activation of intracellular signaling cascade, Ras-Raf-MEK-ERK or as also known mitogen-activated protein kinase (MAPK) pathway, which normally transmits extracellular growth signals. In normal cells, BRAF codes protein kinase called B-RAF, whose activation by Ras causes phosphorylation of MEK and eventually this leads to changes in gene expression. Generally, genes activated by MAPK pathway cause increased growth and proliferation. Mutations in BRAF are thought to resemble phosphorylation of B-RAF and thus causing its activation and subsequent signal transduction (Davies et al., 2002, Mandalà, Voit, 2013). As BRAF mutations cause activation of MAPK, it is not surprising that these mutations are associated with increased ulceration and a more advanced disease at the time of diagnosis (Ellerhorst et al., 2011). They have therefore become an interesting therapeutic target. Inhibition of the mutated BRAF in metastatic melanoma slows down disease progression in approximately half of the patients (Flaherty et al., 2010, Sosman et al., 2012). Some patients with metastatic disease have been reported to have long survival times (Puzanov et al., 2015).

Mutations in neuroblastoma RAS viral oncogene homolog (NRAS) represent another type of mutation commonly found in melanomas. These mutations also activate the same MAPK pathway as BRAF mutations. Approximately 18 % of melanomas have mutations in NRAS (Lee, J. H., Choi & Kim, 2011). Similar to BRAF mutations, NRAS mutations are associated with negative prognostic factors, such as increased invasion depth (Ellerhorst et al., 2011), nodular type melanoma (Lee, Choi & Kim, 2011) and shorter overall survival time in metastatic melanoma (Stage IV) (Jakob et al., 2012).

Melanoma is regarded as one of the most immunologically active cancers. The immune system affects the progression of cancer and increased immune system activation generally improves the survival of patients. Tumor-infiltrating lymphocytes (TILs) are lymphocytes which localize in the tumor itself or in the tumor stroma. Interest towards them has risen in recent years. In most studies, higher TIL grade in melanoma is associated with better prognosis of the patient (Azimi et al., 2012, Thomas et al., 2013). There

has been some debate, as some studies have shown TILs fail as prognostic markers for patient survival (Taylor et al., 2007). Nevertheless, the absence of TILs is a predictive marker for sentinel lymph node positivity (Taylor et al., 2007). Collectively, the literature suggests a higher number of TILs is mostly seen as a positive prognostic marker in cutaneous melanoma (Lee, N. et al., 2016).

2.11.4 Treatment of melanoma

The primary treatment for a local melanoma is surgery with wide marginals. During the same surgery, a sentinel lymph node biopsy can be performed and if melanoma spreading has been detected, the affected lymph nodes can be removed. Sentinel lymph node biopsy is especially recommended for patients with intermediate thickness of melanoma (1.20-3.50 mm) (Morton et al., 2006). The clinical significance of a sentinel lymph node biopsy in thicker melanomas has not been as well-established because a sentinel lymph node biopsy does not correlate with melanoma specific survival, but it does correlate with increased disease-free survival (Boada et al., 2018). The treatments for metastatic melanoma depend largely on whether the melanoma has a BRAF mutation, and if the mutation is detected, medications inactivating BRAF are generally recommended. Other treatment options are cytotoxic drugs, radiation or immunological drugs which activate the immune system of patients to recognize cancer cells. Immunological drugs, including antibodies against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death-1 (PD-1), are the newest treatment options in metastatic melanoma. These drugs change the T-cell response of patients. Antibodies against CTLA-4 activate the immune system and antibodies against PD-1 prevent the binding of programmed death ligand-1 (PD-L1), thus preventing the inactivation of T-cells attacking the cancer cells. Both overall and progression-free survival rates of metastatic melanoma patients have improved with immunological drugs in randomized trials (Ribas et al., 2015, Robert et al., 2015, Schachter et al., 2017). PD-1 antibody treatment seems to superior compared to treatment targeting CTLA-4 (Schachter et al., 2017) .

2.12 BENIGN MELANOCYTIC NEVI, DYSPLASTIC NEVUS AND *IN SITU* MELANOMA

2.12.1 Benign nevi and dysplastic nevus

Benign melanocytic nevi are a common type of nevi which are seen in most of humans. Macroscopically, they look like dark moles which can either be elevated from the skin or even be at the skin level. These nevi are formed by benign melanocytic tumor cells (nevus cells) and are divided into three different classes depending on the localization of melanocytic cells: junctional, intradermal and compound nevi. In a junctional or intradermal nevus, the melanocytic cells localize in the epidermis and dermis, respectively (Fig. 5). As the name suggests, a compound nevus is a mixture of these two (Fig. 5).

Dysplastic nevi are also melanocytic nevi. Histologically, the melanocytic cells in benign nevi have no atypia, whereas dysplastic nevi are characterized with architectural disorder and at least low-level nuclear atypia of the melanocytic cells (Elder, 2010). Macroscopically, dysplastic nevi tend to look more irregular than benign nevi, but the diagnosis of dysplastic nevi is done on a histological basis.

Benign and dysplastic nevi are not treated as direct precursor lesions for melanoma, although approximately 20-54 % of melanomas are thought to emerge from previously existing nevi (Stolz et al., 1989, Kaddu et al., 2002, Bevona et al., 2003, Haenssle et al., 2015). The dysplastic nevi are often the nevi type from which melanomas arise, compared to benign nevi (Kaddu et al., 2002, Bevona et al., 2003). Congenital nevi are another type of melanocytic nevi that are associated with melanomas arising from previously normal nevi (Kaddu et al., 2002). Even though some of the melanomas are associated with nevi, the risk of a nevus seen on a 20 year old transforming into melanoma is rather low; lifetime risk is 0.03% and 0.009% for men and women, respectively (Tsao et al., 2003). This risk increases with the age, but even at its highest the risk for a nevus evolving into melanoma is 0.05 % in 40 year old men (Tsao et al., 2003).

Melanomas can certainly evolve from nevi, but it is nearly impossible to identify which nevi are in danger to transform. Most of the nevi remain benign throughout the lives of humans.

Interestingly, nevi possess some similar genetic mutations to melanomas as both BRAF and NRAS mutations are commonly detected in benign melanocytic nevi (Kumar et al., 2004). Thus, these mutations are not solely enough for the development of melanoma (Kumar et al., 2004).

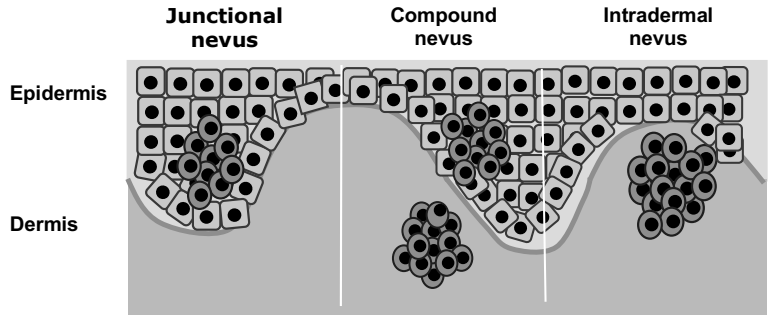

Figure 5. Benign nevi types. Junctional nevi reside in the junction of epidermis, intradermal nevi in the dermis and compound nevi are mixture of these two. Dysplastic nevi differ from benign nevi in that they have both architectural disorder and atypia of the melanocytic cells.

2.12.2 *In situ* melanoma

In situ melanomas resemble clinically invasive melanomas, but they are melanoma precursor lesions which have not penetrated the basement membrane of epidermis. These melanomas have not got an invasive component and thus Breslow thickness is not recorded from these tumors (Table 2). As they are not invasive lesions, they are non-metastatic. From 1997 to 2011 the incidence of *in situ* melanoma has risen from 1.97 to 8.7 per 100 000 in the Danish population indicating that the increasing incidence trend of cutaneous melanomas over the last decade can also be seen in *in situ* melanomas (Toender, Kjær & Jensen, 2014). The treatment of *in situ* melanoma is surgery with wide marginals.

2.13 DIFFUSELY INFILTRATING ASTROCYTOMAS

2.13.1 Epidemiology and etiology

From 2011 to 2015 the incidence of CNS tumors was 16.33 and 19.4 per 100 000 for males and females in Finland, respectively (The Finnish Cancer Registry, 2018). In the United States, the incidence rates for primary brain and CNS tumors were 27.86 per 100 000 from 2007 to 2011 (Ostrom et al., 2014). These rates are composed of both malignant and non-malignant CNS tumors. Approximately 33.7 % of primary CNS tumors are malignant (Ostrom et al., 2014). The most common group of malignant CNS tumors are astrocytic gliomas and glioblastoma (grade IV) is the most common diagnosis among the malignant primary CNS tumors (Ostrom et al., 2014).

Certain rare inheritable conditions increase the risk of developing a glioma. Several such syndromes have been recognized including neurofibromatosis 1 and 2, Li-Fraumeni syndrome and tuberous sclerosis complex (McGaughran et al., 1999, Melean et al., 2004). The clinical appearance of inheritable CNS tumors is highly different from sporadic CNS tumors; for example, neurofibromatosis 1 is associated with an increased risk for low-grade (grades I-II) gliomas in young age with overall survival rate of 95% over a 5.25-year observation time (Hernaiz Driever et al., 2010). One established risk factor is external radiation

which can cause a glioma as a rare complication following previously given radiation-therapy (Elsamadicy et al., 2015). Malignant radiation-induced gliomas develop after a substantially long latency-period of nine years (Elsamadicy et al., 2015).

The risk of CNS tumor is increased especially among first-degree relatives (Hemminki, Li & Collins, 2001, Sadetzki et al., 2013) and also an increased risk for astrocytomas in first-degree relatives has been reported (Malmer et al., 1999). 83% of familial gliomas include two cases of gliomas in the same family (Sadetzki et al., 2013). This suggests that outside of known genetic disorders, gliomas are not easily inheritable (Sadetzki et al., 2013). Reasons for this increased risk of gliomas without any syndromes in family are rarely documented. Mutations in telomere shelterin complex (POT1) have been described in several families (Bainbridge et al., 2015).

2.13.2 Pathogenesis and classification of diffusely infiltrating astrocytomas

Normal neuroglial cells function as supportive cells in the CNS and in the peripheral nervous system. Several distinctive CNS neuroglial cells are recognized: astrocytes, oligodendrocytes, ependymal cells and migroglial cells (Fig. 6). Gliomas originate from these neuroglial cells or their precursor cells. As their name implies, diffusely infiltrating astrocytomas arise form astrocytes or their precursor cells. Other recognized glioma types are oligodendrogliomas, oligoastrocytomas and ependymomas.

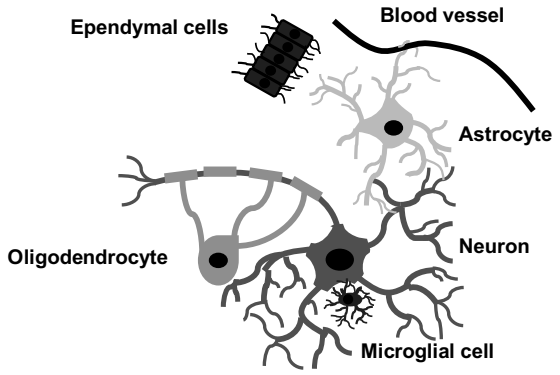

Figure 6. Glial cells of CNS. Astrocytes participate in several different functions in the central nervous system (CNS); they provide nutrients, maintain homeostasis and help in creating the brain-blood barrier. Oligodendrocytes form the myelin sheath around neuronal axons in the CNS. Ependymal cells line the ventricular system of the CNS and participate in creating cerebrospinal fluid. Microglial cells are a part of the brain`s immune system.

The World Health Organization (WHO) classification divides astrocytomas into four categories (grades I-IV) (Louis et al., 2007, Louis et al., 2016). Grades II-IV represent diffusely infiltrating astrocytomas whereas grade I tumors are pilocytic astrocytomas. Pilocytic astrocytomas have a distinct pathology and clinical onset compared to diffusely infiltrating astrocytomas as they generally affect children and young adults. Approximately half of the malignant primary CNS tumors are glioblastomas (grade IV) (Ostrom et al., 2014, Visser et al., 2015). Glioblastomas are notoriously known for their aggressive behavior despite new treatment methods. The five-year survival rate for glioblastoma is estimated to be 5.0 % and 6.3 % in the United States and in Europe, respectively (Ostrom et al., 2014, Visser et al., 2015). A very distinct attribute of gliomas is the progression of tumor grade at the time of recurrence. In other words, previously lower grade gliomas have a tendency to recur as higher grade gliomas.

Macroscopically malignant brain tumors generally manifest in the frontal lobe of brains (Ostrom et al., 2014). All the diffusely infiltrating astrocytomas have a common trait of poorly formed tumor boundaries, and infiltration of tumor cells into the adjacent brain tissue is always present. Grade II tumors are defined as tumors with cytological atypia and in addition grade III tumors manifest anaplasia and mitotic activity

according to the WHO criteria (Louis et al., 2007). Typical histopathological factors for grade IV tumors are high microvascular proliferation, increased amount of necrotic areas and high cellularity (Louis et al., 2007). Still, this categorization does not completely explain the behavior of diffusely infiltrating astrocytomas. With genomic analysis, distinct subtypes of glioblastomas have been recognized (Verhaak et al., 2010). These subtypes have been identified as proneural, classical, mesenchymal and neural glioblastomas (Verhaak et al., 2010). The subtypes differ in their responsiveness for oncological treatments as the treatments do not affect the prognosis of patients with proneural glioblastomas (Verhaak et al., 2010). The most recent 2016 WHO grading of gliomas emphasizes molecular testing as a part of glioma diagnosis and evaluation (Louis et al., 2016).

Through new techniques, new prognostic and predictive markers have been recognized in the pathogenesis of diffusely infiltrating astrocytomas. Mutations in isocitrate dehydrogenase 1/2 (*IDH*) are generally seen in lower grade tumors or in secondary glioblastomas (Yan et al., 2009, Kim, Y. et al., 2010). *IDH* mutations have been shown to correlate with increased survival time of patients (Yan et al., 2009, Hartmann et al., 2013).

O6-methylguanine-DNA methyltransferase (MGMT) promotor-methylation is also associated with longer survival time in glioblastoma patients (Krex et al., 2007, Hartmann et al., 2013). It is also a predictive factor as it leads to increased survival time in patients treated with alkylating chemotherapy (Malmström et al., 2012) . MGMT is a DNA repair enzyme with the ability to repair DNA damage caused on the O6-methylguanine and O6-chloroethylguanine by alkylating chemotherapy (Kaina, Margison & Christmann, 2010). The promotor methylation causes inactivation of this DNA repair enzyme thus increasing the effect of alkylating chemotherapy.

There have been conflicting results regarding the significance of tumor suppressor tp53 mutations in gliomas. In some studies, tp53 mutations did not affect the prognosis of patients (Houillier et al., 2006, Krex et al., 2007, Ogura et al., 2015). Nevertheless, tp53 mutations are associated with decreased survival of patients in grade II gliomas (Kim et al., 2010). Tp53 mutations are generally seen in secondary glioblastomas compared to primary glioblastomas (65% vs 28%) (Ohgaki et al., 2004) and in many cases it is seen together with *IDH1*/2 mutations in grade II diffusely infiltrating astrocytomas (Kim et al., 2010).

Alpha-thalassemia/mental retardation syndrome X-linked (ATRX) mutations cause inactivation of the same gene. Loss of ATRX is generally seen together with *IDH* mutations (Wiestler et al., 2013, Leeper et al., 2015). Grade III anaplastic gliomas with the loss of ARTX have better prognosis compared to patients with normal ATRX expression (Wiestler et al., 2013). 1p/19q deletion (Wiestler et al., 2013) and loss of ARTX are generally mutually exclusive (Wiestler et al., 2013, Leeper et al., 2015). 1p/19q deletion is a hallmark of oligodendrogliomas.

Epidermal growth factor receptor (EGFR) amplification is seen in approximately 40% of glioblastomas (Järvelä et al., 2006, Weller et al., 2009). In genomic analyzes, 57% of glioblastomas had changes in EGFR (Brennan et al., 2013). EGFR is a plasma membrane receptor and its amplification leads to increased signaling through it; this generally leads to activation of genes which regulate cell proliferation and migration. High prevalence of changes in EGFR expression in glioblastomas have made it an interesting research subject; however, its prognostic implication has remained vague. In neural stem cells, overexpression of wild-type EGFR or its commonly mutated variant EGFRvIII, increased cancer cell-like behavior, including increased proliferation and migration of cells (Ayuso-Sacido et al., 2010). However, EGFR amplification has not been shown to affect survival of patients with glioblastomas (Järvelä et al., 2006, Weller et al., 2009). Increased EGFR amplification rate is observed in astrocytomas and correlates with tumor grade; 4 % grade II tumors have an amplification whereas 39 % of grade IV tumors have an amplification (Järvelä et al., 2006). In grade III astrocytomas, EGFR amplification is associated with decreased survival of patients (Järvelä et al., 2006). Summary of histopathological and molecular changes in astrocytomas is provided in Fig. 7.

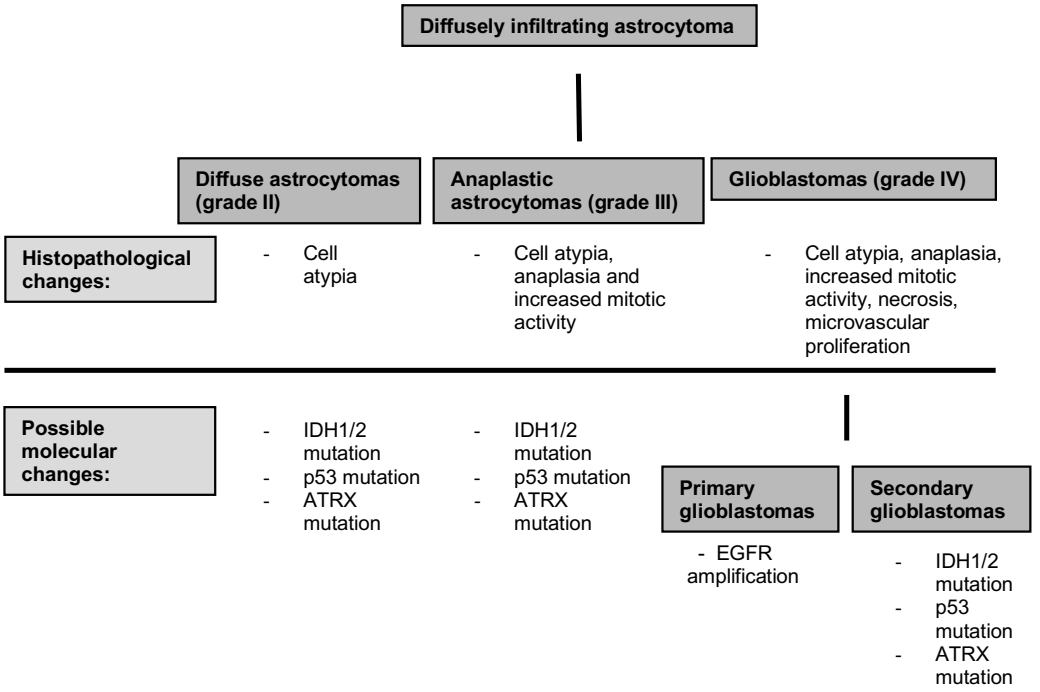

Figure 7. Histopathological and molecular findings in diffusely infiltrating astrocytomas (Ohgaki et al., 2004, Järvelä et al., 2006, Louis et al., 2007, Yan et al., 2009, Kim et al., 2010, Jiao et al., 2012, Brennan et al., 2013, Wiestler et al., 2013, Leeper et al., 2015, Louis et al., 2016) .

The treatment of diffusely infiltrating astrocytomas focuses on a maximal safe surgery. The problem with this approach is the infiltrative nature of glioma cells which makes it difficult to achieve complete resection, especially in high grade gliomas. After surgery, resection radiation therapy and chemotherapy can be given. Gliomas do not send metastasis outside the CNS but tend to recur in the CNS.

3 AIMS OF THE STUDY

Hyaluronan is an abundant extracellular matrix macromolecule in many human tissues. Its metabolism is variable in several human cancers, such as carcinomas. *In vitro* studies have shown that hyaluronan is involved in tumor progression by affecting cellular functions such as cellular invasion, migration and angiogenesis. However, in many cases the regulatory mechanisms behind variable intratumoral hyaluronan content remain obscure and the clinical significance needs to be studied further. In this thesis, the metabolism of hyaluronan was studied in benign and premalignant cutaneous melanocytic tumors and in cutaneous melanoma and diffusely infiltrating astrocytomas.

The main aims of this thesis were:

1. To investigate hyaluronan content and expression of hyaluronidases HYAL1-2, hyaluronan synthases HAS1-3 and CD44 receptor in benign melanocytic tumors, melanomas and lymph node metastases.
2. To investigate whether the possible changes in tumoral hyaluronan content are due to changes in expression of HAS1-3 or HYAL1-2 in melanocytic tumors
3. To inspect whether expression levels of HAS1-3 or HYAL2 are associated with clinical or histopathological parameters or patient outcome in the melanoma.
4. To investigate hyaluronan content and expression of CD44, HYAL2 and HAS1-3 in diffusely infiltrating astrocytomas and any possible correlations with histopathological parameters and prognosis.

4 SUBJECTS AND METHODS

4.1 CLINICAL DATA AND TISSUE MATERIAL

A more detailed version of the methods used can be found in the respective publications I-III.

4.1.1 Clinical data

The clinical data from melanoma patients was collected at the Kuopio University Hospital from patients diagnosed during 1980–2010 (II) (Table 3). The follow-up time ranged from 0.1 to 32.67 years (mean 8.2 years) (II). The clinical data of diffusely infiltrating astrocytoma patients was collected at Tampere University Hospital from patients diagnosed during 1983-2001 (III) (Table 4). The follow-up time ranged from 0.1 to 83.4 months (mean 18.0 months) (III).

Table 3. Clinical data of melanoma patients

	Superficial melanomas (< 1mm)	Deep melanomas (> 4mm)	Lymph node metastasis	Total
Number of cases	41	41	47	129
Relapses	3	23	39	65
Alive				
Yes	31	8	9	48
No	10	33	38	81
Deaths from melanoma	1	20	32	53

Table 4. Clinical data of patients with diffusely infiltrating astrocytoma

	Grade II astrocytomas	Grade III astrocytomas	Grade IV astrocytomas	Total
Number of cases	25	6	119	150
Mean survival time (months)	36.3	29.5	13.4	18.0
Alive				
Yes	9	2	6	17
No	16	3	108	127
No data	0	1	5	6
Deaths from glioma	16	3	108	127

4.1.2 Tissue materials

Cutaneous and lymph node samples (I-II) consisted of standard diagnostic histopathological tissue samples from the laboratory of pathology at the Kuopio University Hospital from patients diagnosed between 2000-2008 (I) and 1980-2010 (II) (Table 5). The diffusely infiltrating astrocytoma samples were diagnosed between 1983-2001 at the Tampere University Hospital (Table 5).

After resection, all tissue samples were formalin-fixed and paraffin-embedded according to standard procedures for diagnostic surgical pathology. After diagnosis, the astrocytoma samples were processed into tissue microarray (TMA) blocks (Nordfors et al., 2015) (III). The samples from melanocytic tumors were whole slide sections (I-II).

Table 5. Tissue samples

	Number	Publication
Benign nevi	29	I
Junctional	5	I
Intradermal	14	I
Compound	10	I
Dysplastic nevi	29	I
In situ melanomas	18	I
Superficial melanomas	41	I-II
Deep melanomas	41	I-II
Lymph node metastases of melanoma	47	I-II
Grade II diffuse astrocytomas	25	III
Grade III anaplastic astrocytomas	6	III
Grade IV glioblastomas	119	III

4.2 METHODS

4.2.1 Immunohistochemistry and hyaluronan staining

After deparaffinization, tissue samples for HAS1-3 and HYAL1-2 immunostainings were cooked in 10 mM citrate buffer (pH 6.0) in a pressure cooker for 15 minutes and after cooling they were washed with 0.1 M phosphate buffer (PB; pH 7.0). The endogenous peroxidase activity was blocked with 1 % H_2O_2. Unspecific binding was blocked with 1 % bovine serum albumin (BSA), 0.05 % Tween-20 and 0.1 % gelatin (Sigma G-2500) (HAS1-3 and HYAL1-2) or with 1% BSA in 0.1 M Na-PB (hyaluronan and CD44). Samples were incubated overnight with their respective primary antibodies (Table 6) or with a biotinylated hyaluronan-binding complex (bHABC) to detect hyaluronan (C. Wang, Tammi & Tammi, 1992, Tammi et al., 1994). Subsequently, CD44, HAS1-3 and HYAL1-2 samples were incubated with their respective secondary antibodies (Table 6). The bound antibodies were visualized with the avidin-biotin-peroxidase method (1:200, Vector Laboratories, Irvine, CA), color was visualized with 0.05 % 3,3-diaminobenzidine (DAB, Sigma, St. Louis, MO) and counterstaining was done with Mayer's hematoxylin.

The specificity of bHABC was tested by predigesting the control samples with *Streptomyces* hyaluronidase (Seikagaku, Kogyo, Tokyo, Japan) in acetate buffer with protease inhibitors. The specificity of CD44 and HYAL1-2 antibodies was tested by omitting the primary antibody in negative control stainings. Negative control stainings of HAS1-3 were done with peptide-blocking of corresponding HASes in study I and for HAS2 in study III.

Table 6. Primary and secondary antibodies and bHABC

Antibody or bHABC	Origin	Publication
Hyaluronan (bHABC)	Manufactured in the Institute of Biomedicine, University of Eastern Finland	I and III
CD44 (Hermes3)	A kind gift from Dr. Sirpa Jalkanen, University of Turku, Finland (Karvinen et al., 2003, Pasonen-Seppänen et al., 2012a, Takabe et al., 2015)	I and III
HAS1	Santa Cruz Biotechnology, Santa Cruz, CA	I-III
HAS2	Santa Cruz Biotechnology, Santa Cruz, CA	I-III
HAS3	Santa Cruz Biotechnology, Santa Cruz, CA	I and III
HYAL1	Atlas Antibodies, Stockholm, Sweden	I
HYAL2	Abcam, Cambridge, UK	I-III
Anti-mouse secondary antibody for CD44	Vector Laboratories, Burlingame, CA, USA	I and III
Anti-goat secondary antibody for HAS1-3	Vector Laboratories, Burlingame, CA, USA	I-III
Anti-rabbit secondary antibody for HYAL1-2	Vector Laboratories, Burlingame, CA, USA	I-III

4.2.2 Histopathological analyses

The stainings were analyzed with light microscopy. The intensity of staining was evaluated with the four-level scoring system: negative (0), weak (1), moderate (2) or strong (3) (studies I-III). If intensity of sample varied considerably, the more frequent intensity score was given for the sample. The coverage of stained cells was divided into five categories: 1 = 0-5 %, 2 = 6-25 %, 3 = 26-50 %, 4 = 51-75 %, 5 = 76-100 % (Auvinen et al., 2013, Auvinen et al., 2014, Tiainen et al., 2015). Stainings of melanocytic tumor cells and stromal cells (i.e. fibroblasts and connective tissue components excluding lymphocytes and endothelium) were analyzed separately (I-II). In the astrocytomas, staining intensity and coverage of cancer cells were evaluated in a similar fashion to the other two studies. The coverage of stained cells and the staining intensity of cells were computed into a staining index by multiplying the numerical values of staining intensity with the numerical value of coverage. This index ranged from 0 to 9 (III).

An experienced pathologist (RS) re-evaluated the histopathological parameters of melanoma and lymph node samples for studies I-II. The astrocytoma samples had been re-evaluated in the Fimlab Laboratories at the Tampere University Hospital (III). Study III included additional, previously evaluated immunostainings: Ki-67 (MIB1) for the proliferation activity, detection of *IDH1* mutation (R132H mutation-specific mouse monoclonal antibody (Dianova GmbH, Hamburg, Germany)), EGFR gene amplification (chromogenic in situ hybridisation (CISH)) and p53 status (antibody: DO-7, Novocastra Laboratories, Newcastle, UK) (Haapasalo et al. 1999, Nordfors et al. 2015).

4.2.3 Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics. A non-parametric Mann–Whitney U test was used to evaluate the differences in staining results between histopathological groups (I-II). Correlations between histopathological groups were tested with Spearman's rho (I). Categorical variables were tested with a $\chi 2$ test to analyze associations between staining results and clinical and histopathological variables (II-III). Continuous variables were tested with a Kruskal-Wallis test (III). Univariate survival tests were performed with a Kaplan-Meier log rank test (II-III). Multivariate survival tests were performed with a Cox regression model (II-III). P-values less than 0.05 were considered significant.

4.2.4 Ethics approvals

All the studies were approved by the Research Ethics Committee of the Northern Savo Hospital District and The Finnish National Supervisory Authority for Welfare and Health (VALVIRA). The protocol for study III was approved by the Ethical Committee of Tampere University Hospital. All the studies were retrospective and new samples were not collected from the patients.

5 RESULTS

According to our results, hyaluronan and CD44 content of cutaneous melanocytic tumors significantly varied between the tumors stages. In the diffusely infiltrating astrocytomas, overall hyaluronan and CD44 content were high. There were also significant differences in the levels of HASes and HYALs in all the tumor types. Summaries for studies I-II staining results can be found in Figures 8 and 10 and Tables 7 and 8. Staining results for melanocytic tumors are in Table 7 and staining results for stroma are in Table 8. A summary of study III staining results can be found in Figure 9 and Table 10.

5.1 HYALURONAN CONTENT AND CD44 EXPRESSION DECREASE IN THE ADVANCED STAGES OF INVASIVE MELANOMA

5.1.1 The content of hyaluronan and CD44 is low in deeply invasive melanomas and lymph node metastasis

Hyaluronan and CD44 stainings were evaluated in benign and dysplastic melanocytic tumors, *in situ* and invasive melanomas and in lymph node (LN) metastasis of melanoma. In all the different melanocytic tumors, hyaluronan was detected predominantly in the pericellular matrix surrounding the plasma membrane of tumor cells, whereas expression of CD44 was localized on the plasma membrane. The levels of hyaluronan content and CD44 immunostaining were similar in the respective tumor types.

Benign nevi displayed high content of hyaluronan and CD44 in tumor cells (I, Fig. 2). Nevertheless, the highest staining intensity of hyaluronan was in tumor cells of *in situ* melanomas (Fig. 8) (Fig. 10). The intensity of hyaluronan in tumor cells was lower in benign and dysplastic nevi, compared to *in situ* melanomas ($p = 0.004$ and $p < 0.01$, respectively) (I, Fig.2) (Table 7). Similarly, the intensity of CD44 was the highest in tumor cells of dysplastic nevi and *in situ* melanomas (I, Fig. 2).

In contrast, staining coverage of hyaluronan and CD44 were lower in the tumor cells of deep melanomas (> 4mm) ($p = 0.000$ and $p < 0.05$, respectively) (Fig. 8) and LN metastases ($p < 0.001$ and $p < 0.001$, respectively), compared to *in situ* melanomas (I, Fig. 2) (Fig. 10). Results were similar for staining intensity (I, Fig. 2). Among the invasive melanomas, the staining coverage of hyaluronan was the highest in the tumor cells of superficial melanomas ($p < 0.01$) (I, Fig. 2) (Table 7). The staining coverage and intensity of CD44 in tumor cells were also decreased in LN metastases compared to superficial ($p < 0.001$) and deep melanomas ($p = 0.006$ for staining coverage and $p = 0.007$ for intensity) (I, Fig. 2) (Fig. 10).

5.1.2. Stromal coverage and intensity of CD44 is the lowest in the deep melanomas and lymph node metastases

Tumor stroma of all lesions showed uniformly high staining coverage of hyaluronan without statistically significant differences (I, Fig. 2). Staining intensity of hyaluronan was the highest in benign nevi (I, Fig. 2).

Staining coverage and intensity of CD44 in stromal cells were the highest in dysplastic and benign nevi, whereas they were significantly lower in deep melanomas and LN metastases (I, Fig. 2). LN metastases had the lowest stromal staining intensity of CD44 ($p = 0.020$ compared to deep melanomas) (I, Fig. 2). *In situ* and invasive melanomas had low CD44 staining intensity in stromal cells compared to benign nevi ($p = 0.010$-0.000) (I, Fig. 2) (Table 8).

5.1.3 Diffusely infiltrating astrocytomas demonstrate high hyaluronan and CD44 content

Hyaluronan and CD44 stainings were analyzed in tumor cells of grade II-IV diffusely infiltrating astrocytomas. Both hyaluronan and CD44 showed intense staining in all astrocytomas (Fig. 9). The stainings were localized intracellularly and on the plasma membrane of tumor cells. Interestingly, CD44 staining was concentrated around vascular structures in some samples (III, Fig. 2D, insert). There were no

negative hyaluronan stainings in any groups and over 80% of tumors had either strong or moderate hyaluronan staining intensity (III, Fig. 4). The results were similar for CD44 albeit there was less strong staining intensity in tumors compared to hyaluronan stainings. There were no statistically significant differences in hyaluronan and CD44 stainings of different grade astrocytomas (III, Fig. 4) (Table 10).

5.2 EXPRESSION OF HAS1 AND HAS2 IS LOW IN ADVANCED MELANOMAS AND HIGH IN HIGH GRADE ASTROCYTOMAS

5.2.1 Expression of HAS2 is high in dysplastic nevi and *in situ* melanomas, but decreased in advanced melanomas

HAS1-3 stainings were evaluated in benign and dysplastic melanocytic tumors, *in situ* and invasive melanomas and LN metastasis. HAS1 and HAS2 stainings were performed in studies I and II, whereas HAS3 stainings were done in study I. HAS2 was the principle HAS isoform stained in melanocytic cells of all the melanocytic tumors. Expression of HAS3 was the weakest of all the HASes and we did not detect any differences in its expression in the melanocytic cells of different tumors (I, Fig.5). All HASes displayed cytoplasmic and membranous staining in melanocytic cells.

In study I, the highest staining coverages of HAS1 and HAS2 were recorded in tumor cells of *in situ* melanomas (Fig. 8) and dysplastic nevi (I, Fig. 5) (Fig. 10). Tumor cells of benign nevi had lower staining coverages of HAS1 and HAS2 than dysplastic nevi ($p = 0.021$ and $p = 0.000$, respectively) (I, Fig. 5). Staining coverage of HAS2 in the tumors cells was also lower in benign nevi compared to *in situ* melanomas ($p = 0.000$) (I, Fig. 5) (Table 7).

Staining coverages of HAS1 and HAS2 were low in the tumor cells of invasive melanomas. HAS2 staining coverage was low in LN metastases, deep (Fig. 8) and superficial melanomas, compared to *in situ* melanomas ($p < 0.001$, $p = 0.007$ and $p = 0.043$, respectively) (I, Fig. 5) (Fig. 10). For HAS1, the difference was also significant between LN metastases and *in situ* melanomas ($p < 0.01$) (I, Fig. 5).

Melanoma cells of superficially invasive melanomas had the highest staining coverages of HAS1 and HAS2 among the invasive melanomas in study II (II, Fig. 2). Both staining coverage and intensity of HAS1 was decreased in tumor cells of LN metastases compared to deep melanomas ($p = 0.039$ for coverage and $p = 0.018$ for intensity) (I, Fig. 5 and II, Fig. 2) (Fig. 10). Staining coverage of HAS1 in tumor cells was also lower in LN metastases, compared to superficial melanomas ($p = 0.002$) (II, Fig. 2) (Table 7). HAS2 staining coverage was decreased in tumors cells of LN metastases and deep melanomas, compared to superficial melanomas ($p = 0.012$ and $p = 0.013$, respectively) (II, Fig 2) (Fig. 10). Similarly, HAS2 staining intensity was lower in tumor cells of deep melanomas than in superficial melanomas ($p = 0.002$) (II, Fig. 2).

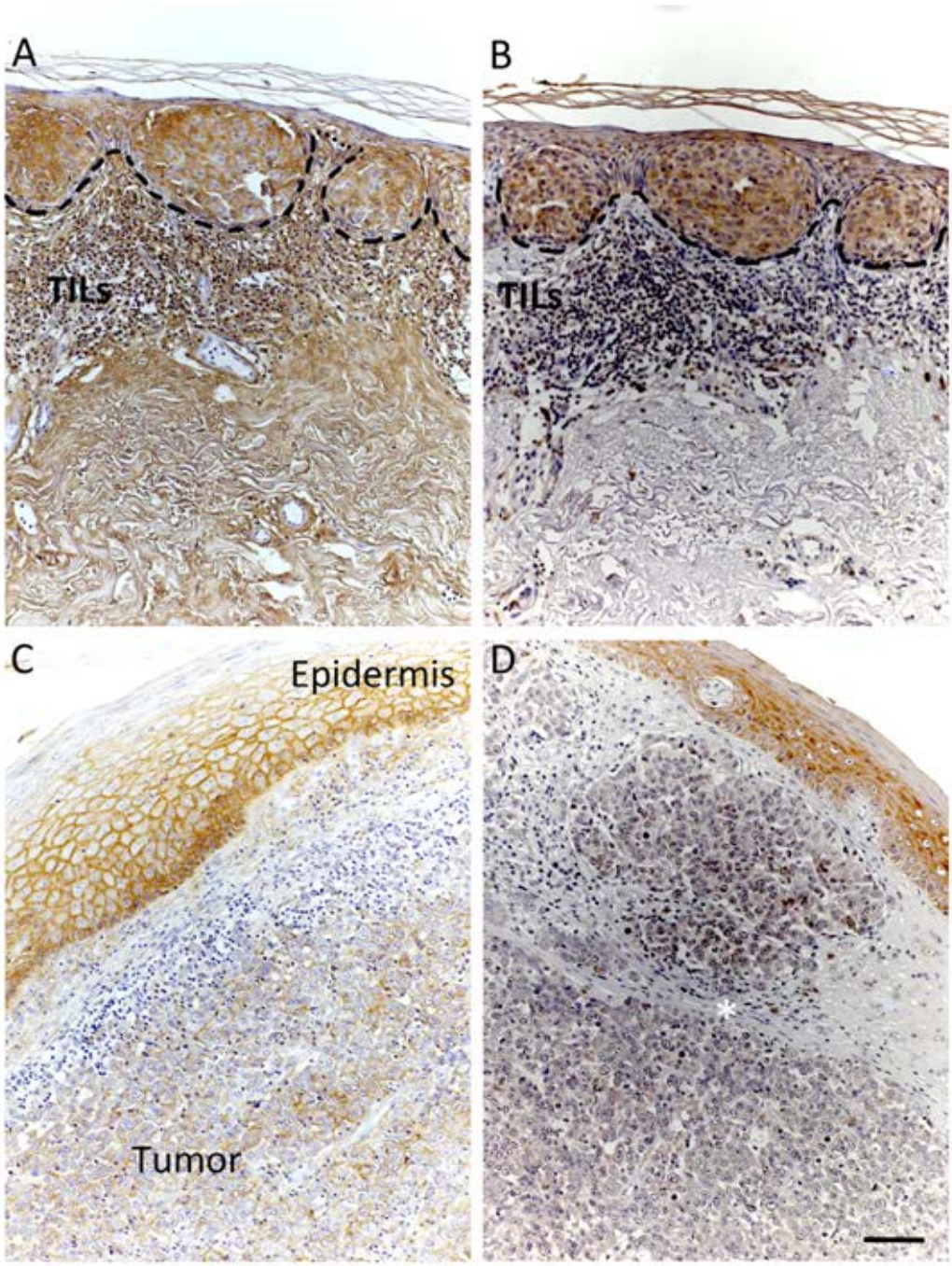

Figure 8. Hyaluronan (A and C) and hyaluronan synthase 2 (HAS2) (B and D) stainings in in situ melanoma (A and B) and deep melanoma (C and D). In situ melanomas displayed both high hyaluronan content and HAS2 expression while in deep melanomas, hyaluronan and HAS2 content of tumors were significantly lower. TILs = Tumor infiltrating lymphocytes * indicates the stroma surrounding the melanoma. Scale bar 50 µm.

5.2.2 HAS1 is the major hyaluronan synthase in stromal cells of melanocytic tumors

HAS1 was the most prevalent HAS isoform in tumor stroma of all melanocytic tumors. The highest staining coverage and intensity of HAS1 in stroma was recorded in benign nevi and the lowest in LN metastases (I, Fig. 5 and II, Supplementary Fig. 3). The intensity of HAS1 in stromal cells was high in benign nevi compared to all other melanocytic tumors ($p = 0.020$-0.000) (I, Fig. 5). Staining coverage of stromal cells was lower in deep melanomas and LN metastases compared to superficial melanomas in study I ($p < 0.05$ and $p < 0.01$, respectively), but the difference was not significant in study II (I, Fig 5 and II, Supplementary Fig. 3).

Expression of HAS2 and HAS3 was low in stromal cells, predominately only 0-5 % of cells were stained in all melanocytic tumors (I, Fig. 5 and II, Supplementary Fig. 3). Benign nevi had the highest staining coverage of HAS3 in stromal cells and this was statistically significant compared to dysplastic nevi and deep melanomas ($p = 0.009$ and $p = 0.030$, respectively) (I, Fig. 5). There were no significant differences in stromal coverage of HAS2 between different tumors (I, Fig. 5 and II, Supplementary Fig. 3). In stromal cells, the highest staining intensities of HAS2 and HAS3 were recorded in benign nevi (I, Fig. 5). The intensity of HAS2 in stromal cells was decreased in LN metastases compared to superficial and deep melanomas ($p < 0.001$ and $p < 0.05$, respectively) (II, Supplementary Fig. 3) (Table 8).

5.2.3 Expression of HAS1 and HAS2 is high in high grade astrocytomas

HAS1-3 stainings were analyzed in grade II-IV astrocytomas. Among them, the main HAS isoform was HAS2, whereas expression of HAS3 was the weakest. Expression of HAS1 and HAS2 was localized on the cell membrane and in the cytoplasm. HAS1 was also detected granularly in the cytoplasm.

Predominately, the tumors had either a weak or negative HAS1 staining intensity (III, Fig 4.). However, intensity of HAS1 tended to be higher in high grade tumors. Most of grade II-III astrocytomas had weak or negative HAS1 staining intensity (over 90%) whereas 20% of grade IV tumors presented moderate staining intensity ($p = 0.026$) (III, Fig 4.).

All astrocytomas displayed at least weak HAS2 staining intensity and no negative stainings were recorded. In high grade tumors, the staining intensity of HAS2 was higher than in low grade tumors (Fig. 9). HAS2 staining intensity was moderate in 53% of grade IV tumors, 33% of grade III tumors and 21% of grade II tumors ($p = 0.044$) (III, Fig. 4). Statistical difference was even clearer when grade II-III tumors were compared to grade IV tumors ($p = 0.009$) (III, Fig. 4) (Table 10).

HAS3 staining intensity was similar in all astrocytomas. Generally, staining intensity was either negative or weak without any statistically significant differences between tumors (III, Fig. 4).

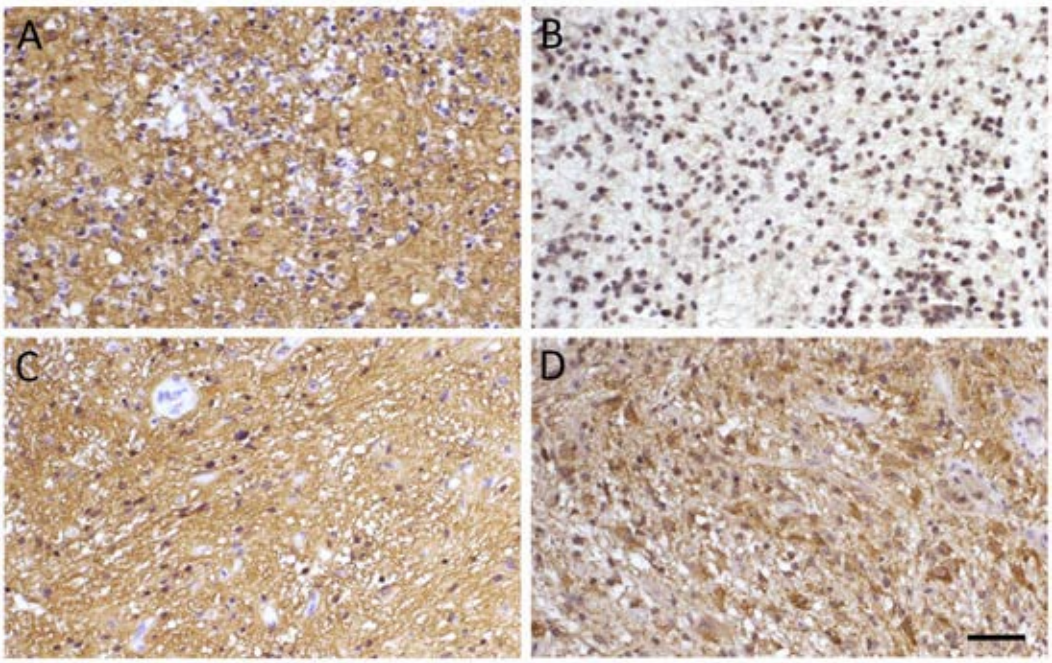

Figure 9. Hyaluronan (A and C) and hyaluronan synthase 2 (HAS2) (B and D) stainings in grade II diffusely infiltrating astrocytoma (A and B) and grade IV glioblastoma (C and D). All diffusely infiltrating astrocytomas had high hyaluronan content, however expression of HAS2 was higher in grade IV tumors compared to grade II tumors. Scale bar 50 µm.

5.3 EXPRESSION OF HYAL2 IS HIGH IN MELANOCYTIC TUMORS AND HIGH GRADE ASTROCYTOMAS

5.3.1 Expression of HYAL2 is lower in benign nevi compared to other melanocytic tumors

HYAL1-2 stainings were evaluated in benign and dysplastic melanocytic tumors, *in situ* and invasive melanomas and LN metastasis. Expression of HYAL1 in melanocytic tumors was recorded in study I, whereas HYAL2 stainings were done in studies I and II. HYAL1 and HYAL2 stainings localized diffusely intracellularly in melanocytic cells.

Staining coverage of HYAL1 and HYAL2 in melanocytic cells was predominantly high (76-100% of melanocytic cells stained) in all melanocytic tumors (I, Fig. 3 and II, Fig. 4). Both staining coverage and intensity of HYAL2 were the lowest in tumor cells of benign nevi compared to dysplastic nevi, *in situ* melanomas and all invasive melanomas ($p = 0.042$-0.006 for coverage and $p = 0.009$-0.000 for intensity) (I, Fig. 3) (Fig. 10). Otherwise, melanocytic tumors had no differences in HYAL2 stainings of tumor cells. Staining intensity of HYAL1 in tumor cells was the highest in *in situ* melanomas and significantly lower staining intensity was recorded in superficial ($p = 0.008$) and deep ($p = 0.029$) melanomas and LN metastases ($p = 0.005$) (I, Fig. 3) (Fig. 10).

5.3.2 Expression of HYALs is low in stromal cells of melanocytic tumors

Hyaluronidase immunoreactivity was low in tumor stroma of all melanocytic tumors. Predominately, the coverage of stained stromal cells was 0-5% and intensities of stainings were negative or weak for both HYALs (I, Fig. 3 and II, Fig. 4).

LN metastases had slightly higher HYAL1 staining coverage and staining intensity in stroma than superficial melanomas ($p < 0.05$ for both) (I, Fig. 3) (Table 8). Expression of HYAL2 in stromal cells was the highest in deep melanomas and LN metastases. HYAL2 staining coverage of stromal cells was higher in deep melanomas, compared to benign nevi ($p = 0.028$) (I, Fig. 3). LN metastases had higher HYAL2 staining intensity, compared to *in situ* melanomas ($p < 0.01$). In benign nevi, stromal intensity of HYAL2 was lower than in dysplastic nevi ($p = 0.006$) and superficial ($p = 0.006$) and deep melanomas ($p = 0.010$) (I, Fig. 3).

Table 7. Staining results of melanocytic cells. Dysplastic nevi, in situ melanomas and superficial melanomas have been compared to benign nevi. Deep melanomas and LN metastases have been compared to superficial melanomas.

	Dysplastic nevi	***In situ* melanomas**	**Superficial melanomas**	**Deep melanomas**	**LN metastases**
Hyaluronan					
Coverage	ns	↑*	ns	↓	↓
Intensity	ns	↑	ns	ns	ns
CD44					
Coverage	ns	ns	ns	ns	↓↓
Intensity	↑	↑	ns	ns	↓↓
HAS1					
Coverage	↑	ns	ns	ns	↓
Intensity	ns	ns	ns	ns	↓**
HAS2					
Coverage	↑↑	↑↑	ns	↓	↓
Intensity	ns	ns	ns	↓	ns
HAS3					
Coverage	ns	ns	ns	ns	ns
Intensity	ns	ns	ns	ns	ns
HYAL1					
Coverage	ns	ns	ns	ns	ns
Intensity	ns	ns	ns	ns	ns
HYAL2					
Coverage	↑	↑	↑	ns	ns
Intensity	↑↑	↑	↑	ns	ns

ns = not significant, ↑/↓ statistically significant increase/decrease, two arrows indicate $p < 0.001$, * *in situ* melanomas were compared to dysplastic nevi, ** LN metastases compared to deep melanomas

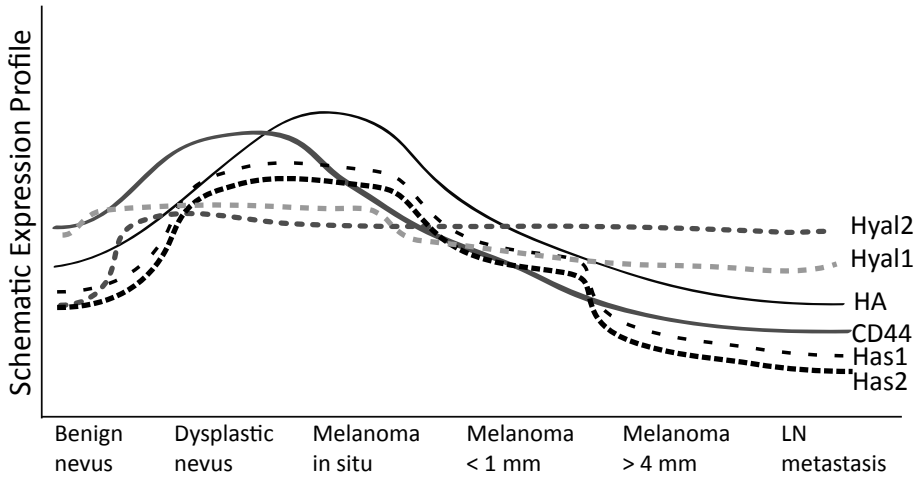

Figure 10. Changes in hyaluronan (HA) metabolism in melanocytic cells. HAS1-3 = Hyaluronan synthases 1-2, HYAL1-2 = Hyaluronidase 1-2.

Table 8. Staining results of stromal cells. Dysplastic nevi, in situ melanomas and superficial melanomas have been compared to benign nevi. Deep melanomas and LN metastases have been compared to superficial melanomas.

	Dysplastic nevi	*In situ* melanomas	Superficial melanomas	Deep melanomas	LN metastases
Hyaluronan					
Coverage	ns	ns	ns	ns	ns
Intensity	↓↓	↓	↓	ns	ns
CD44					
Coverage	ns	ns	ns	↓↓	↓↓
Intensity	ns	↓	↓↓	ns	↓
HAS1					
Coverage	↓	ns	ns	↓	↓
Intensity	↓	↓	↓	↓	↓
HAS2					
Coverage	ns	ns	ns	ns	ns
Intensity	ns	ns	↓	ns	↓↓
HAS3					
Coverage	↓	ns	ns	ns	ns
Intensity	↓↓	↓	↓	ns	ns
HYAL1					
Coverage	ns	ns	ns	ns	↑
Intensity	ns	ns	ns	ns	↑
HYAL2					
Coverage	ns	ns	ns	ns	ns
Intensity	↑	ns	↑	ns	ns

ns = not significant, ↑/↓ statistically significant increase/decrease, two arrows indicate $p < 0.001$, * *in situ* melanomas were compared to dysplastic nevi.

5.3.3 Expression of HYAL2 is higher in high grade astrocytomas than in low grade astrocytomas

Predominantly, weak HYAL2 expression was detected in the cytoplasm of astrocytomas. Besides tumor cells, endothelial cells showed expression of HYAL2. HYAL2 staining intensity and HYAL2-INDEX were

significantly higher in tumor cells of high grade tumors as HYAL2 staining intensity was moderate in 9.5% of grade II tumors, 0% in grade III tumors and 25% in grade IV tumors ($p = 0.049$ for intensity and $p = 0.001$ for HYAL2-INDEX) (III, Fig 4.) (Table 10).

5.4 HYALURONAN LOCALIZES MAINLY IN WHITE MATTER OF NORMAL BRAIN TISSUE WHEREAS HYAL2 SHOWS STRONGER EXPRESSION IN CORTICAL AREAS

Hyaluronan and CD44 staining showed abundant staining of white matter in cerebral brain (III, Fig. 1). The content of hyaluronan and expression of CD44 were significantly lower in neurons in cortical areas. Overall expression of HAS2 was low in normal brain samples, but some neurons and glial cells stained positively with HAS2 (III, Fig. 1). Interestingly, the localization of HYAL2 staining was the opposite to hyaluronan and CD44. HYAL2 demonstrated stronger staining in cortical areas whereas white matter was negative (III, Fig. 1). Intense CD44 staining was also seen near perivascular areas, while endothelial cells were positive for HYAL2 (III, Fig. 1).

5.5 LOW EXPRESSION OF HAS2 IN INVASIVE MELANOMAS AND HIGH EXPRESSION OF HAS2 IN DIFFUSELY INFILTRATING ASTROCYTOMAS ARE ASSOCIATED WITH NEGATIVE PROGNOSIS

Summaries of clinicopathological correlations can be found in tables 9 (II) and 10 (III).

5.5.1 Decreased expression of HAS1 and HAS2 correlates with short survival time in melanoma

Histopathological parameters that were analyzed in study II were ulceration, TILs (evaluated either low, moderate or high amount), mitotic count (mitosis/mm^2), horizontal diameter (mm) and growth type. HAS2 immunostaining results (both coverage and intensity) in melanoma cells were the only ones that correlated with these histopathological variables. Low HAS2 staining coverage and intensity associated with both increased horizontal diameter of primary melanoma ($p = 0.002$ and $p = 0.042$, respectively) and lower number of TILs ($p = 0.036$ and $p = 0.040$, respectively) (II, Table 2). High HAS2 intensity in melanoma cells correlated with superficial type of melanoma ($p = 0.047$). Low HAS2 staining intensity in melanoma cells associated with increased mitotic count and nodular melanoma ($p = 0.018$ and $p = 0.001$, respectively) (II, Table 2).

Decreased HAS1 staining coverage and decreased HAS2 staining intensity in melanoma cells was associated with increased stage of melanoma ($p = 0.006$ and $p = 0.047$, respectively) (II, Table 2). Furthermore, low expression of HASes were associated with both increased regional and distal recurrence of melanoma. Low HAS1 and HAS2 coverage and low HAS1 intensity in melanoma cells was associated with regional recurrence ($p = 0.006$, $p = 0.007$ and $p = 0.023$, respectively) (II, Table 2). Increased distal recurrence was associated with low HAS1 and HAS2 coverage and low HAS2 staining intensity in melanoma cells ($p = 0.012$, $p = 0.001$ and $p = 0.004$, respectively) (II, Table 2).

The mean follow-up time of melanoma patients was 8.2 years (from 0.1 to 32.67 years). Low HAS1 and HAS2 staining coverage and low HAS2 staining intensity in melanoma cells was associated with poorer disease-specific survival (DSS) ($p = 0.013$, $p = 0.001$ and $p = 0.014$, respectively) (II, Fig. 3). Shortened recurrence-free survival (RFS) time was associated with low HAS1 and HAS2 staining coverage in melanoma cells ($p = 0.041$ and $p = 0.006$, respectively) (II, Fig. 3).

In multivariate analyses, low HAS2 intensity in melanoma cells was associated with shortened DSS and RFS ($p = 0.011$ and $p = 0.014$, respectively) when cutaneous melanomas were included (LN metastases were excluded) (II). Low HAS1 intensity in melanoma cells was associated with shortened DSS in cutaneous melanomas ($p = 0.019$) (II). When all the tumors were included in multivariate analyses, low HAS2 coverage in melanoma cells was associated with shorter DSS ($p = 0.039$) (II).

Table 9. Associations of HAS1 and HAS2 staining results in melanoma cells with histopathological factors and survival times of patients (II). Arrows indicate changes in staining results.

	HAS1 coverage	HAS1 intensity	HAS2 coverage	HAS2 intensity
Ulceration	ns	ns	ns	ns
Mitotic count	ns	ns	ns	↓
Horizontal diameter	ns	ns	↓	↓
TIL	ns	ns	↑	↑
Nodular growth	ns	ns	ns	↓
Superficial type	ns	ns	ns	↑
Stage (pT1, pT4 or pN1-3)	↓	ns	ns	↓
Regional recurrence	↓	↓	↓	ns
Distal recurrence	↓	ns	↓	↓
Decreased DSS (Univariate)	↓	ns	↓	↓
Decreased RFS (Univariate)	↓	ns	↓	ns
Decreased DSS (Multivariate)	ns	↓	↓	↓
Decreased RFS (Multivariate)	ns	ns	ns	↓

ns = not significant, ↑/↓ statistically significant increase/decrease

Stromal staining results did not correlate with any histopathological variables. Low HAS2 staining intensity in stromal cells was associated with shortened DSS and RFS in univariate analyses ($p = 0.049$ and $p = 0.008$, respectively) (II).

5.5.2 High HAS2 staining intensity is associated with short survival time in diffusely infiltrating astrocytomas

Histopathological parameters that were analyzed were *IDH1* mutation, p53, proliferation (Ki-67 staining) and EGFR amplification. Astrocytomas with EGFR amplification were observed to have mostly weak HAS1 staining intensity, whereas tumors without EGFR amplification showed similar HAS1 staining results as the whole staining material ($p = 0.04$) (III, Fig. 5). Low HAS2 staining intensity was associated with a positive prognostic marker, *IDH1* mutation ($p = 0.003$) (III, Fig. 5). Furthermore, high HAS2 staining intensity was associated with increased proliferation ($p = 0.013$), similar to high HYAL2 staining intensity ($p = 0.010$) (III, Fig. 5). Staining results of hyaluronan, CD44 and HAS3 was not associated with any histopathological factors.

The mean follow-up time of patients was 18.0 months (from 0.1 to 83.4 months). High HAS2 staining intensity was associated with decreased overall survival time of patients (only primary tumors were included) in univariate ($p = 0.001$) (III, Fig. 6) and multivariate analyses ($p = 0.008$) (III). Similarly, high HAS2-INDEX was associated with decreased overall survival time of patients in univariate analyses ($p = 0.009$) (III, Fig. 6).

Table 10. Associations of staining intensities with histopathological factors and survival times of patients (III). Arrows indicate changes in staining intensity.

	Hyaluronan	CD44	HAS1	HAS2	HYAL2
WHO grade	ns	ns	↑*	↑	↑
IHD1	ns	ns	ns	↓	ns
Proliferation (KI-67)	ns	ns	ns	↑	↑
EFGR	ns	ns	**	ns	ns
p53	ns	ns	ns	ns	ns
Decreased overall survival (Univariate)	ns	ns	ns	↑	ns
Decreased overall survival (Multivariate)	Not tested	Not tested	Not tested	↑	Not tested

ns = not significant, ↑/↓ statistically significant increase/decrease, * HAS1 associated when grade IV tumors were compared to grade II-III tumors, ** correlation with weak staining intensity

6 DISCUSSION

6.1 HYALURONAN IN THE NON-INVASIVE CUTANEOUS MELANOCYTIC TUMORS

Hyaluronan content of benign and dysplastic nevi have not been previously studied; our work provides novel information about hyaluronan metabolism in these tumours (I). Hyaluronan content and CD44 immunopositivity were high in benign and dysplastic nevi (I). This is in agreement with the knowledge that hyaluronan is an abundant molecule in the skin. Interestingly, the expression of HYAL2 and HAS1-2 were increased in dysplastic nevi compared to benign nevi, which highlights the possible differences in hyaluronan metabolism (I).

Tumoral hyaluronan content is increased in the *in situ* carcinomas of stratified epithelium, similar to *in situ* carcinomas of the esophagus and skin (Wang et al., 1996, Karvinen et al., 2003). The chronic inflammatory condition, oral lichen planus, has in some cases been suggested to be a pre-cancerous change (Fitzpatrick, Hirsch & Gordon, 2014). This disease has increased hyaluronan staining in basal cells, compared to the normal mucosal stratified epithelium (Siponen et al., 2015). Similarly, our results (I) showed that precancerous *in situ* melanomas had the highest hyaluronan and CD44 staining in melanocytic cells. Increased hyaluronan synthesis could explain the accumulation of hyaluronan in the early stages of melanoma. Thus, HAS immunostaining was high in *in situ* melanomas compared to benign nevi potentially explaining the increased accumulation of hyaluronan (I). High level HAS1-2 expression was also observed in dysplastic nevi, although hyaluronan levels remained similar as in benign nevi (I). Although the etiology of inflammation in *in situ* melanomas differs from oral lichen planus, both of these diseases have similar high hyaluronan content which is generally seen together with inflammation. Thus, it is possible that increased hyaluronan accumulation in *in situ* melanomas is related to increased inflammation caused by the tumor. This might be the reason for increased hyaluronan synthesis in *in situ* melanomas. Some of the *in situ* melanomas in study I had a high number of subepithelial lymphocytes, indicating possible inflammation (Fig. 8). Similarly, a high number of TILs has been reported in HER2-positive ductal carcinoma *in situ* (DCIS) of breast (Pruneri et al., 2017).

UV radiation is a known risk factor for cutaneous melanoma and squamous cell carcinoma (SCC). UV radiation causes epidermal hyperplasia and well-differentiated squamous cell carcinomas in mice (Siiskonen et al., 2011). Hyaluronan accumulation and increased immunostaining of HASes has been observed in these areas (Siiskonen et al., 2011). UV radiation has been shown to up-regulate mRNA levels of all three HASes in the epidermal keratinocytes (Kakizaki et al., 2008, Rauhala et al., 2013). Our results indicate that *in situ* melanomas display increased hyaluronan accumulation, which is possibly due to increased hyaluronan synthesis and is similar in this regard to *in situ* carcinomas arising from the stratified epithelium. Thus, it is possible that UV radiation causes similar reactions in melanocytes and leads to increased hyaluronan synthesis and hyaluronan accumulation. This increased expression of HASes and subsequent hyaluronan accumulation in the early stages of tumor progression may inhibit further dissemination of melanocytes as high hyaluronan levels have been shown to inhibit tumor formation in naked mole rates (Tian et al., 2013). In addition, Takabe et all have shown that hyaluronan upregulation in MV3 melanoma cells inhibits proliferation and migration of these cells (Takabe et al., 2015).

Despite hyaluronan accumulation in the precancerous stages of melanoma, it is interesting that we also detected increased HYAL2 immunostaining in dysplastic nevi and *in situ* melanomas, compared to benign nevi (I). This suggests that while there is increased hyaluronan synthesis and subsequent hyaluronan accumulation in *in situ* melanomas there might also be increased hyaluronan catabolism. Similar results have been reported in keratinocytes in which UVB radiation up-regulates mRNA levels of HYAL1-2 and HAS1-3 (Averbeck et al., 2007, Rauhala et al., 2013). It is possible that some of the hyaluronan detected in our samples represents catabolized hyaluronan as the hyaluronan binding probe used in our studies detects all the hyaluronan oligosaccharides larger than HA10 (Tammi et al., 1994). Hyaluronan is degraded

by HYAL2 into oligosaccharides of ~50 disaccharide units (Stern, Jedrzejas, 2006). Thus, some of the detected hyaluronan accumulation in *in situ* melanomas may emerge from hyaluronan oligosaccharides. Hyaluronan oligosaccharides and LMW hyaluronan can promote both angiogenesis and lymphangiogenesis, which facilitate tumor progression (Gao et al., 2008, Wu et al., 2014). Increased HYAL2 expression may favor melanoma progression in the early stages by creating these hyaluronan oligosaccharides.

6.2 EXPRESSION OF HYALURONAN SYNTHASES AND HYALURONIDASES CAN EITHER PROMOTE OR INHIBIT CANCER PROGRESSION

6.2.1 Decreased expression of HAS1 and HAS2 is associated with declined survival of melanoma patients

Poorer prognosis of melanoma was correlated with lower expression of HAS1 and HAS2 in tumor cells (I and II). Immunostaining of HAS1 was decreased in LN metastases and immunostaining of HAS2 was decreased in deep melanomas and LN metastases, compared to superficial melanomas with lower hyaluronan staining (I and II). Our results suggest that low tumoral hyaluronan content is likely due to reduced expression of HAS1 and HAS2.

Another possible reason for declining hyaluronan content is elevated catabolism of hyaluronan. High HYAL2 expression was observed in all melanocytic tumors except in the benign nevi. There were no differences between superficial melanomas, deep melanomas or LN metastases (I and II). There was a slight decrease in the intensities of HYAL1 in the invasive melanomas compared to *in situ* melanomas, but as the hyaluronan content in melanomas declined significantly, this decrease in HYAL1 does not seem to markedly affect hyaluronan content of these invasive tumors (I). Hyaluronan accumulation in breast carcinomas is associated with high HAS1-3 immunoreactivity in cancer cells and stroma indicating that up-regulation of the synthases can increase tumoral hyaluronan accumulation (Auvinen et al., 2014). In the invasive melanomas, decline of hyaluronan content occurs in deep melanomas and LN metastases, while HYAL2 expression is similar in all the invasive melanomas. HYAL2 expression was high in dysplastic nevi, *in situ* melanomas and there were no differences in HYAL2 expression compared to invasive melanomas. Thus, increased expression of HYAL2 and declined amount of hyaluronan do not seem to be simultaneous events. These results suggest that decreased expression of HASes has a more significant role in hyaluronan depletion. Our results are based on immunostaining results, the enzyme activities or mRNA levels were not recorded. However, hyaluronan content of advanced melanomas is decreased and according to our results this occurs concurrently with reduced HAS1 and HAS2 immunostaining, suggesting reduced expression of these synthases.

Decreased HAS1 and HAS2 coverage and staining intensity in melanoma cells was associated with shorter disease-specific survival (II). This is in line with a previous study in which decreased tumoral hyaluronan and CD44 accumulation was associated with reduced survival (Karjalainen et al., 2000). Thus, poor survival seems to be associated with reduced hyaluronan synthesis and a subsequent decrease in hyaluronan. Similar results have been obtained in poorly differentiated tumors arising from stratified squamous epithelia. Decreased or patchy staining of hyaluronan was associated with poorly differentiated SCCs of skin, larynx, esophagus and mouth (Wang et al., 1996, Hirvikoski et al., 1999, Karvinen et al., 2003, Kosunen et al., 2004). The results are similar for CD44 (Hirvikoski et al., 1999, Karvinen et al., 2003). On the other hand, well differentiated squamous tumors of larynx and esophagus retain strong hyaluronan staining (Wang et al., 1996, Hirvikoski et al., 1999). Patchy staining of hyaluronan and CD44 correlate with lymph node and distant metastasis (Hirvikoski et al., 1999). Similarly, decreased immunostaining of HAS1 and HAS2 in melanoma cells correlates with regional and distant recurrence and shorter recurrence-free time (II). This suggests that progression of cutaneous melanoma involves similar metabolic patterns as poorly differentiated squamous cancers. In line with this contention, depletion of HMW hyaluronan induces tumor formation of naked-mole cells, which are normally cancer resistant (Tian et al., 2013). HMW hyaluronan, produced by naked-mole rats HAS2, declines proliferation and causes enhanced cell apoptosis

in breast cancer cells (Zhao et al., 2019). Similarly, breast cancer cells tumor formation was reduced with overexpression of naked-mole rats HAS2 in mouse model (Zhao et al., 2019) . Furthermore, overexpression of HAS3 generates a thick hyaluronan coat around melanoma cells which decreases migration and proliferation (Takabe et al., 2015). Depletion of hyaluronan and CD44 seems to be associated with aggressive tumor progression and this is largely due to decreased expression of HAS1 and HAS2 in melanoma cells. Decreased HAS2 staining intensity is associated with a nodular type of melanoma and increased HAS2 staining intensity was observed in superficially invasive tumors. This is consistent with observed hyaluronan depletion in the more advanced forms of melanoma while less advanced ones have a hyaluronan rich microenvironment (II).

New oncological treatments based on activation of the immune system have recently been developed. The role of hyaluronan in inflammatory processes and in the recruitment of immune system cells makes it an interesting molecule to investigate. According to our results, low HAS2 immunostaining is associated with a lower number of TILs which is regarded as a negative prognostic factor for melanoma (II). It is shown that during inflammation hyaluronan tends to form cable -like structures, which are capable of recruiting and binding leucocytes (de la Motte, C. A. et al., 2003). Low hyaluronan content due to low amount of HASes may prevent the formation of these cables in tumor stroma of melanomas. This might hamper the normal recruitment of TILs, which enable further spreading of melanoma. TILs express CD44v10 isoform in primary melanomas with tumor regression, which means at least the partial disappearance of malignant tumors (Weimann et al., 2003). This suggests that CD44v10 expression in TILs is advantageous for the patient. This fits together with our results that expression of CD44 is high in superficial melanomas, indicating its expression is advantageous for the patient.

6.2.2 The stromal hyaluronan metabolism in the invasive melanomas

The overall levels of stromal hyaluronan were high in all melanocytic tumors (I). The highest hyaluronan intensity was observed in benign nevi (I). In contrast, hyaluronan intensity in stromal cells was lower in all other melanocytic tumors (I). Similarly, the expression of CD44 and all three HASes was also lower (I). Although the expression of hyaluronidases in stroma was quite moderate, we detected higher expression of HYAL2 in invasive melanomas which also explains the decrease of stromal hyaluronan intensity (I). According to our results, HAS1 seems to be the most prevalent HAS isoform in the stromal cells of melanomas and its expression was notably lower in all other melanocytic tumors compared to benign nevi (I and II). Our results imply that hyaluronan is produced by both melanocytic tumor cells and stromal cells. Hyaluronan content remained high in the stroma of melanomas, whereas hyaluronan levels in melanoma cells were lower in deep melanomas and LN metastases, compared to superficial melanomas. Fibroblasts are the main cell type in the dermal part of the skin and they most likely synthesize the main part of the stromal hyaluronan in melanomas. It is possible that high stromal hyaluronan content is needed for melanoma cell migration into the surrounding stroma. Melanoma cells can induce the expression of various cytokines and chemokines in fibroblasts (Whipple, Brinckerhoff, 2014). Similarly, melanoma cells induce the expression of HAS2 in fibroblasts causing a subsequent increase in hyaluronan synthesis, indicating that the tumor cells can change surrounding hyaluronan content by affecting the stromal cells (Pasonen-Seppänen et al., 2012b, Willenberg et al., 2012). Interestingly, this upregulated HAS2 expression correlated with elevated MMP expression in fibroblasts (Pasonen-Seppänen et al., 2012b). Induction of HAS2 in fibroblasts can also contribute to angiogenesis. Kobayashi et al showed that low stromal levels of hyaluronan due to decreased expression of HAS2 in fibroblasts from mammary tumors led to diminished neovascularization and lymphangiogenesis of tumors in a mouse model (Kobayashi et al., 2010). This reduced neovascularization could hamper tumor growth as blood vessels are part of the tumor stroma and support tumor growth via an increased supply of oxygen and nutrients.

High stromal hyaluronan content profoundly worsens the survival of breast carcinoma patients (Auvinen et al., 2000, Auvinen et al., 2013). According to our results, reduced HAS2 staining intensity is associated with shorter disease-specific survival and recurrence-free times (II). The expression of HAS2 by

stromal cells was low, so their role in hyaluronan synthesis seems to not be relevant for tumoral hyaluronan content. Neveretheless, this finding highlights the relevance of HAS2 in tumor progression, as its decreased expression in the melanoma and stromal cells seems to significantly worsen the prognosis.

6.2.3 High expression of HAS2 is associated with short overall survival time in the diffusely infiltrating astrocytomas

The ECM of the brain contains high amounts of hyaluronan and thus its influence on the pathogenesis of gliomas has been studied previously. In previous studies, hyaluronan and CD44 have been shown to have a strong, increasing effect on glioma cells aggressiveness (Yoshida et al., 2012, DeSouza et al., 2013, Kim, Kumar, 2014). In agreement with these results, we found that these astrocytomas display abundant hyaluronan (III). High HAS2 staining intensity in glioma cells correlated with poor prognosis (III). Although expressions of HAS1 and HAS2 were both increased in grade IV glioblastomas, only HAS2 had an impact on survival (III). Previously, elevated HAS2 immunostaining has been showed to correlate with poor prognosis in carcinomas of breast and pancreas (Cheng et al., 2013, Auvinen et al., 2014, Lien et al., 2014). Elevated HAS2 expression is also associated with aggressive triple-negative and basal-like phenotypes of breast carcinoma (Lien et al., 2014). These results suggest that HAS2 has a major impact on carcinogenesis of several cancers, including diffusely infiltrating astrocytomas. Nevertheless, other hyaluronan synthases have been shown to contribute to carcinogenesis. Overexpression of HAS2 and HAS3 increased tumor growth in xenograft tumors of pancreatic cancers in mice (Kultti et al., 2014). Increased mRNA levels of HAS1 was associated with increased metastasis of bladder carcinoma and decreased disease-specific survival of patients (Kramer et al., 2011). Even though HAS1 did not correlate with prognosis in astrocytomas, its elevated expression might contribute to the aggressiveness of glioblastomas. Our results indicate a significant impact of high expression HAS2 and a slightly less significant impact of high expression HAS1 in the aggressiveness of diffusely infiltrating astrocytomas.

Expression of HYAL2 was high in astrocytomas but it had no influence on survival (III). Although the enzyme activities were not measured because our tumor samples were formalin-fixed, the expression of hyaluronan synthases and hyaluronidases was higher in high grade astrocytomas, which may explain why hyaluronan content remained stable in them (III). Increased enzymatic activity of hyaluronidases has been reported in colorectal cancer (Bouga et al., 2010) although high hyaluronan content is associated with poor survival in colorectal cancer (Ropponen et al., 1998). Thus, elevated HYAL2 expression could be significant for astrocytoma aggressiveness without affecting the tumoral hyaluronan content, for example through creation of hyaluronan oligosaccharides which enhance angiogenesis (Gao et al., 2008). Measurement of hyaluronidase activities in astrocytomas would be interesting as this would further elaborate hyaluronan metabolism in them.

Even though the immunostainings of HAS1, HAS2 and HYAL2 varied significantly, we were unable to detect any significant changes in hyaluronan or CD44 content. High CD44 immunoreactivity has been reported in grade III-IV astrocytomas (Yoshida et al., 2012). Low expression of CD44 has been linked with better prognosis in glioblastomas and especially in proneural subtype (Pietras et al., 2014). Still, the prognostic role of CD44 has remained unclear as studies have yielded opposite results (Wei et al., 2010). In our material, CD44 staining intensity was increased in grade IV tumors, but the difference was not statistically significant (III). Even though hyaluronan and CD44 were prognostically insignificant, we observed high HAS1, HAS2 and HYAL2 staining intensities in high grade astrocytomas. This implies that hyaluronan metabolism is different in high grade astrocytomas.

Elevated HAS2 and HYAL2 staining intensities were associated with increased proliferation of astrocytoma cells (III). In contrast, glioma cell proliferation has not been affected by HAS2 overexpression *in vitro* (Enegd et al., 2002). Yet, blocking of HAS2 decreases cell proliferation in several other types of cancer cells (Li et al., 2007, Twarock et al., 2010, Wang, S. J. et al., 2013). *In vitro* co-expression of HAS2 and HYAL1 has been shown to restore proliferation of 22Rv1 prostate cancer cells, compared to mere overexpression of HAS2 (Simpson, 2006). In diffusely infiltrating astrocytomas, the cellular proliferation may be increased with co-expression of HYAL2 and HAS2.

In our material, low HAS2 staining intensity was associated with the presence of the *IDH1*-mutation, a known positive prognostic factor (III). This correlation may indicate that low HAS2 intensity and *IDH1*-mutation coexist in low-grade diffusely infiltrating astrocytomas without physiological connection. Nevertheless, this association indicates that high HAS2 expression is associated with poor prognosis in diffusely infiltrating astrocytomas.

6.3 THE EFFECTS OF HYALURONAN SYNTHASES AND HYALURONIDASES ON TUMOR PROGGRESSION IN MELANOMAS AND ASTROCYTOMAS

6.3.1 Simultaneous expression of HASes and HYALs favors carcinogenesis

We observed increased immunoreactivities of HAS1, HAS2 and HYAL2 in high grade astrocytomas (III). Similar results have been reported previously. Human glioblastoma cells lines express HASes and HYALs (Enegd et al., 2002). In vivo, overexpression of HYAL2 in a murine astrocytoma cell line forms larger and more invasive intracranial tumors (Novak et al., 1999). Interestingly, overexpression of HAS2 caused substantial inhibition of intracranial tumor growth in a murine glioma cell line incapable of producing hyaluronidase (Enegd et al., 2002). These studies suggest that both hyaluronan synthesis and hyaluronidase activity seem to be relevant for tumor growth. We also detected high levels of HAS1, HAS2 and HYAL2 in the high grade astrocytomas (III). Similar results have been reported in cancers of epithelial origin, in which high expression levels of HASes and HYAL1 has been observed in bladder carcinoma tissues, compared to normal urothelium (Kramer et al., 2011). Depending on the tumor type, co-expression of both HASes and HYALs seems to be favorable for tumor progression. This co-expression seems to be relevant also in diffusely infiltrating astrocytomas. A hyaluronan rich microenvironment enhances the migration of glioma cells (DeSouza et al., 2013). Nevertheless, high expression of HAS2 without hyaluronidases seems to inhibit tumor growth in murine glioma cells, indicating that both of them are needed for tumor growth (Enegd et al., 2002). The expression of HAS2 and HYAL2 also correlated with increased proliferation activity of tumor cells (III). Of course, it is possible that increased tumor cell number produced higher amounts of HAS2 and HYAL2 which showed as a correlation in our results (III).

Co-expression of HAS2 and HYAL1 has been showed to favor tumor growth and angiogenesis in murine prostate carcinomas (Simpson, 2006). Enhanced angiogenesis caused by hyaluronan oligosaccharides may be due to the observed expression of HASes and HYALs in the astrocytomas. Hyaluronan oligosaccharides are angiogenetic (Gao et al., 2008) and their production demands both HASes and HYALs. Thus, increased turn-over of hyaluronan could favor carcinogenesis via angiogenesis in diffusely infiltrating astrocytomas.

6.3.2 The effects of increased hyaluronan turn over on carcinogenesis

Many *in vitro*-studies indicate that increased hyaluronan synthesis is a favorable feature in glioma cells. Hyaluronan and CD44 induce increased migration and invasion of glioma cells making CD44 the main receptor of hyaluronan in glioma cells (Yoshida et al., 2012, DeSouza et al., 2013, Kim, Kumar, 2014). In our results, hyaluronan and CD44 demonstrated high staining intensities without statistically significant differences (III). Although the levels of hyaluronan and CD44 did not change significantly, elevated expression of HAS1 and HAS2 was associated with increased grade of astrocytomas (III). Thus, high expression of HAS1 and HAS2 seems to be a favorable feature of *in vivo* and *in vitro* astrocytomas. The stable hyaluronan content of high grade astrocytomas could be explained with simultaneously increased hyaluronidase activity but verifying this would demand fresh samples of tumors from which the hyaluronidase activity could be measured.

The association of increased HAS2 with the worse prognosis of astrocytomas may indicate that HAS2 has some hyaluronan-independent mechanisms through which it can affect carcinogenesis. HAS2 is involved in TGF-β-induced EMT which is independent of CD44 and hyaluronan (Porsch et al., 2013). Expression of TIMP-1 is also elevated in breast carcinoma cells in which HAS2 has been silenced thus

reducing the invasion of carcinoma cells (Bernert, Porsch & Heldin, 2011). These studies suggest that HAS2 expression might be beneficial for cancer cells for other reasons than hyaluronan production. Thus, it is possible that HAS2 affects the prognosis of diffusely infiltrating astrocytomas through hyaluronan-independent mechanisms.

Our results indicate that declined hyaluronan synthesis is a favorable feature for the melanoma dissemination as deep melanomas and LN metastases stained less with hyaluronan and HASes. Our results differ from some *in vitro*-studies, in which high hyaluronan levels seem to favor proliferation of melanoma cells (Ahrens et al., 2001). However, Takabe and others showed that increased production of HMW hyaluronan by overexpression of HAS3 decreases migration, cell adhesion and proliferation of MV3 melanoma cells (Takabe et al., 2015). According to our results the function of hyaluronan and HASes seems to be twofold in melanoma. High levels of HASes are detected in the non-invasive phase (*in situ* melanomas) and invasive melanomas demonstrate lower expression. Similar results have been reported in tumors arising from stratified epithelia. Accumulation of HMW hyaluronan in naked mole rat fibroblasts prevents anchorage-independent growth induced by viral oncoproteins (Tian et al., 2013). Degrading HMW hyaluronan with hyaluronidase triggered anchorage-independent growth of these fibroblasts indicating that HMW hyaluronan could prevent malignant characteristics in these cells (Tian et al., 2013). Thus, it is possible that high hyaluronan accumulation around melanoma cells might prevent further spreading and growth in the early stages of melanoma. Once melanoma cells acquire invasive capacities our results indicate that decreased hyaluronan synthesis might be an unfavorable feature.

As an ECM molecule, hyaluronan interacts with other ECM molecules. Some of the changes in hyaluronan content might affect carcinogenesis via other ECM molecules. Versican is a hyaluronan binding protein in ECM and its silencing decreases melanoma cell proliferation and migration (Hernandez et al., 2011). Melanomas also express versican, whereas benign nevi do not (Touab et al., 2002). Thus, hyaluronan might affect the prognosis of melanomas via versican instead of CD44. Hyaluronan has also been shown to induce expression of another ECM protein, osteopontin in glioma cells (Kim et al., 2005). Inhibition of osteopontin suppresses hyaluronan-mediated glioma cell migration (Kim et al., 2005). High hyaluronan content in astrocytomas could enable this osteopontin-dependent glioma cell migration.

6.3.3 The effects of HYAL2 on tumor progression

Invasive melanomas and high grade diffusely infiltrating astrocytomas displayed high HYAL2 expression compared to benign nevi or lower grade astrocytomas, respectively (I and III). In survival analyses, HYAL2 did not affect the prognosis in either group (II and III). In the development of cutaneous melanomas, increased HYAL2 expression is an early phenomenon (I). High hyaluronidase activity may enhance tumor cell spreading due to eradicating the hyaluronan rich environment surrounding the cancer cells. This kind of effect has been reported in naked-mole rat cells, in which the depletion of hyaluronan rich ECM induces tumor formation (Tian et al., 2013). The breakage of hyaluronan rich ECM can also cause opposite effects. Hyaluronidase, PEGPH20, has been combined with gemcitabine and this combination inhibits pancreatic adenocarcinomas tumor growth in mice more than gemcitabine alone (Jacobetz et al., 2013). The effect of PEGPH20 comes from breaking the hyaluronan rich matrix surrounding the pancreatic tumor cells, thus increasing drug delivery of gemcitabine (Jacobetz et al., 2013). High HYAL2 expression may release cancer cells from the tight hyaluronan-rich matrix enabling further cancer cell dissemination because HYAL2 can directly cleave CD44 (Duterme et al., 2009). Cleavage of CD44 with MMPs can disrupt the hyaluronan-CD44 interaction and promote motility of cancer cells (Okamoto et al., 1999, Chetty et al., 2012). Furthermore, LMW hyaluronan have been shown to cleave CD44 in pancreatic carcinoma cell line without any stimulation (Sugahara et al., 2006). This cleavage of CD44 increased tumor cells migration (Sugahara et al., 2006). Thus, high hyaluronidase activity could favor cancer progression in different cancers. High expression of HYAL2 in melanomas and astrocytomas could indicate the acquirement of a motile cancer cell type.

High HYAL2 expression may enhance aggressiveness of tumors by the creation of small hyaluronan fragments. In melanoma, LMW hyaluronan disrupts the lymphatic endothelial barrier and increase LN metastases in mice (Du et al., 2016). LMW hyaluronan can also induce angiogenesis, which is vital for tumor formation (Lennon et al., 2014). One of the features of glioblastomas is active microvascular proliferation. Elevated expression of HYAL2 in high grade astrocytomas could be a favorable feature for the cancer cells as it could partake in the generation of lower molecular weight hyaluronan (III). In our material, endothelial cells of diffusely infiltrating astrocytomas were stained with HYAL2 and CD44 staining was seen perivascularly in glioblastomas (III). This result indicates that they may participate in the angiogenesis of glioblastomas. Similar perivascular CD44 staining has been reported in glioblastomas (Yoshida et al., 2012). CD44 participates in LMW hyaluronan-induced angiogenesis (Lennon et al., 2014). Overexpression of HYAL1 has been showed to increase microvessel density in bladder carcinoma (Lokeshwar, Cerwinka & Lokeshwar, 2005) It is possible that HYAL2 and CD44 could take part in similar processes in glioblastomas, whose hallmark is increased microvascular proliferation.

7 CONCLUSIONS

The main results in this thesis are:

- Benign cutaneous nevi had high hyaluronan content and expressed high amounts of CD44; HAS1 and HAS2 were the main hyaluronan synthases found in them
- High level staining of hyaluronan, CD44, HAS2 and HYAL2 was observed in melanocytic cells of *in situ* melanomas
- Low level staining of hyaluronan, HAS1 and HAS2 was observed in invasive melanomas
- Low immunostaining of HAS2 was associated with known negative prognostic histopathological markers in melanoma; nodular type melanoma, increased mitotic count and decreased number of TILs
- Decreased immunostaining of HAS1 and HAS2 was associated with shorter disease-specific survival and recurrence-free time of melanoma patients
- In the normal brain samples, hyaluronan and CD44 were detected in white matter while HYAL2 was found in cortical areas and overall expression of HAS2 was low
- High immunostaining intensities of HAS1, HAS2 and HYAL2 associate histologically with high tumor grade in diffusely infiltrating astrocytomas
- Low HAS2 staining intensity was associated with high appearance of a positive prognostic marker *IDH1*-mutation in diffusely infiltrating astrocytomas
- Increased proliferation of astrocytoma cells was associated with increased immunostainings of HAS2 and HYAL2
- Increased HAS2 immunostaining intensity was associated with poor prognosis of patients with diffusely infiltrating astrocytomas

This thesis work provides us with new information about hyaluronan metabolism in melanocytic tumors and diffusely infiltrating astrocytomas. Staining results were correlated with clinical data in order to demonstrate the clinical significance. These results increase knowledge of hyaluronan and hyaluronan related proteins in cutaneous melanomas and diffusely infiltrating astrocytomas and how their expression associate with clinical outcome. However, further studies are needed to reveal exact molecular mechanisms how hyaluronan is involved in tumor progression.

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The thesis showed that high hyaluronan content and elevated expression of hyaluronan synthases are observed in in situ melanomas while in invasive melanomas the results were opposite. Instead, diffusely infiltrating astrocytomas showed increased expression of hyaluronan synthases and hyaluronan metabolizing enzyme in aggressive tumors. Thus, these results provide novel information about hyaluronan metabolism in these aggressive tumors and its impact on prognosis of patients.

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