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HLA-A*02 AND ITS PROGNOSTIC TRAITS IN CANCER.

–AN IMMUNOLOGICAL BIOMARKER AS A TOOL TOWARDS INDIVIDUALISED CANCER THERAPY

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Stockholm 2016

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For my (very large) family

ABSTRACT

This thesis is based on the findings that HLA-A*02, a common allele in the Swedish population, has a decreasing frequency in the European continent in relation to geographic latitude. Furthermore, mortality of ovarian cancer correlates to the HLA-A*02 frequency and geographic latitude.

The aims of the thesis were to study the HLA haplotypes and overrepresentation of HLA-A*02 in ovarian cancer patients, to determine the prognostic traits of HLA-A*02 in combination with MHC class I expression, as well as to analyse the role of HLA-A*02 in colon cancer patients together with MHC class I expression and CD8+ lymphocyte infiltration.

In the first paper, HLA-A*02 is determined in 97 ovarian cancer patients and correlated with overall survival. HLA-A*02 was analysed from FFPE tumour samples. Patients with serous ovarian cancer and HLA-A*02 positivity had a poorer survival compared to those who were HLA-A*02 negative.

In the second paper, blood samples were collected from 32 patients with advanced epithelial ovarian cancer (EOC) to determine HLA-A, -B, -Cw and -DRB1. The results showed an unusual representation of HLA alleles in EOC compared to healthy Swedish bone-marrow donors where HLA-A*02 was overrepresented, also HLA-A*02 homozygotes were two-fold higher among EOC patients. The frequency of HLA-A*01 was also increased whereas HLA-A*03 was decreased. Combinations of A*02 with B*05, B*15, DR1*03, DRB1*04, Cw*3 and DRB1*03 were also significantly increased, but only when considered as single, non-corrected analysis. These results indicated that the prognostic trait was restricted to HLA-A*02.

In the third paper, blood samples and FFPE tumour samples were collected from 162 EOC patients and analysed for HLA-A*02 genotype as well as MHC class I and β2 microglobulin expression. Patients were with advanced stage, serous histology and HLA-A*02 positivity were grouped and named "worse prognosis group". In this group there was an increased downregulation of MHC class I, which also correlated to a poorer prognosis. However the level of MHC expression was independent of prognosis when excluded from the worse prognosis group, suggesting that the prognostic trait of HLA-A*02 was not dependent on MHC class I expression.

In the fourth paper, FFPE tumour samples were collected from 520 patients with colon cancer randomised to surgery and adjuvant chemotherapy or surgery alone. Tumour slides were analysed for HLA-A*02 genotype, MHC class I expression, HLA-G expression and CD8+ lymphocyte infiltration. Expression of HLA-G was a negative prognostic marker for the male patients. Also a high infiltration of CD8+ lymphocytes was important in the male patients, where a high frequency of infiltration correlated with a good prognosis. The HLA-A*02 genotype was, on the other hand, a superior negative prognostic marker for the female patients compared to CD8+ lymphocyte infiltration.

In the fifth paper, blood samples and FFPE tumour samples were collected from 16 EOC patients. Blood samples were HLA typed at the clinical immunology laboratory. DNA was extracted from the FFPE samples and primers were designed to amplify exons 2, 3 and 4 of HLA-A. The amplification product was then sequenced and the sequence was aligned against all known HLA-alleles. Complete HLA-A alleles could be assigned to 14/16 patients, in one case only one allele could be assigned and in one case no alleles could be assigned. The conclusion is thus that HLA-A typing can be performed in fragmented FFPE-derived DNA using this approach.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer är en samling sjukdomar som har det gemensamma att celler delar sig okontrollerat och sprider sig i kroppen. För att cancercellerna ska lyckas med detta, måste de bland annat undvika att upptäckas av kroppens immunförsvar. I denna avhandling undersöks två olika tumörtyper, äggstockscancer och tjocktarmscancer. Båda tumörgrupperna har det gemensamma att för att uppnå bot, krävs ofta stora kirurgiska ingrepp och ibland även tillägg av cellgiftsbehandling med mycket biverkningar. I dagsläget är det fortfarande svårt att bedöma vilken patient som gagnas bäst av en specifik behandling.

Humant Leukocytantigen (HLA) är en grupp gener med stor variabilitet belägna på kromosom 6. Dessa gener kodar för en del av proteinerna som kallas MHC, vilka är viktiga när immunförsvaret ska känna igen kroppens egna fungerande celler från invaderande organismer eller kroppsegna celler i stress. Detta sker genom det som kallas för antigenpresentation, när MHC presenterar proteinfragment till immunförsvarets celler som sedan väljer att agera och skapa en immunologisk reaktion, eller avstå. HLA-generna kan delas in i två större grupper, klass I och klass II. Klass I generna kallas HLA-A, -B och -C och kodar för proteiner som presenterar proteinfragment från cellernas insida. Klass II generna kallas HLA-DP, -DQ och -DR och kodar för proteiner som presenterar proteinfragment från cellernas utsida. När en cell är ansatt av exempelvis stress som vid cancer, signaleras detta genom att MHC presenterar proteinfragment till vita blodkroppar (lymfocyter) som tvingar cellen att gå i apoptos, dvs. cellulärt självmord. I denna avhandling diskuteras även HLA-G som är en gen och ett protein som benämns under gruppen icke-klassiska HLA. HLA-G har till skillnad från tidigare nämnda HLA en immunhämmande egenskap och agerar för att avbryta det immunologiska svaret.

HLA-A*02 är en genvariant av typen HLA klass I. Varianten är vanlig i den skandinaviska befolkningen, ca 57 % svenskar bär på denna genvariant, och frekvensen sjunker med den geografiska latituden i Europa. HLA-A*02 har tidigare visat ha koppling till autoimmuna sjukdomar och tillstånd, men det är även visat att HLA-A*02 är vanligare hos äggstockscancerpatienter jämfört med friska individer. Vidare har man även visat att HLA-A*02 har koppling till dålig prognos vid lungcancer, äggstocks- och prostatacancer samt malignt melanom. Denna avhandling syftar till att vidare undersöka HLA-A*02s egenskaper att prognostisera cancersjukdomar och föra en diskussion om den eventuella mekanismen bakom detta.

I **delarbete 1** var syftet att undersöka överrepresentationen av HLA-A*02 bland äggstockcancerpatienter. Därför samlades tumörmaterial från 97 patienter med konstaterad äggstockscancer och undersöktes för närvaro av HLA-A*02 genen. Man kunde se en klar överrepresentation av HLA-A*02 genen bland patienterna med avancerad sjukdom i jämförelse med patienterna med sjukdom lokalt. Vidare kunde man se att patienterna med HLA-A*02 hade en mycket sämre prognos än de som hade en annan HLA-A genvariant, speciellt bland de patienter med en serös äggstockscancer som är den vanligaste typen. Man kunde således dra slutsatsen att HLA-A*02 är en negativ prognostisk faktor för patienter med avancerad äggstockscancer, framför allt för patienter med serös typ.

I **delarbete 2** var syftet att undersöka om andra HLA-typer hade liknande överrepresentation bland patienter med avancerad äggstockscancer. Således samlades blodprov från 32 patienter med avancerad eller återkommande äggstockscancer för att analyseras för HLA-A, -B, -C samt -DRB1. Genfrekvenserna bland patienterna jämfördes med genfrekvenserna bland friska svenska benmärgsdonatorer. Hos patienterna såg man fler HLA-A*01, A*02 och B*08, men färre HLA-A*03 jämfört med kontrollgruppen. Särskilt såg man en fördubbling av patienter som ärvt HLA-A*02 av båda föräldrarna (homozygoter) i patientgruppen. Man kunde således dra slutsatsen att det fanns en ovanlig representation av HLA gener bland patienterna, och att HLA-A*02 var helt klart överrepresenterat.

I **delarbete 3** var syftet att undersöka om det var HLA-A*02 genen eller proteinet den kodar för, MHC klass I, uttryckt på tumörens yta som hade störst inverkan på prognosen i patienter med äggstockscancer. Således samlades tumörmaterial från 162 kvinnor med konstaterad äggstockscancer. Uttrycket av MHC klass I analyserades på tumörmaterialet och HLA-A*02 genen analyserades antingen i tumörmaterialet eller i ett blodprov från patienten. Ett nedsatt uttryck av MHC klass I var förenligt med dålig prognos. Patienterna delades in i två grupper, där den ena som kallades "sämst prognosgruppen" bestod av patienter med HLA-A*02 positivitet, avancerad sjukdom och serös tumörtyp och den andra "bäst prognosgruppen" bestod av resterande. När man sedan jämförde MHC uttryck mellan dessa grupper såg man att det inte hade någon betydelse då patienterna i "sämst prognosgruppen" fortfarande hade sämst prognos oavsett om MHC uttrycket var nedsatt eller ej. Man kunde således dra slutsatsen att HLA-A*02 som gen är mer betydande för den dåliga prognosen än nedsatt uttryck av MHC klass I protein.

I **delarbete 4** var syftet att undersöka om HLA-A*02s prognostiska egenskaper även var aktuella hos patienter med tjocktarmscancer som slumpmässigt valts till kirurgi och cellgiftsbehandling eller enbart kirurgi. Dessutom var syftet att undersöka om MHC klass I uttryck, HLA-G uttryck och närvaro av lymfocyter i tumören påverkade dessa patienters prognos. Således samlades tumörmaterial från 520 män och kvinnor med konstaterad tjocktarmscancer. HLA-A*02 genen, uttrycket av MHC klass I och HLA-G samt närvaro av lymfocyter analyserades i tumörmaterialet. De manliga patienterna hade sämre prognos om de hade få lymfocyter i tumören och om tumörcellerna uttryckte HLA-G. De kvinnliga patienterna å andra sidan hade ingen påverkan i prognos av varken lymfocyter i tumören eller om tumörcellerna uttryckte HLA-G, däremot hade de en dålig prognos om de var positiva för HLA-A*02, oavsett vilken behandling de fått. Man kunde således dra slutsatsen att i HLA-A*02 är en markör för dålig prognos i tjocktarmscancer hos kvinnor.

I **delarbete 5** var syftet att utveckla en ny metod för HLA-A typning i formalin-fixerat paraffininbäddat tumörmaterial. Vid formalinfixering och paraffininbäddning som sker på majoriteten av bortopererat tumörmaterial, sker stora skador på cellernas DNA. Det är således svårt att göra vissa analyser på denna typ av material. HLA-typning är en metod som används framför allt inför stamcellstransplantation. De metoder som används är utvecklade för färska blodprover där patienternas DNA är relativt helt. Tumörmaterial och blodprover samlades in från 16 patienter med äggstockscancer. Blodproverna skickades för HLA-typning på klinisk immunologi laboratoriet. För tumörmaterialet användes DNA amplifiering och sekvensering av de regionerna på HLA-A genen med mest variation för att med den informationen kunna bestämma patientens HLA-A typ. I 13/16 (81 %) av patienterna fick man en komplett HLA-A typning. Man kunde således dra slutsatsen att HLA-A typning är möjligt i formalinfixerat paraffininbäddat material.

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iv.   Tertipis N, **Villabona L**, Nordfors C, Näsman A, Ramqvist T, Vlastos A, Masucci GV, Dalianis T.
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v.   Andersson E, Poschke I, **Villabona L**, Carlson J. W, Lundqvist A, Kiessling R, Seliger B, Masucci GV.
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"A combined analysis of HER3, MMR and HLA-A*02 in primary colon cancer and prognosis"
Submitted manuscript January 2016

CONTENTS

LIST OF ABBREVIATIONS

3'UTR	3' UnTranslated Region
ABC	Avadin-Biotin Complex
APM	Antigen Presenting Machinery
CCR7	C-C Chemokine Receptor 7
CD	Cluster of Differentiation
CI	Confidence Interval
CRC	Colorectal Cancer
CTL	Cytotoxic T Lymphocyte
DAB	3'DiAminoBenzyne
DC	Dendritic Cell
ddNTPs	diDeoxyNucleoTides
DNA	DeoxyriboNucleic Acid
dNTPs	DeoxyNucleoTides
ECM	Extracellular Matrix
EFI	European Federation of Immunogenetics
EOC	Epithelial Ovarian Cancer
ER	Endoplasmatic Reticulum
FFPE	Formalin-Fixed Paraffin-Embedded
FFPE	Formalin-Fixed Paraffin Embedded
HAM-TSP	HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis
HC	Heavy Chain
HFE	Human Hemochromatosis (gene)
HIPEC	Hyperthermic IntraPeritoneal Chemotherapy
HLA	Human Leukocyte Antigen
HNSCC	Head and Neck Squamous Cell Carcinoma
HPR	Horse Radish Peroxidase
HR	Hazard Ratio
HTLV	Human T-Lymphotrophic Virus
IDO	Indoleamine 2,3-doxygenase
IFNγ	Interferon gamma
IGKC	Immunoglobulin G Kappa Chain
IHC	Immunohistochemistry
Ij	Invariant chain
IL	Interleukin
MDSC	Myeloid-Derived Supressor Cell
MHC	Major Histocompatibility Complex
miRNA	Micro RNA
MLC	Mixed Lymphocyte Culture
NK	Natural Killer
NKT	Natural Killer T-cell
OS	Overall Survival
PCR	Polymerase Chain Reaction
PCR-SSOP	PCR with Sequence-Specific Oligonucleotide Probes

PCR-SSP	PCR with Sequence-Specific Primers
RFLP	Restriction-Fragment-Length-Polymorphism
RNA	RiboNucleic Acid
ROS	Reactive Oxygen Species
SAP	Shrimp Alkaline Phosphatase
SBT	Sequence-Based Typing
SNP	Single Nucleotide Polymorphism
TAM	Tumour Associated Macrophages
TAP	Transporter-Associated Protein
TCR	T-cell receptor
TDO	Tryptophan 2,3-dioxygenase
TGFβ	Transforming Growth Factor beta
TH	T Helper cell
TIL	Tumour Infiltrating Lymphocyte
TME	Tumour microenvironment
Treg	Regulatory T-cell
VEGF	Vascular Endothelial Growth Factor
WP	Worse Prognosis group
β_2M	Beta-2-microglobulin
$\gamma\delta$T-cell	gammadelta T cell

1 INTRODUCTION

1.1 CANCER – SIMPLY A COMPLEX DISEASE

"Cure sometimes, treat often, comfort always."

-Hippocrates

Cancer is a collection of diseases, which have the same fundamental finding, an uncontrolled growth and metastasis of cells. In order for the cells to succeed with this, there are a set of properties they must master, and these were summarized as the hallmarks of cancer. These hallmarks are sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (1). Progress in tumour biology research has added two additional hallmarks: reprogramming of energy metabolism and evading immune destruction (2).

1.1.1 The immune response

The immune response is the organisms' way to protect itself against foreign pathogens, but to avoid reacting against its 'normal' self. The response has evolved to destroy whatever has been triggered by pathogens, or pathogenic changes of the organism, and thus protect the body as a whole. This response, carried out by a variety of cells, has also been shown to protect us against malignancies. It can generally be divided into two systems, innate immunity being the first and quickest response, and adaptive immunity, which is the slower but more effective response.

1.1.1.1 Innate immunity

The cells of the innate immune system are usually triggered by recognising a pattern of components conserved among a broad group of microorganisms, or when damaged, injured or stressed cells send out alarm signals. This recognition is mediated by different pattern recognition receptors (PPRs) inherited through generations (3). The cells of the innate immunity consist of macrophages, neutrophils, dendritic cells (DCs), mast cells, eosinophils, basophils and natural killer (NK) cells. Macrophages engulf invading micro-organisms; degrade them into peptides, which are then presented to the cells of the adaptive immunity in order to activate them. DCs perform constant pinocytosis, where they filtrates residual peptides from the surrounding fluid in order to present these to the cells of the adaptive immune response. The presentation of these peptides is dependent on the HLA antigen, which is described in section 1.2.1.

Natural killer (NK) cells are a type of cytotoxic lymphocytes, which require both an activating signal and the absence of an inhibitory signal. The activating receptors of NK cells can be activated by cytokines or antibodies. The inhibiting receptors are activated by the major histocompatibility complex (MHC) on other cells, making the NK cell distinguish between "self" and "missing self" (4).

1.1.1.2   Adaptive immunity

The adaptive immune response is activated by the innate immune response and upon its activation is a much more effective way of protection. Not only is the response more specific due to improved recognition, the response is also memorised allowing the response to start much faster if the pathogen is re-encountered. The cells involved in the adaptive immune response are lymphocytes named B- and T-cells (5).

B-cells are in charge of the humoral immune response and produce large quantities of antibodies, which can act either as neutralising or opsonising. Neutralising antibodies attach to toxins secreted by e.g. bacteria and neutralise the effect of the specific toxin. Opsonising antibodies form a coat around the entire pathogen and thus inactivating them until they are destroyed or engulfed by cells of the immune system (5).

T-cells consist of different subgroups with different functions in the adaptive immune response. CD4+ T helper cells and CD8+ T cytotoxic cells are activated by interaction between their T-cell receptor (TCR) and MHC class I or II antigens. Upon this activation the antigen presenting cell produces either IL-12 or IL-4 in order to make the response takes one of two motifs. IL-12 production leads to a **Th1 response** characterised by production of IFNγ, which activates macrophages, opsonising antibody production by B cells and differentiation of CD8+ cytotoxic T-cells. This response is generally more effective against intracellular pathogens such as viruses. IL-4 leads to a **Th2 response** which is characterised by IL-4, IL-5 and IL-13 production which activates eosinophils and neutralising antibody production in B-cells. This response is generally more effective against extracellular pathogens such as bacteria and parasites (5).

Other subgroups of T-cells are regulatory T-cells (Tregs), which act as inhibitors of the immune response by producing immunosuppressive cytokines, disrupt the CD8+ T-cell mediated killing and inhibit DC maturation (6). Gamma-delta T-cells (γδT-cell), which abide in the gut mucosa, are assumed to be a link between the innate and adaptive response (7). Together with natural killer T (NKT)-cells, which are T-cells expressing similar markers as NK-cells (8), they all belong to a group of T-cell subsets with characteristics border-line to the innate and adaptive response.

1.1.2   Tumour microenvironment

Cancers are surrounded by a complex environment, which they depend on for sustained growth, invasion and metastasis. This tumour microenvironment (TME) comprises not only of cancer cells, but also extracellular matrix, blood and lymphatic vascular networks, innate and adaptive immune cells. These components collectively have critical modulatory functions in tumour development and metastasis and can also be referred to as the tumour stroma (Figure 1).

1.1.2.1   Extracellular matrix

In order for the tumour to grow into its surroundings it needs to take control of the extracellular matrix (ECM) around it. It does that by recruiting fibroblasts to join its cause. Tumour associated fibroblasts produce tumour-promoting growth factors, chemokines, cytokines, ECM components and ECM remodelling enzymes. Furthermore, it has also been shown that they have important immunosuppressive activity e.g. by producing TGFβ (9) which stimulates induction of Tregs (10). Adipocytes usually surround the TME. In some cancers they aid recruitment of malignant cells through the secretion of adipokinases. They also provide fatty acids as fuel for cancer cells (11).

1.1.2.2 Vasculature networks

The growth of the tumour requires oxygen and nutrients to succeed. Vascular endothelial cells are thus recruited by the production of angiogenic factors, e.g. vascular endothelial growth factor (VEGF)-A. These factors form new blood vessels which have a chaotic branching and uneven vessel lumina. They are also leaky, raising interstitial pressure with uneven blood flow, oxygen, and nutrient and drug delivery (12). Pericytes are perivascular stromal cells which provide support for the blood vessels. Tumours with a low abundance of these cells tend to have a poor prognosis and increased metastasis (13).
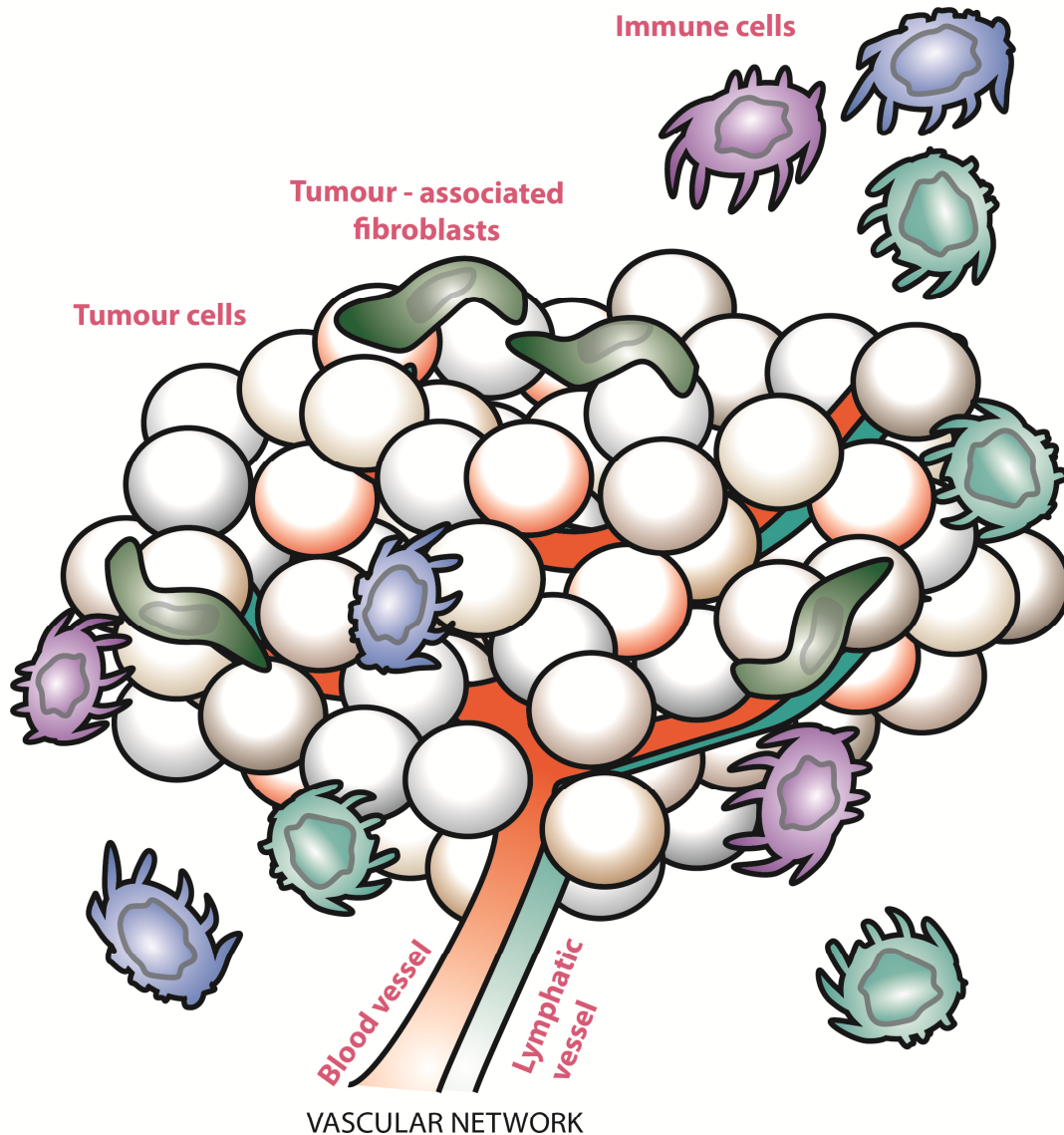
Figure 1. The TME consists of not only malignant cells, but also fibroblasts which produce ECM components, endothelial cells which make up the blood and lymph vessels and immune cells which are either pro- or anti-tumour.

To have a successful dissemination of malignant cells, the lymphatic system is invaded or stimulation of lymphatic vessels sprouting can be achieved by production of factors such as VEGF-C and –D. The lymph vessels are also important to alter the hosts' immune response to the tumour (14). Mesenchymal stem cells can be recruited from the bone marrow and reach the TME via the blood stream. They give rise to new fibroblasts, pericytes, adipocytes and smooth muscle cells in the TME (15).

1.1.2.3   Immune cells

When the immune system is alerted to the presence of the tumour, several immune related cells migrate to the TME. T-cells are abundant in the majority of human cancers. They are found within and surrounding the tumour mass. The variation of pro- and anti-tumour subsets varies. CD8+ cytotoxic T-cells, CD4+ Th1 helper T-cells and γδ T-cells are usually associated with a good prognosis. FOXP3+ Tregs, CD4+ Th2 T helper cells and Th17 cells are usually associated with a poor prognosis (16). B-cells are sometimes found in the invasive margin, but they are more often discovered in secondary or tertiary structures adjacent to the TME (17). Tumour associated macrophages (TAMs) are typically tumour-promoting. They produce angiogenic factors and accumulate in hypoxic or necrotic areas of the TME (18). Myeloid-derived suppressor cells (MDSCs) are inhibitory immune cells and produce large amounts of IL-10. They thus inhibit cytotoxic T-cells and polarize TAMs to a tumour-promoting phenotype (19). Tumour-associated neutrophils can have both pro- and anti-tumour activity (20). Terminally-differentiated myeloid dendritic cells may be defective in the TME and cannot stimulate an immune response to tumour associated antigens. NK cells are usually found outside the tumour area (15).
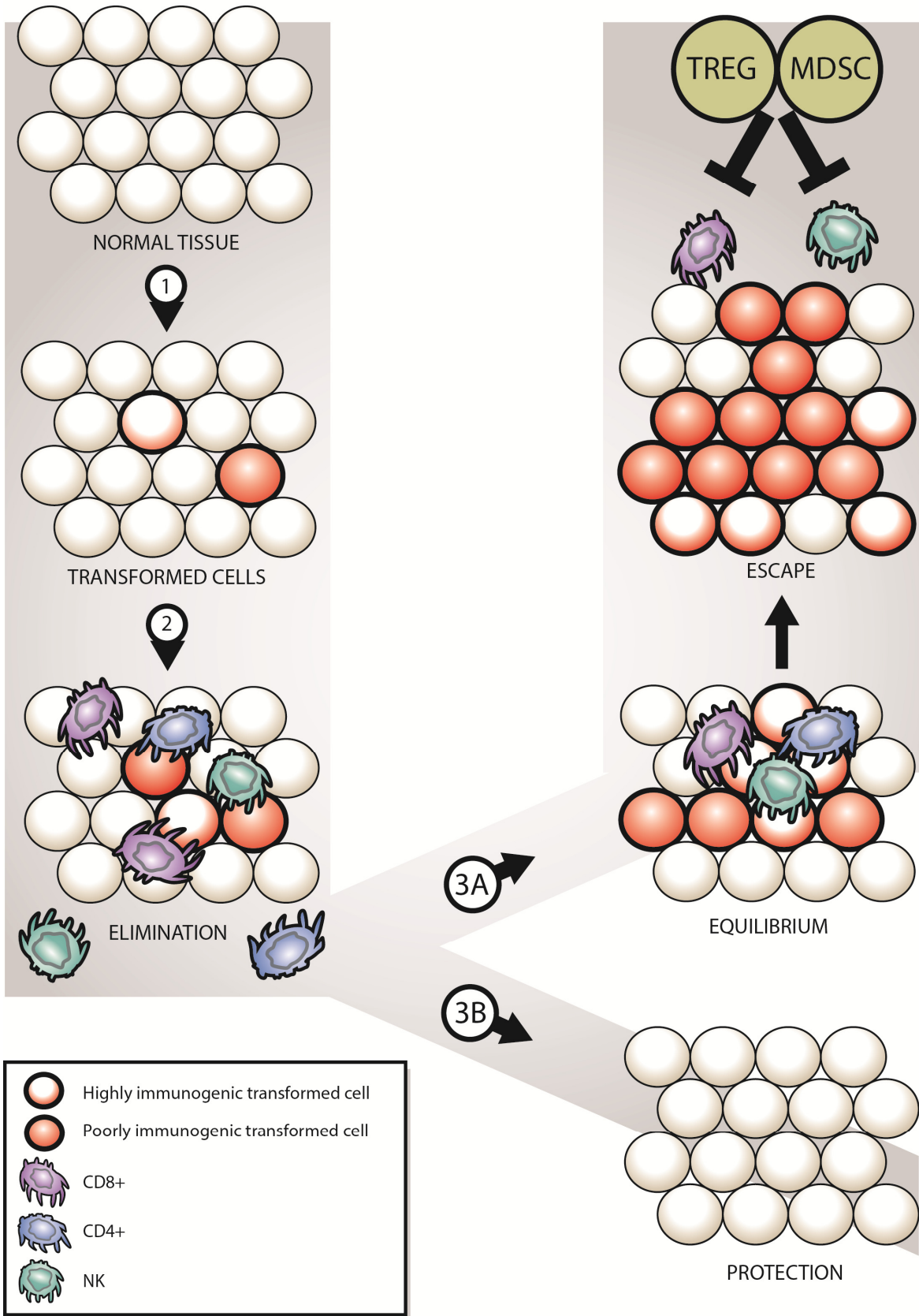
1.1.3   Tumour immunology

Already in 1957 the immune system was considered to have an effect on cancer, when Burnet et al., hypothesised of an immune surveillance against malignant cells (21). However, not until recently has the complex interaction between the immune system and malignant cells become clearer. Immunoediting is the process by which the tumour develops during the immune response of the body. This process is a complicated balance of mechanisms that is believed to tilt back and forth, but to simplify it has been divided into three steps: elimination, equilibrium, escape (22).

1.1.3.1   Elimination

During elimination, the cells of the innate immune system recognize the growing tumour due to local tissue damage, distressed cells or stromal remodelling. This is followed by the introduction of inflammation in order to recruit other immune cells (NK cells, macrophages, dendritic cells) to the tumour site. There is a high production of IFNγ to eliminate cancer cells. Debris from the battle is taken up by macrophages and dendritic cells and presented in the local lymph node to T-cells in order to educate the adaptive immune response. This education triggers the differentiation of Th1 cells which elicit the development of cytotoxic CD8+ T lymphocytes, also known as killer T-cells. Macrophages and NK cells may also transactivate one another via IFNγ and IL-12 production, which promotes more killing of tumour cells by apoptosis and reactive oxygen and nitrogen intermediates (22, 23).

4

NORMAL TISSUE

1

TRANSFORMED CELLS

2

ELIMINATION

3A

EQUILIBRIUM

TREG MDSC

ESCAPE

3B

PROTECTION

Highly immunogenic transformed cell

Poorly immunogenic transformed cell

CD8+

CD4+

NK

1.1.3.2 Equilibrium

Sometime during the elimination phase the tumour either is eliminated or adapts to its surroundings and initiates an equilibrium phase. This phase is thought to be the longest phase in solid malignancies, and one may envision a Darwinian selection of tumour cells where many are destroyed and new variants arise carrying new mutations that provide yet another immune resistance. During this phase it is believed that the immune system holds the tumour in a state of functional dormancy with a balance between anti-tumour (IL-12, IFNγ) and tumour promoting cytokines (IL-10, IL-23). Due to the constant immune pressure, tumour cells must evolve to resist immune recognition by antigen loss or defects in the antigen presenting machinery (APM) (22, 23).

1.1.3.3 Escape

The elimination gradually swishes to the escape phase. Tumour cells can escape the immune system by many different mechanisms: reduced immune recognition, increased resistance and survival or development of an immunosuppressive tumour microenvironment. In order to evade immune recognition, MHC class I, class I-like or co-stimulatory molecules need to be lost. Tumour cells with increased resistance intensify expression of STAT-3, a transcription factor that mediates process for cellular growth. In order to increase survival, anti-apoptotic signals are enhanced, such as bcl2. An immune suppressive environment can be achieved by several mechanisms. Either cytokines VEGF, TGFβ, IL-6 and M-CSF can be released which inhibit differentiation, proliferation and activation of immune cells (22, 23), create a tryptophan depraved milieu which inhibits clonal T cell expansion (24), or inhibitory molecules can be expressed.

There are many examples of inhibitory molecules that can be expressed in an immunosuppressive milieu. Some examples of these are IDP, TDO, PD-L1, CD39, CD73, HLA-E and HLA-G. IDO and TDO are tryptophan catabolic enzymes that suppress T and NK cells. They also activate immune suppressive cells such as Tregs and MDSC. Another example is PD-L1, which binds to a receptor on B and T-cells, and initiates apoptosis in antigen specific T-cells, while it simultaneously reduces apoptosis in Tregs. CD39 and CD73 are enzymes that drive the metabolic environment towards an immune suppressive one with high concentrations of adenosine, making it beneficial for Tregs (22, 23). Finally, HLA-E and -G which are NK cell inhibiting molecules may be expressed (25).

Figure 2. When normal cells transform into malignant cells (step 1), they are recognised by the immune system which leads to an immune response and the Elimination of malignant cells (step 2). This response may be successful and eliminate all malignancy (step 3a) or the malignant cells may adapt to the pressure of the immune system and an Equilibrium is reached where the malignant cells create an immune suppressive environment by increased levels of IFNγ (Step 3b). When enough malignant cells with the ability to evade the immune system have been selected and the tumour can Escape the immune system and grow without immune interference (step 4).

6

1.1.4 Immunological biomarkers in cancer

1.1.4.1 T-cells and immune score

Finding immunological biomarkers which correlate to cancer progression is not a new ambition in the scientific world. The field has however, in recent years shown more progress due to technological advances.

Calculating tumour infiltrating T lymphocytes (TILs) with immunohistochemistry (IHC) in tumour material was initially used to establish a correlation to prognosis (26, 27). The primary ambition was to use TILs as a therapeutic intervention by adoptive T-cell transfer. This was strategy was successful to some extent with the co-stimulation of IL2 (28). However, the correlation of TILs and clinical outcome gave inconsistent results in different studies, probably due to differences in protocols and manual cell count. Consequently a debate arose on the prognostic implication of TILs, was it good or bad for the patient? In 2006 however, a prognostic benefit in TILs for colorectal cancer (CRC) was reported (29). In that study an objective scoring system based on image analysis was used, and it showed that the type, density and location of immune cells within tumours predicted positive clinical outcome. Furthermore, other groups demonstrated by immunostaining of hundreds of CRC specimens that a strong local immune reaction correlated with a favourable prognosis regardless of the local extent of the tumour or the regional lymph node involvement. In these studies analysis for CD3+, CD8+, and memory CD45RO+ T-cells were included (16, 30). T-cell infiltrates emerged as the stronger independent prognostic factor, when compared to the conventional clinicopathological criteria such as tumour size, depth of infiltration, differentiation, or nodal status (31). Using these results, "an immune score" can be calculated, that takes consideration to of the density, location, phenotype, and function of T-cells for each tumour as a part of the standard pathologic examination. This "immune score" emerges as the first immunologic marker of risk in cancer with the potential to be incorporated into a prognostically relevant immune classification of human CRC equal to or better than the conventional TNM classification (31).

Evaluating circulating T-cells is another approach that has been studied as a potential biomarker. Unfortunately no convincing evidence has shown that circulating T-cells can predict patient outcome (32). The reason for this may be that the assays used in these studies, i.e. ELISPOT and CellSearch, which use single antigen epitopes for detection. However, the differentiation of circulating T-cells has been correlated to prognosis. The optimal immune response in a tumour situation is to drive T-cells to differentiate to CD8+, also known as cytotoxic, T-cells. This drive can be mediated by chemokines which bind to CCR7 expressed on T-cells. Low frequencies of circulating CD8+ CCR7+ T-cells have been shown to be a recurrence risk factor in head and neck squamous cell carcinoma (HNSCC) (33). The hypothesis is therefore that the breadth of an immune response to tumour associated peptides rather than a response to a single peptide may correlate better with clinical response (34, 35). This makes it even more important to establish an immune score rather than looking for single immunological markers.

Every war has its casualties, even the immune response against cancer ends in cell death for many T-cells. The frequency of CD8+ T-cells undergoing spontaneous apoptosis in the blood of patients with cancer has been shown to be significantly elevated compared to healthy controls (36). Apoptosis occurs preferentially via the Fas/FasL or Trail/TRAILR pathways, or indirectly through the release of tumour-derived exosomes. Spontaneous apoptosis can be measured by analysing Annexin V using flow cytometry. Using this approach attempts have been made to both screen for

cancer, and also to correlate Annexin V elevation to prognosis, however without success for both (37).

1.1.4.2 B-cells

In comparison to T-cells, fewer studies have been conducted researching the prognostic value of B-cells. There are; however interesting results concerning B-cells in cancer. Both B- and T-cell infiltration has been shown to correlate to a better prognosis in malignant melanoma (38) and serous ovarian carcinoma (39). Also in oro- and hypopharyngeal carcinoma a high frequency of peritumoral B-cells together with intraepithelial CD8+ T-cells in metastatic tumours was associated with a favourable in patient outcome (40). Immunoglobulin G kappa chain (IGKC) in the tumour stroma was associated with metastasis-free survival in node negative breast cancer and predicted response to anthracycline-based neoadjuvant chemotherapy. Similar results have been shown for CRC and non-small cell lung cancer, but could not be shown in ovarian cancer (41).

1.1.4.3 NK cells

NK-cells have also shown interesting results as prognostic markers in cancer. Infiltration of tumours with NK cells was shown to represent a positive prognostic marker CRC, gastric carcinoma and squamous cell lung cancer (42-44). Unfortunately, there were often only a few infiltrating NK cells, making their contribution to tumour elimination questionable (45). The inability of NK cells to reach the tumour may be due to the immunosuppressive milieu that the TME creates.

1.1.4.4 Immune suppressors

Two types of immune suppressive cells have been the most studied in correlation to cancer prognosis, regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs). A high frequency of Tregs has been linked with poor prognosis in ovarian carcinoma, melanoma, breast cancer and glioblastoma (46). The marker used to detect Tregs has most often been FOXP3+, which unfortunately has been shown to be somewhat unreliable, since Tregs are a very heterogeneous and consist of many subsets (46). The prognostic value of Tregs is yet to be proven. MDSCs also consist of several subsets, making it also here difficult to determine which cell subset to focus on. The commonly used markers have been CD33+, CD11b+, CD15+ and CD14+, which differ in their mechanisms of immune suppression (47). After cryopreservation CD15+ and CD33+ are significantly decreased and the remaining CD14+ and CD11b+ lose their ability to produce reactive oxygen species (ROS). These findings have led to the conclusion that MDSCs should only be studied in fresh blood samples (48), making their potential as a prognostic biomarker somewhat limited.

1.1.4.5 Cytokine expression

Cancer is considered to be a Th2-dominant disease with an increased IL-4, IL-5, IL-10 and TGFβ production (49). A shift back towards the Th1 profile may thus correlate to a better clinical outcome (50). Increased levels of IL-6 in the circulation have been associated with decreased survival (51). Inflammatory cytokines, IL-1β, IL-6 and TNFα, facilitate tumour growth possibly by STAT3 hyperactivation in both tumour and immune cells (52). Cytokines as prognostic markers have similar technical difficulties as MDSCs, since the storage of samples greatly affects the cytokine concentration. The analyses thus require fresh blood or tumour samples, again making their biomarker potential limited.

1.1.5 Ovarian cancer

Ovarian cancer is a malignant tumour originating from ovarian tissue. The majority of ovarian cancer is epithelial ovarian cancer (EOC), but there are also other forms originating from the stroma or follicular cells. These much smaller groups of cancers are not addressed in this thesis. About 750 patients are diagnosed yearly with EOC in Sweden, and the incidence has decreased since the 1980's with 2.7% per year (53). One theory behind the decrease of cases is that there is a protective effect from hormonal contraception. Another theory is that there has been an optimisation in diagnosis. Women of any age are at risk of developing ovarian cancer, but the risk increases with age, giving the highest risk in women of ages 75-89 years.

1.1.5.1 Diagnosis and staging

The classification of ovarian cancer has been difficult to establish due to the fact that the ovaries are not easily accessible and biopsies are rarely made without clear pathological indication. Previously the classification has been based on the histological characteristics of the tumour, and ovarian carcinoma was thus divided into serous, endometrioid, mucinous and clear cell carcinoma. In recent years, however, the scientific community has however established a consensus, which groups these malignancies according to mutational characteristics rather than morphology. Therefore EOC was divided into two major groups: type I and II. **Type I** includes tumours of low-grade serous, low-grade endometrioid, clear cell and mucinous carcinoma. These are usually diagnosed at an early stage and have a slow progression. **Type II** includes high-grade serous, high-grade endometrioid, carcinosarcoma and undifferentiated carcinomas. These carcinomas are usually aggressive and diagnosed in late clinical stages (54). High-grade serous cancer is the most common type and accounts for approximately 45-55% of all EOC cases in Sweden.

1.1.5.2 Staging

The purpose of staging is both to describe the disease manifestation, but also to give prognostic information and determine further treatment. In ovarian cancer, the staging is made according to the FIGO criteria. In **Stage I** the cancer is limited to the ovary. This is further divided into IA) the cancer involves one ovary with an intact capsule; IB) the cancer involves both ovaries with intact capsules; IC) the tumour involves one or both ovaries with additional capsule rupture or malignant cells in the peritoneal cavity. In **Stage II** the cancer extends to one or both ovaries, peritoneal cavity and pelvis. Also here there are subgroups: in IIA there is cancer found in the uterus and fallopian tubes; in IIB there is tumour elsewhere in the pelvis. In **Stage III** the cancer is found outside the pelvis or in the retroperitoneal lymph nodes and more specifically, the cancer has metastasised to the retroperitoneal lymph nodes (IIIA), has microscopic metastasis in the peritoneum <2cm (IIIB) or has macroscopic metastasis in the peritoneum >2cm (IIIC). Finally, in the final **stage IV** the cancer has spread outside the peritoneum and caused distant metastasis, either shown as pleural effusion with cancer cells (IVA) or metastasis to distant organs or the inguinal and extra-abdominal lymph nodes (IVB) (55).

1.1.5.3 Treatment and prognosis

Primary treatment of ovarian cancer is surgery. Macroscopic radical surgery is clearly correlated with overall survival (56). This surgery consists of bilateral salpingoophorectomy, total hysterectomy, omentectomy, appendectomy and several excisions from the peritoneum. Also the

abdominal cavity is rinsed for cytological analysis in order to detect presence of malignant cells. Lymphadenectomy is not performed if the tumour is a low-grade serous carcinoma or endometrioid carcinoma with a high differentiation due to the low risk of metastasis. In advanced cases, bowel resection, splenectomy, extensive peritoneal resection, cholesystectomy and resection of the diaphragm and pleura may also come in question. Adjuvant chemotherapy is usually added to the treatment, with some few exceptions. Platinum-based treatment with carboplatin and paclitaxel intravenously has been a standard treatment since the late 1970s. In Sweden, neoadjuvant chemotherapy is recommended to patients with inoperable disease, i.e. stages IIIC-IV with good general condition. If the chemotherapy is successful in decreasing the tumour load to the extent of surgery being possible, patents undergo surgery as described above.

Intraperitoneal chemotherapy treatment is a recent approach offered to patients with stage III disease with a minimal residual tumour post primary surgery. The treatment is infused through a port in the abdomen. The objective is to increase the local dosage in the peritoneum (57). Hyperthermic intraperitoneal chemotherapy (HIPEC) was first established to treat peritoneal carcinoma in colon cancer patients, but has recently also been performed on EOC patients. Heated, sterile chemotherapy solution is circulated throughout the peritoneal cavity for up to 90 minutes after surgery. The objective here is to increase circulation and oxygen intake of the tumour and consequently increase the absorption and sensitivity of chemotherapy (58). Unfortunately for both intraperitoneal treatments, there are no randomized studies which show a benefit in patient outcome.

Other, more targeted cancer therapies are also used in EOC patients. Bevacizumab is an antibody, which binds to VEGF-A, an endothelial growth factor needed for the formation of blood vessels to the tumour. Upon binding the effect of VEGF-A is inhibited, and as a consequence it becomes impossible for the tumour to grow further, since it cannot receive nutrients. Adjuvant treatment with Bevaciumab in combination with carboplatin and paclitaxel has shown an increase in progression free survival in EOC patients (59). Poly-ADP-ribos-polymerase (PARP) inhibitors are the newest addition to EOC treatment. PARP is a base-excision-repair protein which when inhibited leads to double stranded DNA breaks causing an apoptotic reaction of the cell. Olaparib, a PARP-inhibiting antibody has been shown to effectively decrease the tumour burden in patients with BRCA-mutation and breast cancer and/or EOC (60).

The mortality of ovarian cancer is the highest of all gynaecological cancers, most likely due to the diffuse symptoms and thus results in a late diagnosis. The majority of cases are diagnosed in FIGO stage III-IV when the tumour already has spread to local lymph nodes or metastasised to other organs.

1.1.6 Colon cancer

Colon cancer is the third most common cause of cancer in men and women In Sweden about 4000 new cases, divided about the same in both genders, are diagnosed per year. The incidence has increased with 70% over the past 40 years, and this increase is assumed to be due to a successively aging population (53). Most of the cancers (90-95%) spring from the glandular cells and are adenocarcinomas. In the Stockholm region, a population screening has been in use since 2008 which has a calculated mortality decrease in both colon and rectal cancer of 16% (61). Risk factors are high fat/low fibre diet, high levels of red meat consumption and smoking. Individuals

with an inflammatory bowel disease are also at greater risk of developing colon cancer, whereas acetylsalicylic acid intake has been shown to have a protective role.

1.1.6.1 Staging

Staging in colon cancer is currently made according to the TNM staging system. T describes the primary tumour and its growth and/or invasion into neighbouring tissue. N describes the amount of metastasis in the regional lymph nodes. M describes the presence of distant metastasis.

Previous to the TNM classification, another system was used developed by pathologist Cuthbert Dukes in 1932. Dukes A: invasion into but not through the bowel wall. B: Invasion through the bowel wall penetrating the muscle layer but not involving lymph nodes. C: Involvement of lymph nodes. D: Widespread metastases. The Dukes classification system has in recent years been replaced by the more detailed TNM system, but is still usable when performing retrospective analysis.

1.1.6.2 Treatment and prognosis

Similar to ovarian cancer, surgery is the primary treatment for colon cancer. The main objective with the surgery is to remove all tumour bearing segments with sufficient margin along and around the bowel. In addition, the lymph vessels connected to the tumour must be extracted and a thorough lymphadenectomy is required since at least 12 negative lymph nodes is sufficient to exclude lymph node metastasis (62). If the tumour is found mid-surgery to be unresectable (10-15% of cases), the Swedish recommendations state that no resection is to be attempted and the patient may be alleviated by a stoma (63). These patients may become resectable after neoadjuvant chemotherapy. In stage I colon cancer, no chemotherapy is offered and surgery is the definitive treatment. The role of chemotherapy in stage II colon cancer is debatable. There is evidence that adjuvant chemotherapy treatment in stage II colon cancer patients increased disease-free survival (DFS) (64). It is therefore reasonable to offer treatment to patients with high risk features such as obstruction, perforation of the tumour or inadequate lymph node sampling. Adjuvant treatment usually consists of fluorouracil or capacetabine and in some cases in combination with oxaliplatin. Also irinotecan and tegafur/uracil have shown to increase overall survival in colon cancer patients.

Successful targeted therapies in colon cancer have been few. Cetuximab inhibits the epidermal growth factor receptor (EGFR) and thus inhibits the cancer cells uncontrolled mitosis. Cetuximab has shown to increase resectability of liver metastasis in a neoadjuvant setting when given in combination with oxaliplatin- or irinotecan-based therapies (65). Also Bevacizumab was used for colon cancer in combination with oxaliplatin and fluorouracil to increased resectablilty of liver metastasis (66), however due to adverse events as bowl perforation, increased bleeding, thromboembolic events and increased wound healing time it is no longer given as a routine.

The possibility for a curative treatment in colon cancer is strongly correlated to survival. Patients with tumour stage I-II tumours have >90% chance of 5-year survival. Patients with tumour stage III and IV have a 75% and 26% chance of 5-year survival respectively.

## 1.2 HUMAN LEUKOCYTE ANTIGEN – THE CELLULAR ID TAG

"The human mind treats a new idea the same way the body treats a strange protein; it rejects it"

- Peter Medewar

A crucial part in the immune system is to recognize when to defend. Many steps are assigned to the recognition of normal cells in the body and to distinguish them from invaders of dysfunctional cells. One of the mechanisms of signalling is called antigen presentation, an event where the cell presents peptides to the outside by binding them to a protein and transporting them to the surface. The general name for the antigen presenting proteins in vertebrates is the major histocompatibility complex (MHC). Antigens of the human MHC were initially studied using antibody reactions with white blood cells and they were therefore called the human leukocyte antigens (HLA).

1.2.1 HLA molecules

All HLA antigens share some similarities and have the capacity to bind a wide range of peptides, which attach to the cleft of the HLA molecules. When a "foreign" peptide is bound, the HLA-peptide complex interacts with the T-cell receptor (TCR). Interaction between the two is a first step in the activation of the adaptive immune response. HLA antigens are divided into three structurally and functionally distinct groups, class I, class II and non-classical HLA antigens.
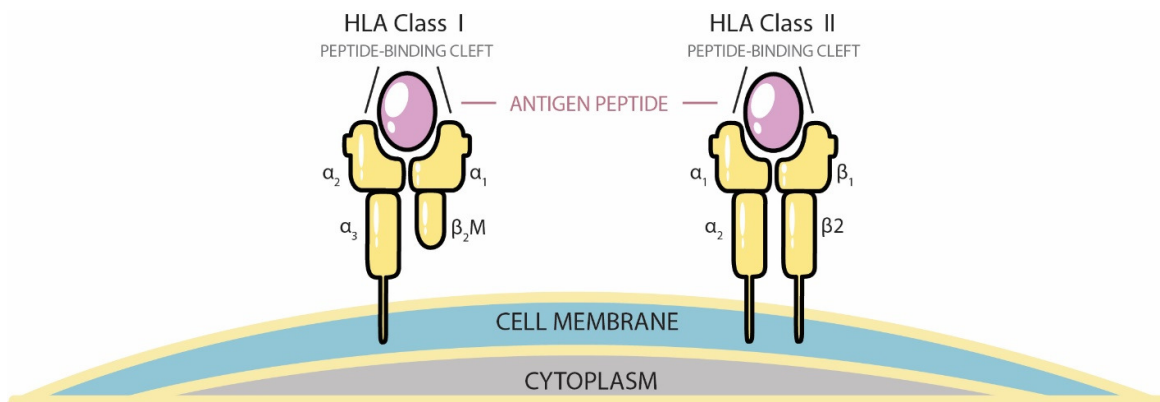
Figure 3. HLA class I antigens consist of dimers of a transmembrane glycoprotein and a soluble protein β2-microglobulin. HLA class II antigens are heterodimers of two transmembrane glycoproteins.

1.2.1.1 HLA class I antigens

HLA class I antigens consist of dimers of a transmembrane glycoprotein and a soluble protein $\beta 2$-microglobulin (β_2M). HLA class I antigens are expressed on most somatic nucleated cells with, depending on cell type, some discrepancies in the level of expression. For example, muscle cells and neurons have very low expression, whereas lymphocytes express high levels of class I antigens. The peptide bound by HLA class I molecules are mainly generated by proteasome-mediated cleavage of cytosolic proteins. Peptides are translocated into the endoplasmatic reticulum (ER) by transporter associated protein (TAP). Inside the ER the incomplete HLA class I

molecule is stabilized by Calnexin. Upon the binding of β_2M, Calnexin disassociates, and the HLA class I molecule is nearly complete. In order to bind the peptide, the HLA class I molecule is stabilised by a TAP complex including Calreticulin, Erps and Tapasin. Upon binding a "foreign" peptide, the HLA class I molecule leaves the ER via an endosome. The class I peptide complex is transported to the cell surface via the Golgi, where it is recognised by CD8+ T-cells. Upon activation these cells become cytotoxic (5).

1.2.1.2   HLA class II antigens

HLA class II antigens are heterodimers of two transmembrane glycoproteins. Unlike HLA class I antigens, they are only expressed by a limited number of cells, dendritic cells, macrophages and B-cells. After having been synthesised in the ER, the HLA class II molecule complexes with the invariant chain (Ii) to stabilize it in the absence of a bound peptide and to target it to the endocytic pathway. The Ii blocks the binding of antigenic peptides to the HLA class II molecule and is removed by stepwise proteolysis in endosomes, before the antigenic "foreign" peptide is loaded. For removal of the final fragment of the Ii, the CLIP segment, HLA-DM is required. HLA-DM catalysis the peptide-exchange, proves and favours the presentation of stably binding peptides. HLA class II molecules bind peptides predominantly derived from proteins internalised from the outside the HLA class II-bearing cell. These can be cell-surface proteins, soluble proteins or proteins derived from whole organisms such as viruses, bacteria or protozoan parasites that invade or are phagocytosed by the cell. HLA class II-peptide complexes are transported to the cell surface where CD4+ T-cells recognise them. CD4-positive T-cells mainly function as helper cells (T_H), releasing cytokines (e.g. L-2, IL-4, IL-5 or γ-interferon) which serve to amplify the overall immune response by introducing T and B-cell proliferation, macrophage activation or B-cell differentiation (5).

1.2.1.3   Non-classical HLA antigens

HLA-E, -F, -G are part of the non-classical HLA antigen system and do not play a prominent role in presenting antigens to T-cells. Instead they interact with natural killer (NK) cells as part of the innate immune response and are involved in suppression of the immune system (67). HLA-G exists as both a membrane bound and a soluble protein, whereas HLA-E and –F have a similar structure to HLA class I (5). High levels of HLA-G expression in trophoblast cells during pregnancy are assumed to participate in the establishment of maternal-foetal tolerance. In contrast to HLA-G, the HLA-E gene is transcribed in most-cells and tissues. Additionally, HLA-F also exhibits a restricted pattern of mRNA expression and is reported to be expressed mostly in B-cells (68).

Figure 4. Class I antigen presentation is illustrated on the left. Cytosolic proteins considered to be foreign are digested by the Proteasome into peptide fragments. These peptide fragments are translocated into the ER where they bind to the peptide binding cleft of the MHC class I. Upon the binding of the peptide, the MHC class I complex is translocated to the cell surface by vesicles. Upon arrival, it interacts with the TCR on a CD8+ T cell in order to activate an immune response. Class II antigen presentation is illustrated on the right. Antigens are endocytosed from the outside of the cell and translocated by endosomes to an MHC class I compartment. The MHC class II, residing in the ER, has its peptide binding cleft blocked by Ii. Ii is removed leaving the final piece, CLIP which is removed by HLA-DM in the MHC class II compartment. This results in the binding of a peptide which can be presented on the cell surface with the MHC class II. This complex interacts with a CD4+ T cell in order to activate an immune response.
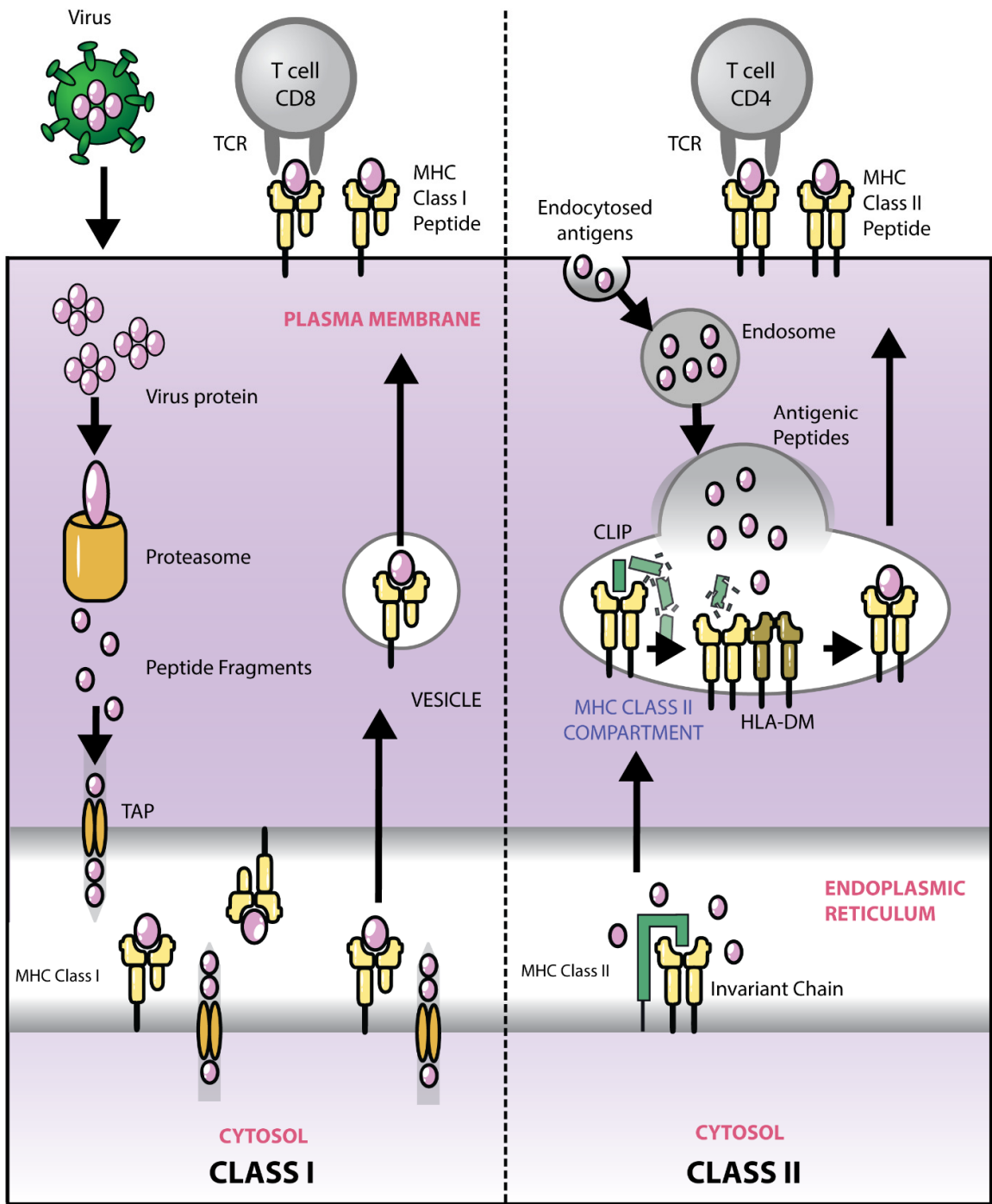
1.2.2 HLA genetics

The HLA locus is located on chromosome 6, on position 6p21.1-21.3. This locus spans 3,6Mb and extends to 7,6Mb with the telomeric repeats (69, 70).

HLA class I genes are grouped on the HLA supercluster. This region comprises the classical and non-classical class I genes (-A, -B, -C and –E, -F, -G respectively), and class I-like genes (MICA and MICB) (70).

Classical HLA class I genes are highly polymorphic and this genetic variation is principally based on single nucleotide polymorphisms (SNPs). The most polymorphic gene is HLA-B, with a high quantity of variants making it the most polymorphic human gene. Besides the SNPs, insertion/deletion polymorphisms are important (70). Nucleotide sequence analysis of alleles shows that the variation between alleles is restricted predominantly to the exons that encode the HLA peptide-binding domain. It implies that this high polymorphism on theses alleles defines the repertoire of peptides that can bind to HLA allotypes. Additionally, the expression of the different genes is co-dominant, which means that a heterozygous person can express six different classical HLA class I and class II allotypes (71). This kind of variety defines an individual's ability to respond to exposure to many infectious agents meaning that the HLA polymorphism is favourable to the species survival (72).

Non-classical HLA class I genes, i.e. HLA-E, -F and -G are related in sequence identity and molecular structure to classical genes, but have lower expression levels, tissue specific expression, and low levels of polymorphism (73).
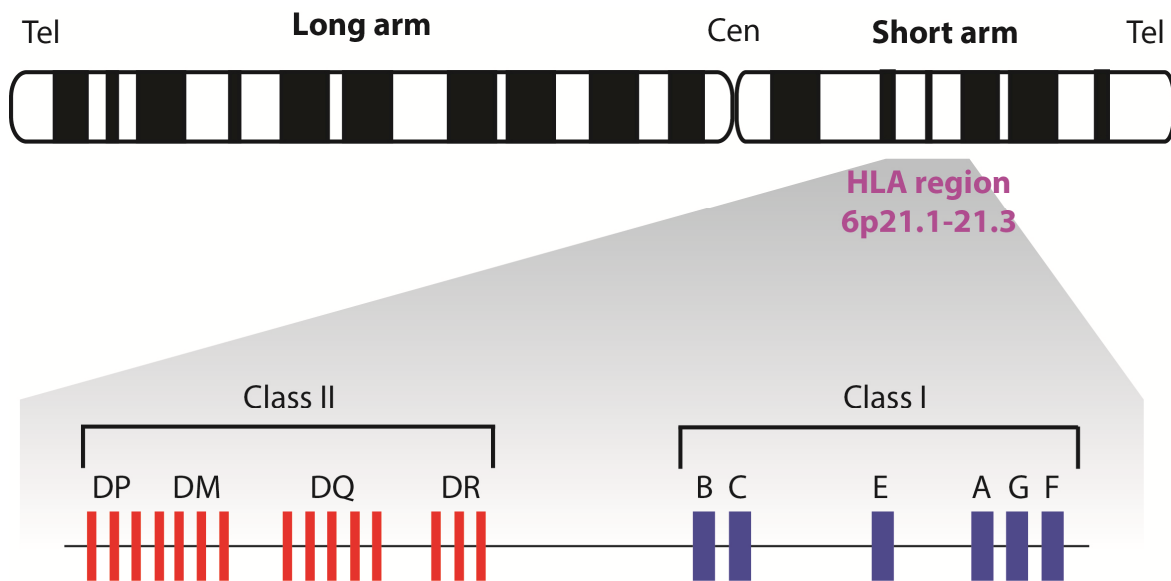
Figure 5. The HLA region is located on the short arm of chromosome 6. HLA class I consists of classical HLA-A, -B and -C; as well as non-classical HLA-E and -G. HLA class II consists of HA-DP, -DQ and -DR; as well as non-classical HLA-DM.

The number of different phenotypes that are possible from random combination of the known HLA alleles is greater than the earth's population. However, HLA genes are inherited together as haplotype, which is the combination of alleles encoded by different loci on the same chromosome. Certain alleles of different loci are more frequently inherited together than expected by random combination. This phenomenon is called positive linkage disequilibrium. It makes certain haplotypes, e.g. the A2, B7, DR2 haplotype or the A1, B8, DR3, are over-represented in a given population.

1.2.2.2 HLA nomenclature

The nomenclature used to describe different HLA antigens has been confusing and partly explained by the development of different typing techniques. In the cellular assays, initially used, cells were sorted into groups depending on their inability to respond to homozygous stimulator cells. Since this is a functional test of all HLA molecules on the cell surface the cellular specificities, the series of HLA-D types were influenced not only by DRB1 genes but also by other DRB genes (the DRB3, DRB4 and DRB5 genes) and also by DQ genes as well as DP.

HLA-B, -C and -D alleles previously maintained the additional "w" to distinguish them from the loci for complement factors B, C and D. The genes coding for these are also located in the HLA region. HLA-D has since then been renamed –DR and HLA-B and -C are now usually indicated with "*".

Using serological for typing, the assignment of HLA alleles was based on the reaction of specific antisera with human cells. However, since antibodies cannot detect all variability, each serologically identified specificity could include many subtypes (e.g. HLA-A*0201).

The recent use of genomic typing techniques, DNA sequencing and PCR-based typing techniques, has made it possible to assign each HLA class I and II allele uniquely. Genomic typing was previously indicated by "*", e.g. HLA-A*020101, however, this suffix has in recent years been used for all typing methods. The consensus of the nomenclature currently used is illustrated in Figure 6.
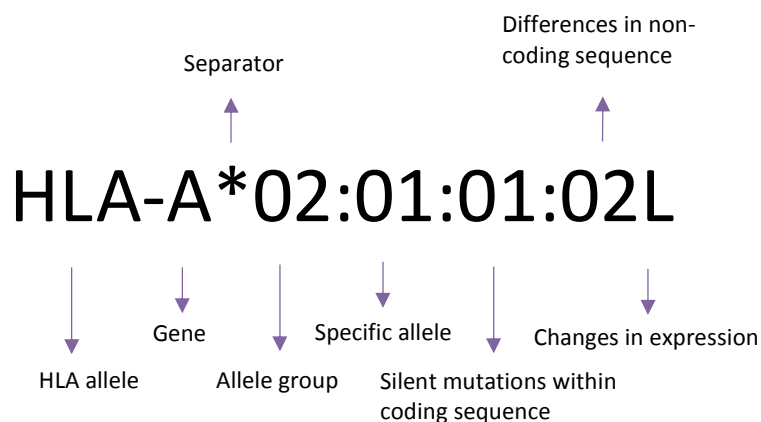
Figure 6. Explanation of HLA nomenclature. Adaptation from hla.alleles.org.

1.2.3 HLA typing

1.2.3.1 Cellular methods

The discovery of HLA class II molecules was made using mixed lymphocyte culture (MLC). The method uses increased DNA synthesis as a sign of cell division in a "responder" CD4+ T-cell recognising MHC class II differences of a "stimulator" lymphocyte. This increase was measured by tritiated thymidine (^{3}H-TdR) incorporation. The stimulator cells were inhibited to proliferate by previously administering mitomycin C treatment or irradiation. When typing HLA class I antigens using this method, CD8+ T-cells were often used as "responders" since they could induce cell-mediated lysis which could be detected by the release of Cr^{51} in labelled target cells. The response was compared to a maximum and minimum lysis of cells (74).

1.2.3.2 Serological typing

Serological HLA typing was based on the detection of expressed HLA class I and II molecules on the surface of T- and B-cells. The assay required live lymphocytes and a large panel of different antisera (75). Serological typing methods can still be used as a more crude measure of cell identity. The limitations are still considerable as the allelic variation of the HLA molecule is mainly in the peptide binding cleft and thus inaccessible to antibodies, making polymorphisms undetectable with serologic typing.

1.2.3.3 Genomic typing

In the end of the 1980s, the DNA based typing methods started to replace the serological typing especially for HLA class I and II antigens. The first DNA analysis was restriction-fragment-length-polymorphism (**RFLP**) where genomic DNA was cleaved with restriction enzymes. The digested DNA was then separated according to size by gel electrophoresis giving a fragment pattern that could be analysed (76-78). Unfortunately the resolution was not on the allele level.

With the birth of the polymerase chain reaction (PCR) in 1983 (79), the door for new DNA based typing methods opened. One of these methods was PCR with sequence-specific oligonucleotide probes (**PCR-SSOP**). This assay was based on hybridisation of labelled oligonucleotide probes to the amplification product of the HLA locus. The probes were detected by radioactivity, or enzymatic labelling and many samples could be processed at the same time (80, 81). Another method was PCR with sequence specific primers (**PCR-SSP**) where a series of PCR amplifications using perfect complementary group specific primers were used. The results were then visualised by gel electrophoresis and based on the pattern of the presence or absence of product, an allele could be determined (82, 83).

When using sequence-based typing (**SBT**), the sequences of the polymorphic exons were determined. A PCR with dye-labelled primers was performed, amplifying both alleles of the specific locus. The alleles were sequenced as a mixture with fluorescent automated sequencing, and the results analysed and identified heterozygous positions and assigning alleles (84). Some combinations were however ambiguous, especially for the HLA-B locus. In these cases a more selective PCR reaction was performed. This method was quite expensive and was thus mostly used to determine allele resolution and when a new allele gave an unexpected result.

1.2.3.4   Typing in FFPE derived material

Routine tissue processing has generated banks of formalin-fixed paraffin embedded (FFPE) tissue from patients. These biobanks can be a valuable material source when performing retrospective studies. Unfortunately there are some difficulties when working with this material. The yield of DNA from FFPE tissue is four times less than that from fresh tissue and 30% of the mount that can be extracted from frozen tissue due to autolytic or bacterial degradation (85). Another problem with FFPE derived DNA is fragmentation due to formation of covalent bindings formed by formaldehyde. Fragmentation makes amplification reactions difficult, since there is a limit to the size of amplification product, anything from <150bp (86) to 400bp (87) have been reported. Furthermore, artificial base alterations can occur during the fixation step (88), making it difficult for primers to adhere during amplification and also to interpret sequencing results. Finally, inhibiting substance remnants from the extraction reactions and contamination can be an obstacle in receiving an amplification product or sequence. Despite these limitations, successful HLA typing in FFPE material has been reported by several authors. Ota et al (89) used a PCR-SSOP method and successfully typed HLA class I and II in different types of tissue after 24-96h formalin fixation. Also PCR-SSP has been reported to be a successful approach, Bateman et al (90, 91) typed for HLA class II with a nested PCR successfully. Finally Lee et al (92) showed that a PCR-SSOP method could be used but needed to be confirmed by a nested PCR-SSP. The fact that a nested PCR amplification was needed gave an indication that there may be unfragmented DNA strands present in the samples. However, the DNA fragmentation of FFPE samples increases with time, giving a maximum age to the samples one could include. In paper V of this thesis, a method for HLA-typing is presented in which the above problems are circumvented.

1.2.4   HLA and disease

To find correlation between HLA alleles and diseases has been ongoing for as long as we have been able to study the HLA. Most of these connections have been made in the field of autoimmune diseases. It has been shown that 90% of Caucasians with ankylosing spondylitis are HLA-B*27:02 or B*27:05 positive. Also 90-100% of the narcoleptic Caucasians patients carry HLA-DRB1*06:02. In type 1 diabetes, >90% of the patients are HLA-DRB1*03/DQB1*02:01 or HLA-DRB1*04/DQB1*03:02 as compared to 40% in the control group. The mechanisms behind these associations have been heatedly discussed over the years, and in general the debate can be divided into three major theories:

1) *The mistaken identity-theory,* where it is believed that the HLA allele appears to associate with the disease, although the actual culprit belongs to a different locus in the haplotype and associates through linkage disequilibrium. Hemochromatosis was initially associated with HLA-A*03 and A*29 positivity. It was later discovered that there was a two point mutation in a non-classical HLA, now called HFE which was inherited together with the HLA-A alleles mentioned due to linkage disequilibrium (93, 94).
2) *The immune reactivity to self-antigens theory*, which is proposed to depend on different mechanisms such as an aberrant T-cell repertoire selection, immune cross-reactivity with foreign antigens or immune attack on "altered-self" antigens.
3) *The MCH cusp theory* proposes a role in the disease outcome for a tri-dimensional cusp-like structural motif common to all products of the HLA gene family (95). This cusp-like structure is located in the α2 domain of HLA class I molecules and a similar structure on β1 domain in class II molecules. Ligands produced by these cusp-like structures encoded

by their HLA alleles interact with the receptors in the non-adaptive immune system and activate pathways, aberrations or amplifications.

There are several associations between HLA-A*02 and specific medical conditions. This genotype has been associated with spontaneous abortion in infertile couples, thought to be due to the increased maternal immune response to the foetus (96). Other autoimmune conditions that have also reported to have an association to HLA-A*02 are juvenile rheumatoid arthritis (97), vitiligo (98) and uveitis (99, 100). There has also been a debate on the association of HLA-A*02 to Alzheimer disease, however more than one study, has shown this to be the case in different populations (101-103). Further studies show HLA-A*02 as a risk factor for multiple sclerosis (104). Finally a study by Torres et al(105) report an increased frequency of HLA-A*02 in patients with autism, which could be explained by the fact that HLA-A*02 positivity affects prenatally.

1.2.5 HLA and cancer

1.2.5.1 MHC class I expression and cancer

The downregulation or loss of MHC class I expression on tumour cells is believed to be an important immune escape mechanism (106, 107). The alteration of the malignant cells MHC class I phenotype has been shown to be both reversible and irreversible. Reversible downregulation of MHC class I is believed to be on a transcriptional level and the MHC class I expression can be restored by cytokines, e.g. IFNγ or TNFα (108, 109). Also, down-regulation of different components of the APM have also been demonstrated (110, 111). Irreversible alterations to MHC class I expression is believed to be due to genetic changes such as mutations and chromosomal defects. HLA haplotype loss is the most frequently described, where either one or both copies of HLA-A, -B and -C genes are lost (112).

The prognostic value of MHC class I down-regulation or loss is still under debate. Low expression of MHC class I has been shown to correlate to prognosis in different malignancies, while several studies fail to show the same correlation (107, 113, 114). A reason for this may be the usage of FFPE samples and monoclonal antibodies which does not distinguish between intracellular and cell surface proteins. Also, the MHC class I staining is sometimes difficult to assess since the expression of MHC class I in tumours can sometimes be intermediate. Different groups have therefore invented different scoring systems, making results difficult to compare.

1.2.5.2 HLA-G protein expression and cancer

Numerous studies have been performed on more than one thousand malignant lesions, showing that although turned off in surrounding normal areas, HLA-G gene transcription and protein expression is switched on in various tumour lesions. In light of the known tolerogenic or immune response suppressive properties of HLA-G, their role in cancer were is believed to be a tumour-driven immune escape mechanism. HLA-G expression by tumour cells protects them from NK- and CD8+ lymphocyte mediated cytolysis (115). Expression of HLA-G has been linked to poorer prognosis in several malignancies. However, it has also been observed to correlate with a better prognosis n haematological malignancies (116-121). This is believed to be due to the suppressive properties of HLA-G may function as a negative feedback loop on the malignant immune-cell clone and thus inhibit its proliferation (122).

1.2.5.3   HLA genotype and cancer

There are an increasing number of reports showing an association between HLA-genotype and cancer. In breast cancer HLA-DQB*03032 and DRB1*11 positive patents were less frequent compared to the control group, suggesting that these two alleles may be "protective" (123). In patients with ulcerative colitis who had developed CRC, HLA-DR17 was overrepresented compared to the control group of ulcerative colitis patients who had not developed colorectal cancer (124). Also in virus-related malignancies HLA-allele associations have been studied. Women carrying the HLA-DRB1*0301 allele had an increased risk of developing cervical intraepithelial neoplasia when infected by HPV 16. This allele was also overrepresented in cervical intraepithelial neoplasia grade III cases compared to grade II cases (125).

*1.2.5.4   HLA-A*02 genotype and cancer*

HLA-A*02 has been associated with a reduced risk to develop EBV positive Hodgkin's lymphoma, whereas HLA-A*01 has been associated with increased risk (126). HLA-A*02 has also been associated with in situ cancer of the vulva (127), cervical cancer (128) and nasopharyngeal cancer (129).

HTLV-1 is known to cause adult T-cell leukaemia, but also independently, the neurological disorder HTLV-1-associated myelopathy/tropical spastic paralysis (HAM/TSP). In HLA-A*02 positive individuals have been shown to have a twofold reduction of the proviral load in HAM/TSP patients compared to asymptomatic carriers (130).

HLA-A*02 has been shown to be correlated to poor prognosis in non-small cell lung cancer (131), ovarian (132) and prostate cancer (133) and malignant melanoma (134). In fact HLA-A*02 is a very common allele in the Swedish population, and the allele frequency decreases with geographical latitude. HLA-A*02 genotype is a strong prognostic factor linked to the mortality of ovarian, prostate cancer and malignant melanoma (133).

2 AIMS

The aims of this thesis were:

✓ To determine the prognostic traits of HLA-A*02 genotype in epithelial ovarian cancer.

✓ To evaluate if the prognostic value of the genotype HLA-A*02 and the expressed protein MHC.

✓ To investigate the prognostic traits of HLA-A*02 in colorectal cancer which is known to be responsive to immunological activity.

✓ To design an HLA-typing method for FFPE material.

✓ To continue the search for the mechanism behind the prognostic traits of HLA-A*02 in cancer.

3 MATERIALS AND METHODS

"I was taught that the way of progress was neither swift nor easy."

- Marie Curie

3.1 PATIENTS AND SAMPLE MATERIAL

The patient cohorts are summarised in Table 1. All patient material used in this thesis has either been tissue from tumour material fixed in formalin and embedded in paraffin, or peripheral blood samples.

In **paper I**, all patients admitted to the oncology department at Karolinska University Hospital with ovarian cancer were recruited during the year 1995. All data, such as age, clinical stage, tumour grade, treatment received were recorded in a database. FFPE tissue blocks from all patients were collected and analysed for the HLA-A*02 genotype. A total of 97 patient FFPE samples were collected, of which seven were unamplifiable and these patients were thus excluded. Of the remaining 88 patients the histology was as follows: 44% serous, 28% endometrioid, 13% clear cell, 6% mucinous, 7% undifferentiated and 2% unclassified. 33% were in clinical stage I-II, 67% were in clinical stage III-IV.

The cohort of thirty-two patients described in **paper II** were considered for recruitment to an HLA-A*02 restricted peptide vaccination trial. The main inclusion criterion was relapsing or advanced epithelial ovarian carcinoma. Of these patients 12.5% were diagnosed in stages I-II and 75% in stage III and 12.5% in stage IV. Most patients had received more than two lines of chemotherapy and 75% were diagnosed with serous histology. Patients were asked for a blood sample in order to analyse their full HLA haplotype.

Initially 182 patients were considered for inclusion in **paper III**. All patients were referred to the oncology department at Radiumhemmet, Karolinska University Hospital 1995-2004. Only 162/182 had available FFPE blocks for immunohistochemical analysis. HLA-typing was performed on peripheral blood samples from living patients and HLA-A*02 genotype was determined from FFPE-derived DNA in deceased patients. The histology distribution was as follows: 52% serous, 28% endometrioid, 9% mucinous, 6% clear cell and 6% undifferetiated and mixed epithelial. Furthermore, 34% were in clinical stage I-II and 66% were in clinical stage III-IV.

The 520 patients in **paper IV** were recruited from a Nordic adjuvant colorectal cancer trial of stage II and III, enrolled from 1991 to 1997. Patients were randomly assigned to surgery or surgery and adjuvant 5-FU-based chemotherapy. Inclusion criteria were colon cancer diagnosis and age under 75 years. The distribution between men and women was 52% and 48% respectively. Parameters of clinical outcome were obtained from centres of epidemiologic oncology in Sweden. The Dukes staging system and the stage 0-IV system for colon cancer was used in pathology reports. FFPE tissue blocks from patient's primary tumours were collected.

The 16 patients described in **paper V** were randomly selected from the patient cohort in paper II. FFPE tissue blocks were collected for each patient.

Table 1. Patient material overview

	Patients	**Year of sample**	**Patient material**	**HLA-Typing**
Paper I	97 patients with epithelial ovarian cancer	1995	FFPE tissue blocks	HLA-A*02 specific primers
Paper II	32 patient with epithelial ovarian cancer	2004-2005	Peripheral blood sample	Complete HLA typing
Paper III	162 patients with epithelial ovarian cancer	1995-2004	Peripheral blood sample and FFPE tissue blocks	Complete HLA typing and HLA-A*02 specific primers
Paper IV	520 patients with colon cancer stages Duke B and C	1992-1995	FFPE tissue blocks	HLA-A*02 specific primers
Paper V	16 patients with ovarian cancer with known HLA-type.	2004-2005	Peripheral blood sample and FFPE tissue blocks	Complete HLA typing and sequence based HLA-A typing

3.2 GENETIC ANALYSIS

3.2.1 DNA extraction

Three different methods of DNA extraction have been used in this thesis, mostly depending on the origin of the sample. For material derived from FFPE tissue blocks, "High-pure RNA paraffin" kit from Roche was used. For material derived from blood samples both "DNeasy Blood & Tissue" kit from Quiagen and "High-pure DNA extraction" kit from Roche were used. The benefit of using kit solutions is that everything comes prepared and most reagents are simple to add without much pre-treatment. The disadvantage of using kits is that if the kit does not function, it is hard to find the malfunctioning step and most protocols do not give much room for adjustment.

3.2.1.1 DNA extraction from FFPE material

To prepare DNA from FFPE-samples the High-pure RNA paraffin kit (Roche) was used. 10 μm thick sections were cut on a microtome and placed in a microcentrifuge tube. The samples were deparaffinised with xylene and 99% ethanol. Tissue lysis buffer, proteinase K and 10% SDS were then added to the samples and incubated at 56°C over-night. The following day the samples were treated with binding buffer and placed in a filter tube where they were washed. To complete the extraction the nucleic acids were eluted from the filter tube and the concentration was measured using Nanodrop. The DNAse step was removed from the protocol hence the mix of RNA and DNA was obtained.

3.2.1.2   DNA extraction from blood samples

The DNA from the one blood sample which was used in paper V was extracted using Quiagen DNeasy Blood Tissue kit (Quiagen AB, Sollentuna, Sweden).

3.2.2 DNA amplification

The polymerase chain reaction (PCR) was developed by Mullins in 1983 (79) and has become a fundamental method in molecular biology. The method is based on utilizing thermal cycling to enable a continuous enzymatic replication of DNA strands. In order to guide the enzyme to amplify the desired gene, primers complementary to the start of the reading frame are designed.

*3.2.2.1   HLA-A*02 PCR*

In the first four papers HLA-A*02 was determined by using primers specific for most HLA-A*02 subtypes, with the exception of HLA-A*02109, *0248, *0250 and *0255. Primers were kindly provided by Associate professor Olle Olerup (Olerup SSP AB, Stockholm). The expected size of the amplicon is 124bp. To determine if the extracted DNA was amplifiable, another set of primers were used to amplify the house-keeping gene S14.

Example of PCR protocol layout:

No of samples: 1

Master Mix (Olerup SSP)	6µl
Sample (30ng/µl)	4µl
Primer F (1pml/µl)	2µl
Primer R (1pml/µl)	2µl
H2O	6µl
Total	20µl

PCR cycling program:

94°C 2min	1 hold
94°C 10s	10 cycles
65°C 60s	
94°C 10s	20 cycles
61°C 50s	
72°C 30s	
4°C	1 hold

Positive amplification was detected by gel electrophoresis using a 2% gel run on 120V for around 20 min. Product size was determined by 1kbPlus ladder (Thermo Fisher Scientific, Stockholm, Sweden).

Table 2. Primers for HLA-A*02 determination

Name	Target	Direction	
A*55LL	HLA-A2	F	5'GGA GCC CCG CTT CAT CGC A3'
A*503invLL	HLA-A2	R	5'CTC CCC GTC CCA ATA CTC CGG A3'
S14sense	S14	F	5'TCA AAA GGG GAA GGA AAA GA3'
S14antisense	S14	R	5'CAG TGA CAT GA CAA AAG TG3'

3.2.2.2   HLA-A PCR

To circumvent the problems with DNA fragmentation in FFPE samples, primers were designed to amplify exons 2, 3 and 4 separately of all HLA-A alleles, with the exception of A*23:11N. Exon 4 was divided into a first and second half. The expected amplification products were 292bp for exon 2, 258bp for exon 3, 166bp for exon 4 1st half, 201bp for exon 4 2nd half. Primers used can be viewed in Table 3.

Example of PCR protocol layout:

No of samples: 1

10x PCR buffer	5.0µl
dNTPs (1.25mM)	8.0µl
MgCl (25mM) 3.0µl	
Primer F (1pmol/µl)	3.0µl
Primer R (1pmol/µl)	3.0µl
Taq polymerase	0.2µl
dH2O	22.8µl
Sample (30ng/µl)	5.0µl
Total	50µl

PCR cycling program:

94℃ 2min	1 hold	
94℃ 30sec	40 cycles	
60℃ 30sec		
72℃ 45sec		
4℃	1 hold	

Table 3. Primers for HLA-A typing

Name	Target	Direction	
A2-1F	HLA-A Exon 2	F	5' TCT CAG CCA CTG CTC GCC 3'
A2-2F	HLA-A Exon 2	F	5' TCT CAG CCA CTC CGT C 3'
A3-1F	HLA-A Exon 3	F	5' GGT TCT CAC ACC GTC CAG A 3'
A3-2F	HLA-A Exon 3	F	5' GGT TCT CAC ACC ATC CAG A 3'
A4-1F	HLA-A Exon 4 1st half	F	5' GCG CCC CAA AAC GCA TAT G 3'
A4-2F	HLA-A Exon 4 1st half	F	5' CCC CCC CCC CAA GAC ACA TAT G 3'
A4-3FH	HLA-A Exon 4 2nd half	F	5' TTC TAC CCT GCG GAG ATC AC 3'
A4-3FH	HLA-A Exon 4 2nd half	F	5' GTC CTC GCT CTG GTT GTA G 3'
A4-3FH	HLA-A Exon 4 2nd half	F	5' GCC CTC GCT CTG GTT GTA G 3'
A2-1R	HLA Exon 2	R	5' GTC CTC GCT CTG GTT GTA G 3'
A2-2R	HLA Exon 2	R	5' GCC CTC GCT CTG GTT GTA G 3'
A3-2R	HLA-A exon 3	R	5' CTT CCC GTT CTC CAG GTC TC 3'
A4-1R	HLA-A Exon 4 1st half	R	5' ATC CCC TGC AGG CCT GGT 3'
A4-2RH	HLA-A Exon 4 2nd half	R	5' CCA TCT CAG GGT GAG GGG 3'

3.2.3 DNA sequencing

In 1977, Fredrik Sanger developed an effective way to determine a DNA sequence (135). This method involved PCR amplification of the DNA region of interest. This was followed by a subsequent sequencing reaction using dideoxynucleotides (ddNTPs) mixed with deoxynycleotides (dNTPs). ddNTPs terminates the polymerases reaction, resulting in a premature stop upon its binding. As the ddNTP's are randomly incorporated, the DNA fragments of different lengths are accumulated. These fragments are then run through a capillary system to sort them according to size, and exposed to an argon ion laser. As each ddNTP holds a different fluorescent property, the result can be interpreted as chromatogram, with each curve representing a nucleic base in the

DNA sequence. In paper five of this thesis Sanger sequencing was used to find the sequence of exons 2, 3 and 4 of the HLA-A gene in order to determine a correct HLA-A allele.

PCR products, acquired from above described method, were cleaned from excess primers and nucleotides by using ExoSAP-IT®. This reagent contains two enzymes, endonuclease I which removes remaining single-stranded primers and left over single-stranded DNA; and Shrimp Alkaline Phosphatase (SAP) which dephosphorylates dNTPs. The enzymes are activated by heating the mixture to 37°C for 15 min and deactivated by heating to 80°C for 15 min.

For the sequencing PCR, automated dye-terminator sequencing was used, where a fluorescent dye is incorporated onto the ddNTPs. This dye emits light at different wavelengths which can be recorded on an Applied Biosystems 3730 DNA analyser. Each sample sequenced using both forwards and backwards primers in order to analyse the complete product.

Example of sequencing protocol layout:		PCR cycling program:	
No of samples: 1			
		96°C 30sec	25 cycles
Big Dye mix	4µl	50°C 5sec	
Sequencing dilution buffer	2µl	60°C 4min	
Primer F or R (1pmol/µl)	3µl		
dH2O	6µl		
Template DNA	5µl		
Total	20µl		

3.2.4 DNA sequence analysis

The sequences were obtained by analysis in Chromas Lite software version 2.01 (Technelysium Pty Ltd). The two sequences per sample, forward and reverse, were exported to a FASTA format and the reverse sequence was translated using Gene Runner Software version 3.05 (Hasring Software, Inc.). Forward and translated reverse sequences were then aligned using *clustalw 2* tool (136), forming a consensus sequence which could be analysed using IMGT-HLA Blast engine (137). An HLA-A allele was thus assigned. By searching for double peaks in the chromatogram, heterozygotes were identified.

3.3 PROTEIN ANALYSIS

3.3.1 Immunohistochemistry

In order to detect specific proteins, molecular biology has exploited antibodies which are immunoglobulins produced by the immune system. By producing antibodies to target desired proteins and attaching reagents to their tail is a good way to visualize protein targets.
When applying this method on histologically intact tissue it is called immunohistochemistry (IHC). In this thesis IHC was used to detect MCH class I, HLA-G, and CD8+ lymphocytes (papers III and IV).

3.3.1.1 Summary of standard protocol

Tissues were fixated and cut into slides, approximately 4µm thick. Slides were de-paraffinised in xylene for 5 min, followed by lowering concentrations of ethanol, 70%, 95% and 100%. Slides

were then washed and placed in citrate buffer in a water bath. The bath was then brought to boil and kept just below boiling point for 30 min in order to make the antigen accessible to the antibody. Following this, slides were cooled and washed. Blocking of unspecific binding was performed by adding 1.5% horse serum for 30 min, followed by adding the primary antibody and incubating overnight.

Day two, slides were washed and a secondary antibody was added for 45 min and then again washed. The secondary antibody was biotinylated with avadin-biotin complex (ABC). Horse radish peroxidase (HPR) was added to visualise the antibody staining. After 40 min of incubation, slides were washed and developed with chromogen 3'diaminobenzydine (DAB). Counterstaining was obtained by placing slides in haematoxyin for 30 sec. Sides were again washed for 5 min in running water and dehydrated in increasing concentrations of ethanol and xylene. Visualisation was performed under light microscopy.

3.3.1.2   Antibodies

In paper III:
   • HC-A2 recognises β_2M -free HLA-A (excluding –A24), -B7301 and –G heavy chain.
   • HC-10 recognises β_2M -free HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33 and –B (excluding -B5702, -B5804 and –B73) heavy chain.
   • L368 recognises β2-microglobulin.

In paper IV:
   • EP1395Y (Abcam) recognised β_2M -free HLA-A.
   • MEM-G/1 (Abcam) recognises human HLA-G heavy chain.
   • Anti-CD8 (Clone C8/44B) detects the cytoplasmic domain of the α chain on the CD8 molecule expressed on cytotoxic T-cells, thymocytes and NK cells.

3.3.1.3   Evaluation

In papers III and IV the percentage of malignant cells stained for MHC class I or β2 microglobulin were scored as follows: 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) or 4 (76-100%).

HLA-G expression, which was analysed in paper IV, is normally not expressed in the colon. Therefore it was determined as positive or negative.

CD8+ lymphocyte infiltration was analysed on three different areas of the tumour sample: inside the tumour, the invasive margin, and the stroma. In each location a hotspot was identified and in an x20 field the amount of CD8+ cells were counted. The samples were scored as follows: I (1-19 cells); II (20-49 cells); III (50+ cells) (26).

All immunohistochemistry analysis was performed by two separate investigators who after analysis compared results and consulted a third investigator if scores differed >2.

3.4   STATISTICAL ANALYSIS

In all papers included in this thesis, statistics were used to analyse the results. The choice of statistical analysis was made by consulting a statistician.

In papers I, III and IV a Chi^2-test was used in order to compare unordered categorical variables, e.g. HLA-A*02 yes/no. In paper II where to sample size was smaller, two-tailed Fishers exact test was used. In this paper we also corrected the test with Bonferroni in order to reduce false-positive results since multiple pair wise tests were performed.

In papers I, III and IV, survival was measured in months from the date of diagnosis until censor date (paper I 10 years; paper III 15 years; paper V 120 months). Overall survival (OS) was defined as the time from diagnosis until death of any cause or censor date.

Cumulative survival plots were constructed using Kaplan-Meier product limit method. To detect difference between the groups, the log-rank test was applied.

In paper II, the Wolf-Haldane method was used to ensure that all HLA-haplotypes were considered even though they were not represented in the patient population. The Hardy-Weinberg disequilibrium test was used to determine the degree of segregation/disequilibrium, since the genotype frequencies of all three genotypes were known.

Adjusted and unadjusted hazard ratios (HR) and confidence intervals (CI) were calculated with Cox regression in papers I, III and IV. This method investigates the effect of several variables upon the time a specific event happens. It provides better estimate of survival probability and cumulative hazard ratios as compared to Kaplan-Meier calculations. It was used in papers I-IV.

P-values <0.05 were considered statistically significant.

In papers I-IV calculations were made using software Statistica version 10 (StatSoft, Tulsa, Oklahoma, USA) and StatView for Windows, SAS Institute Inc. Version 5.0.1.

4 RESULTS AND DISCUSSION

*"One can state, without exaggeration, that the observation of and the search
for similarities and differences are the basis of all human knowledge."*

- Alfred Nobel

4.1 PAPER I

- **Human leukocyte antigen (HLA) A2 as a negative clinical prognostic factor in patients with epithelial ovarian cancer.**

Objective: The aim of this paper was to study the overrepresentation of HLA-A*02 in epithelial ovarian cancer patients.

Results and Discussion. FFPE tumour specimens were from 97 patients, admitted to the oncology department at Karolinska University Hospital in 1995. All patients diagnosed with epithelial ovarian cancer were tested for HLA-A*02 genotype by conventional PCR using primers specific for HLA-A*02. The aim was to study the overrepresentation of HLA-A*02 in epithelial ovarian cancer (EOC patients. Our main finding was that patients with serous ovarian carcinoma stages III-IV positive for HLA-A*02 genotype had a poorer prognosis compared to patients who were negative for HLA-A*02, (HR 7.16, 95% CI:2.04-25.03, p=0.002). None of the HLA-A*02 positive patients survived 5 years, whereas 50% of the HLA-A*02 negative patients were still alive after 5 years. According to the multivariate analysis performed, this finding was independent to age, treatment and tumour grade but not clinical stage, HLA-A*02 phenotype had a HR of 1.71 (95% CI 1-2.86, p<0.001). Finally, we confirmed our previous finding that there was a higher frequency of HLA-A*02 genotype in late stage serous adenocarcinoma patients (67%) compared to the Swedish population in general (58%). This was not the case in early stage disease where the frequency of HLA-A*02 was even lower (50%) compared to the frequency in the Swedish population. There were some limitations in this paper, since eight patients, were excluded due to unamplifiable DNA in their samples, but they were few and not likely to change the conclusions above. Furthermore, the primers used did not cover some HLA-A*02 alleles. This however is not likely to make an impact on the results either, since these alleles, A*02109, *0248, *0250 and *0255, are very rare (<0.0001 allele frequency) in the Swedish population.

The findings above lead to further questions. Did only HLA-A*02 have this prognostic value, or where there other HLA-alleles with an even greater influence on prognosis? If so, where they genetically linked and inherited with linkage disequilibrium or even an ancestral haplotype? Which mechanism was responsible for this prognostic trait, was it genetic or immunological? Genetically there may be a "guilt by association"-situation where the HLA-A*02 correlates to a bad prognosis, but the guilty party is something that co-exists with HLA-A*02. Since the HLA region is known for its linkage disequilibrium, it reasonable to imagine that another genetic entity linked with HLA-A*02 could be responsible for why these patients die prematurely. A more aggressive malignancy could be achieved by a mutated oncogene or tumour suppressor gene. There are no known links between the HLA-genes and oncogenes or tumour suppressor genes,

but when looking generally at the HLA-locus on chromosome 6, Waf1/p21 is located in its proximity.

The role of HLA-A*02 as a protein is however also immunological, since it presents endogenous peptides to T-cells in order to elicit an immune response against foreign/damaged proteins. In fact, HLA-A*02 is one of the alleles mostly used in immunotherapy, both because it is a common allele but also due to its promiscuity which allows presentation of a wide range of antigenic peptides (138). Taking this into consideration, one could envision an immune selection of malignant cells, where the malignant cells are forced to endure an aggressive immune response due to an effective antigen presentation, selecting the malignant cells which have circumvented this process and thus the best adapted cells are saved from the immune system and free to spread throughout the body. The malignancy would then be more aggressive and consequently shorten the life of the patient.

Conclusion. The HLA-A*02 genotype was a more common allele in late stage epithelial ovarian cancer, and it was also linked to a bad prognosis in patients with late stage EOC and serous histology. HLA-A*02 is not a risk factor for developing EOC, since the genetic frequency of HLA-A*02 was not higher in EOC patients with early stage disease compared with the Swedish population in general.

4.2 PAPER II

- **Analysis of HLA class I-II haplotype frequency and segregation in a cohort of patients with advanced stage ovarian cancer.**

Objective. The aim of this paper was to genomically HLA type for HLA-A, -B, -C and DRB1 patients with relapsing or progressive EOC and compare to that of healthy Swedish bone-marrow donors.

Results and Discussion. Blood samples were collected from 32 unrelated Swedish women with relapsing or progressive EOC to genomically HLA-typed for HLA-A, -B, -Cw and -DRB1. The frequency of HLA alleles was then compared to that of 40 162 healthy male and female Swedish bone-marrow donors. We found an increased frequency of HLA-A*01, -A*02 and -B*08 but decreased frequency of A*03. Homozygotes for A*02 were twofold more frequent among the EOC patients when compared to that in healthy donors. Combinations of A*02 with B*05, B*15, DR1*03, DRB1*04, Cw*3 and DRB1*03 had an OR as well as the level of the lower confidence interval above 1 and a significant p-value, but only when considered as single, non-corrected analysis. B*15 and Cw*3 were only detected in combination with A*02. The combination of A*02, B*15, Cw*3 and DRB1*04 was segregated. This combination is a known ancestral haplotype called 62.1.

The shortcoming in this study is mainly the sample size. With an increased number of patients more findings could have yielded statistical significance. However, if the results are analysed carefully some conclusions can still be made.

Conclusions. There was an unusual representation of HLA-haplotypes in patients with relapsing or progressive EOC where HLA-A*02 seemed to be a key player. Interestingly, HLA-A*03 seemed to be underrepresented in the EOC patients, indicating that this allele could have a protective role. Combining HLA-A*02 with the other alleles may be informative, but the mechanism behind the poor prognosis of EOC in HLA-A*02 positive patients cannot be explained by the presence of another HLA allele.

4.3 PAPER III

- **Correlation of HLA-A*02 genotype ad HLA class I antigen down-regulation with the prognosis of epithelial ovarian cancer.**

Objective. The aim of this paper was to investigate the expression of MHC class I in patients with an HLA-A*02 genotype.

Results and Discussion. Blood samples and FFPE tumour specimens were collected from 162 patients diagnosed with ovarian carcinoma between the years 1995-2004, were analysed for the HLA-A*02 genotype and for MHC class I and β2M expression in the tumour tissue. MHC class I expression was analysed using two different antibodies, HCA and HC-10. We found and could confirm previous data (139-141) that down-regulation of MHC class I in the tumour tissue correlated with poorer prognosis.

Furthermore, we could demonstrate for the first time that the frequency of down-regulation of MHC class I was higher in patients with an HLA-A*02 genotype, serous histology and advanced clinical stage (HCA 52%, HC-10 48%). This group of patients was identified as "worst prognosis group (WP)". In the best prognosis (BP) group the downregulation was less frequent (HCA 35.5%, HC-10 19%). In a multivariate Cox-analysis, the WP group had a significant higher Hazard Ratio (HR 3.2; 95% CI 2.1-4.9, p<0.001) compared to loss of MHC class I expression (HCA HR 1.4, p=0.64; HC-10 HR 1.37, p=0.64). The only variable with a higher HR was clinical stage.

The most important finding in this study was patients in the WP group had a more frequent loss of MHC class I expression compared to the BP group, giving the impression that HLA-A*02 may in some way be responsible for the loss of expression. Norell et al (142) described that metastasised malignancies from HLA-A*02 positive patients frequently lost their haplotype. This would be a good way for the tumour to evade recognition by the immune system, although at risk of NK destruction. This finding also proves that HLA-A*02 is more important as a prognostic factor compared to MHC expression.

There are several limitations in this study. Firstly two different antibodies were used to detect MHC class I due to the fact that they are separately unable to identify all HLA class I proteins. It would have been of more value to detect only MHC class I derived from HLA-A. This may have given another picture. Also all tumour slides were cut from primary tumours of the patients, giving a variety of aggressiveness of malignancies. It is not unlikely that even more patients may have had a downregulation of MHC class I expression in metastasis. Nevertheless, the fact remains that HLA-A*02 is a strong negative prognostic factor in EOC patients, but the expression of its protein may not fully explain the mechanism behind this.

Conclusion. There was an association between the HLA-A*02 genotype and a decrease or loss of MHC class I expression. HLA-A*02 genotype is a stronger prognostic factor than impaired expression of MHC class I in the tumour.

4.4 PAPER IV

- **Analysis of immune-related prognostic markers in colon cancer patients randomized to surgery or surgery and adjuvant cytostatic treatment.**

Objective. The aim of this paper was to investigate the frequency of HLA-A*02, expression of MCH class I, as well as HLA-G expression and CD8+ lymphocyte infiltration in colon cancer patients randomised to surgery or surgery and cytostatic treatment.

Results and Discussion. FFPE tumour specimens were collected from 520 colon cancer patients randomized to treatment with surgery alone or surgery and adjuvant chemotherapy. Samples were analysed for the HLA-A*02 genotype, MHC class I expression, HLA G expression and CD8+ lymphocyte infiltration in the tumour, invasive margin and stroma. Patients with a Duke C tumour and HLA-A*02 genotype had a better outcome if randomized to adjuvant chemotherapy versus surgery alone (P=0.03). There was an indication that patients with complete absence of MHC class I expression had a better overall survival compared to patients who had a decreased or increased expression, but not statistically significant (P=0.07). Expression of HLA-G was a negative prognostic marker for the male patients (P=0.002). Also a high infiltration of CD8+ lymphocytes was important in the male patients, where a high frequency of infiltration correlated with a good prognosis (P=0.002). These factors were not, however significant in the female population. The superior negative prognostic marker in the female patients was HLA-A*02 genotype, but unfortunately not strong enough statistically (P=0.07).

Several conclusions can be drawn from this study. Firstly, HLA-A*02 seems to have a different behaviour in this group of patients compared to previously described EOC patients. Looking at the results as a whole, two different patterns start to emerge. The male patients have a poorer outcome if they lack infiltration of CD8+ lymphocytes in the tumour and if they have HLA-G expression. This gives the impression that if there is an immune response, the male patient will do better. The female patients, on the other hand, are not influenced by the infiltration of CD8+ lymphocytes or HLA-G expression, but rather the presence of HLA-A*02. If the female patients are HLA-A*02 carriers, they have a high risk to have a poor prognosis.

There are some limitations with this study. Since the cohort is from 1991-95, the staging is according to Dukes tumour staging. Furthermore, due to surgical advancements, lymphadenectomy is more reliable today, since a larger amount of lymph nodes can be extracted today. Thus there is a risk that a few Duke B patients who should have been categorized as Duke C if more lymph nodes would have been extracted.

The behaviour of HLA-A*02 seems to create an alternative and unexpected outcome in the female patients. Colon cancer is known to have an immunological response due to the prognostic value of the immune contexture (29). The female patients have neither worse overall prognosis or are over represented as a group with more aggressive disease, the association to prognostic markers just seems to be different and HLA-A*02 seems to play a key role. Considering then that the mechanism behind its prognostic traits is most likely not immunological, the remaining answer is then that the mechanism is genetic.

Conclusions. HLA-G expression in the tumour and the lack of CD8+ lymphocyte infiltration correlates to poor prognosis in the male patients. HLA-A*02 genotype correlates to poor prognosis in the female patients where the infiltration of CD8+ lymphocytes seems irrelevant.

4.5 PAPER V

- **A novel approach for HLA-A typing in formalin-fixed paraffin-embedded-derived DNA.**

Objective. The aim of this paper was to investigate whether it was possible to HLA-A type by using FFPE material from relapsing or progressive ovarian cancer.

Results and Discussion. FFPE tumour specimens and blood samples were collected from unrelated Swedish women diagnosed with relapsing or progressive ovarian cancer in order to establish a novel HLA-typing approach in FFPE derived material. The blood samples were HLA-typed at the (European Federation of Immunogenetics) EFI certified immunological laboratory at Karolinska University Hospital. FFPE tumour specimens were HLA-A typed using primers amplifying exons 2, 3 and 4 followed by sequencing of amplified product. Sufficient information was obtained to assign two HLA-A alleles to 14/16 patients. One patient could be assigned one allele and the remaining patient no alleles could be determined.

Paper V shows a successful HLA-A typing approach for damaged samples. The main problem with this method was firstly that the FFPE material was sequenced, and it is known that artificial base alterations may occur during and after fixation. However, since the differences between alleles consist of known polymorphisms, the chance of receiving a sequence with just the correct base alterations due to fixation seems unlikely. The method is unfortunately also somewhat time-consuming since the samples must first be extracted, amplified, checked for their products and then sequenced, a process that can run for five days. There are however ways one could improve this, firstly by assessing the DNA quality before subjecting the sample to amplification by e.g. an Agilent Bioanalyzer ™. Secondly, the sequencing could be made by a next generation sequencer, making the process much faster.

Conclusions. It was possible to use FFPE material to sequence HLA-A in a majority of patients with ovarian cancer. The method described has great potential to be expanded to the other HLA-genes but also to other polymorphic regions, e.g. KIR or TCR. Finally, as the steps require relative basic laboratory equipment, the method is suitable also to small research groups with limited funds.

4.6   IN SEARCH OF ANSWERS IN NON-CODING PARTS OF HLA-A*02

According to the conclusions drawn above, the prognostic traits of HLA-A*02 seem more likely to be genetic than immunological. The genetic differences between the HLA-A alleles are predominantly SNPs which are most frequent in exons 2, 3 and 4. The aim of this unpublished project was to find *in silico* a possible regulatory non coding elements that may influence the HLA-A*02 differently from the other HLA-A alleles.

Non-coding RNAs are RNA molecules found in the cell that do not code for a protein. Small non-coding RNA, such as e.g. microRNAs (MiRNAs), has been shown to regulate gene expression.

The project aimed to answer three questions:
- Are there any miRNAs which target HLA-A*02?
- Are there any known miRNAs coded in or near the HLA-A gene?
- Which are the main genetic differences between HLA-A*02 and the other HLA-A alleles?

MiRNAs regulate mRNAs by attaching to the 3'UTR resulting in a translation inhibition and thus a lower expression of a gene. There are reports on miRNAs regulating HLA-G (143) and -C (144). In the case of HLA-A, the 3'UTR does not change considerably between the different alleles. Also the algorithms developed for target prediction factor in sequence conservation. The HLA genes are known for the lack of conservation as the polymorphism is beneficial for the arts survival. When scanning HLA-A sequence in Target Scan (http://www.targetscan.org/vert_70/) there were several suggested results, however with low scores and there were no sequences that differ between the HLA-A alleles.

If there was no miRNA that target HLA-A*02 specifically, could there be a miRNA coded within the HLA-A*02 gene? miRBase (http://www.mirbase.org/) is a database consisting of predicted hairpin sequences of all known miRNAs both confirmed and non-confirmed experimentally. There are 67 known miRNAs on chromosome 6, none of which are coded within the HLA-locus. The nearest one to the HLA-A gene, miR3143, is situated 2Mb centromerically and has been found during screening of small RNAs in melanoma and breast cancer tissue (145, 146).

These results lead to the conclusion that the search should thus be for a genomic feature yet to be described. The HLA-A alleles all consist of 8 exons and 6 introns with similar length. The differences between the alleles are mostly SNPs concentrated to exons 2, 3 and 4. However, when aligning HLA-A*02 to other HLA-As there is a longer sequence which differs between the alleles situated on intron 4 (Figure 7). This sequence is 22bp long and consists mostly of a larger insertion which is present on HLA-A*02. When inserting the sequence into a structure prediction software (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html) the sequence seems to have the possibility to form a hairpin structure (Figure 7).

The sequence certainly indicates the possibility of small regulatory non coding RNAs within the HLA-A*02 gene, however if this sequence is found within the cell and if it is the reason behind the poor prognosis that HLA-A*02 positive patients have is hypothetical. Experimental validation is needed, but also more knowledge of the regulatory effect of small non-coding RNA.

CAGAAGCAGAGGGGTCAGGGCGAAGTCCCAGGGCCCCAGGCGTGGCTCTCAGGGTCTCAGGCCCCGAAGGCGGTGTATGG
ATTGGGGAGTCCCAGCCTTGGGGATTCCCCAACTCCGCAGTTTCTTTTCTCCCTCTCCCAACCTATGTAGGGTCCTTCTT
CCTGGATACTCACGACGCGGACCCAGTTCTCACTCCCATTGGGTGTCGGGTTTCCAGAGAAGCCAATCAGTGTCGTCGCG
GTCGCGGTTCTAAAGTCCGCCACGCACCCACCGGGACTCAGATTCTCCCCAGACGCCGAGGATGGCCGTCATGGCGCCCCG
AACCCTCGTCCTGCTACTCTCGGGGGGCTCTGGCCCTGACCCAGACCTGGGCGGGTGAGTGCGGGGTCGGGAGGGAAACGG
CCTCTGTGGGGAGAAGCAACGGGCCCGCCTGGCGGGGGGCGCAGGACCCGGGAAGCCGCGCCGGGAGGAGGGTCGGGCGGG
TCTCAGCCACTCCTCGTCCCCAGGCTCTCACTCCATGAGGTATTTCTTCACATCCGTGTCCCGGCCCGGCCGCGGGGAGC
CCCGCTTCATCGCAGTGGGCTACGTGGACGACACGCAGTTCGTGCGGTTCGACAGCGACGCCGCGAGCCAGAGGATGGAG
CCGCGGGCGCCGTGGATAGAGCAGGAGGGTCCGGAGTATTGGGACGGGGGAGACACGGAAAGTGAAGGCCCACTCACAGAC
TCACCGAGTGGACCTGGGGACCCTGCGCGGCTACTACAACCAGAGCGAGGCCGGTGAGTGACCCCGGCCCGGGGCGCAGG
TCACGACCTCTCATCCCCCACGGACGGGCCAGGTCGCCCACAGTCTCCGGGTCCGAGATCCGCCCCGAAGCCGCGGGACC
CCGAGACCCTTGCCCCGGGAGAGGCCCAGGCGCCTTTACCCGGTTTCATTTTCAGTTTAGGCCAAAAATCCCCCCAGGTT
GGTCGGGGCGGGGCGGGGCTCGGGGGACCGGGCTGACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAGGATGTAT
GGCTGCGACGTGGGGTCGGACTGGCGCTTCCTCCGCGGGTACCACCAGTACGCCTACGACGGCAAGGATTACATCGCCCT
GAAAGAGGACCTGCGCTCTTGGACCGCGGCGGACATGGCAGCTCAGACCACCAAGCACAAGTGGGAGGCGGCCCATGTGG
CGGAGCAGTTGAGAGCCTACCTGGAGGGCACGTGCGTGGAGTGGCTCCGCAGATACCTGGAGAACGGGAAGGAGACGCTG
CAGCGCACGGGTACCAGGGGCCACGGGGCGCCTCCCTGATCGCCTGTAGATCTCCCGGGCTGGCCTCCCACAAGGAGGGG
AGACAATTGGGACCAACACTAGAATATCGCCCTCCCTCTGGTCCTGAGGGAGAGGAATCCTCCTGGGTTTCCAGATCCTG
TACCAGAGAGTGACTCTGAGGTTCCGCCCTGCTCTCTGACACAATTAAGGGATAAAATCTCTGAAGGAATGACGGGAAGA
CGATCCCTCGAATACTGATGAGTGGTTCCCTTTGACACACACAGGCAGCAGCCTTGGGCCCGTGACTTTTCCTCTCAGGC
CTTGTTCTCTGCTTCACACTCAATGTGTGTGGGGGGTCTGAGTCCAGCACTTCTGAGTCCTTCAGCCTCCACTCAGGTCAG
GACCAGAAGTCGCTGTTCCCTCTTCAGGGACTAGAATTTTCCACGGAATAGGAGATTATCCCAGGTGCCTGTGTCCAGGC
TGGTGTCTGGGTTCTGTGCTCCCTTCCCCATCCCAGGTGTCCTGTGCCATTCTCAAGATAGCCACATGTGTGCTGGAGGAG
TGTCCCATGACAGATGCAAAATGCCTGAATGATCTGACTCTTCCTGCACAGACGCCCAAAACGCATATGACTCACCACG
CTGTCCTGTGACATGAAGCCACCCTGAGGTGCTGGGCCCTGAGGCTTGTACCGTGCCGGAGATCAGACTGCTTTGCCAGCGG
GATCGGGACGACCAGACCCAGAGACACGGAGCTCTCCGACAGCCGCCTCCAGGGATGGAACCTTCCAGAGATGCGGTCGGC

Query 1621	CAATGTGTGTGGGGGTCTGAGTCCAGCACTTCTGAGTCCTTCAGCCTCCACTCAGGTCAG	1680	
Sbjct 1617	CAATGTGTGTGGGGGTCTGAGTCCAGCACTTCTGAGTCTCTCAGCCTCCACTCAGGTCAG	1676	
Query 1681	GACCAGAAGTCGCTGTTCCCTCTTCAGGGACTAGAATTTTCCACGGAATAGGAGATTATC	1740	
Sbjct 1677	GACCAGAAGTCGCTGTTCCCT--T------C--------T-CAGGGAATAGAAGATTATC	1719	
Query 1741	CCAGGTGCCTGTGTCCAGGCTGGTGTCTGGGTTCTGTGCTCCCTTCCCCATCCCAGGTGT	1800	
Sbjct 1720	CCAGGTGCCTGTGTCCAGGCTGGTGTCTGGGTTCTGTGCTCTCTTCCCCATCCCGGGTGT	1779	

AAGGTGAGAGCTG...
CATTGGATGTATTGAGCATGCGATGGGCTGTTTAA...CTGTGACAGATACGAATTTGTTCATGAATAT
TTTTTTCTATAGTGTGAGACAGCTGCCTTGTGTGG...GAGTTGTTCCTGCCCTTCCCTTTGTGACTTGA
AGAACCCTGACTTTGTTTCTGCAAAGGCACCTGC...GTGTAGGCATAATGTGAGGAGGTGGGGGAGACC
ACCCCACCCCCATGTCCACCATGACCCTCTTCCCA...CCCTCCCCAATCATCTTTCCTGTTCCAGAGA
GGTGGGGCTGAGGTGTCTCCATCTCTGTCTCAACT...AGCTGTAACTTCTTCCTTCCCTATTAAAA

Figure 7. After sequence alignment of HLA-A*02 vs HLA-A*01, an insertion is identified (marked red in the HLA-A*02 sequence). The insertion was located in non-coding region between exons 3-4 and run through folding prediction software which predicted a hairpin structure (RNA on the left and DNA on the right).

5 FUTURE PERSPECTIVES

"A doctor can maybe save a few hundred lives in a lifetime. A researcher can save the whole world"

- Craig Venter

This thesis presents evidence that HLA-A*02 acts as prognostic factor in several solid malignancies. Furthermore the evidence points towards a mechanism not fully explained by the expression or absence of expression of MHC class I. Finally, HLA-A*02 correlates to poor prognosis in female colon cancer patients, where the infiltration of CD8+ lymphocytes seems irrelevant.

The mechanism behind the prognostic traits of HLA-A*02 are still up for debate. However, the immunological importance of HLA-A*02 in these patients seems to be secondary since the expression of MHC class I was not as strong a predictor as HLA-A*02. Furthermore, the pattern of two prognostic motifs in colon cancer patients suggests that there is another mechanism in play, mainly concerning gender differences. More studies are required in order to determine if this mechanism can be found genetically or epi-genetically.

The combination of prognostic factors is the key to better cancer survival. To this date, tumour samples are evaluated with focus on histopathological characteristics and tumour staging according to the TNM classification. This information is the basis of decisions made for the patients' surgery and oncological treatment. Ovarian cancer and colon cancer are malignancies with poor prognosis when diagnosed in later stages. Patients undergo massive surgery and receive aggressive oncological treatment, it is therefore imperative that clinicians base their decisions on the best prognostic markers available.

The complexity of the tumour biology and immune response make it unlikely that the measurement of a single biomarker would be highly informative in predicting all patients outcome in response to different therapies. Rather integration of multiple parameters is needed for accurate patient outcome prediction. As it is shown in this thesis, the combination of clinical parameters, biomarkers as well as patient genetics may result in a prognostic motif in an isolated a group of patients. With molecular profiling, large amount of information can select patients to the right kind of treatment. Incorporating this approach into the clinic may significantly aid in the decision making of patient treatment resulting in what is called personalised therapy.

6 ACKNOWLEDGEMENTS

To my supervisor, **Giuseppe Masucci**, for believing in my ability, especially when I do not, and for encouraging me to keep going no matter what.

To my co-supervisor, **Tina Dalians**, for being my role-model and keeping me focused in both science and life.

To my co-supervisor, **Veronica Höiom**, for always making time to give me advice and guidance.

A special thank you also to the Tina Dalianis-group, past and present members for always making me feel welcome. Anders, Cecilia, Mircea, Nichos, Nathalie, Mathilda, David, Kalle. Joar, Karin, Geraldine and probably many more who have come and gone during my years at CCK.

To my fellow PhD student **Emilia** for all the delightful insight into a life as a pathologist.

Also to the extremely talented support staff, especially **Inger Bodin** and **Gunilla Högberg** for always treating the samples with great care and delivering them with a smile.

To the director of studies, associate professor **Andreas Lundqvist** for all the assistance with bureaucracy.

To the co-authors, for making this thesis into the extraordinary and substantial piece I now have to defend.

To my fellow MD colleagues **Theo** and **Felix** for making my internship a little more fun.

To my friends **Jemima**, for enduring adolescence with me and being my good conscience. **Ida**, for all the good times and being the voice of reason. **Sofia**, for making me stronger mentally and physically.

To my beloved family: my **mother** for telling me never to give up, my **father** for teaching me that education is the most important tool in life. My sister **Johanna** for always being proud of me and my brother **Antonio** for always making me strive for more. To the entire Tulenheimo-clan for making feel like a part of something larger than myself and making therefore me a better person. To the entire Villabona-clan for always making me feel welcome anywhere in the world.

Finally, to my husband **Danijel Djuric**, for being my local co-op partner in gaming, in life, in everything <3

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