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METHODOLOGICAL APPROACHES TOWARDS PERSONALISED CANCER MEDICINE

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Methodological Approaches towards Personalised Cancer Medicine
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“Il y a plus de vieux ivrognes que de vieux médecins”

- François Rabelais

ABSTRACT

Despite advances in diagnostics and treatments, many cancer patients have poor survival rates. Tumours develop drug resistance followed by metastasis, and survivors suffer from treatment side-effects. Omics techniques, targeted treatments and immunotherapy offer the prospect of individually adapting treatments for optimal efficacy and minimal side-effects. This requires integration of biomolecular, clinical and drug data to successfully predict optimal treatments for every patient. The aim of this thesis was to evaluate and apply different methodologies important for personalised treatment in a variety of cancer settings.

The first paper showed that E6/E7 mRNA detection through RT-NASBA is more accurate and sensitive than DNA genotyping for classifying HPV infection in cervical adenocarcinoma, showing RNA analysis to be preferable for identifying high-risk patients.

In paper II, HPV16 E2 and E5 mRNA expression in oropharyngeal cancer was analysed in relation to clinical outcomes and tumour immunology. Neither down-regulation of HLA class I nor $CD8^+$ T-cell infiltration, both indicators of good prognosis, were dependent on E2 or E5. However, absence of E2 was related to poor progression-free survival. This allows E2 expression to be combined with HLA class I and $CD8^+$ T-cells when stratifying patients with good prognosis for milder treatment.

The third paper screened combinations of growth factors and drugs for impact on proliferation of breast cancer cells, creating a two-dimensional space to simulate tumours in different signalling states interacting with drugs. In MDA-MB-231 cells, TGF-β in combination with EGF and oestrogen inhibited growth, with the effect strengthened by Tamoxifen. In MCF7 cells, Tamoxifen inhibited growth when added to both EGF and oestrogen.

In paper IV, the immunoproteome in urinary bladder cancer was analysed. Proteomics and network analysis of regulatory (Treg) and effector T-cells (Teff) of lymph nodes showed that Tregs in sentinel nodes (SN) up-regulate growth and immune signalling networks. IL-16, previously not shown to be expressed by Tregs, was predicted as central to SN-Treg signalling. IL-16 expression in Tregs was validated, shown to be higher in lymph nodes than peripheral blood and inhibited by tumour cell supernatant.

In conclusion, this thesis has shown methods to improve patient stratification in cervical adenocarcinoma and HPV-positive oropharyngeal cancer. The utility of proliferation screenings replicating tumour heterogeneity for optimising drug combinations was demonstrated, and finally, lymph node proteomics revealed individual differences in T-cell signalling, important for optimising immunotherapy. Integration of these and other methods will be key to arrive at personalised cancer medicine – application of the optimal treatment combination for every patient.

LIST OF SCIENTIFIC PAPERS

I. Hovland S, Muller S, Skomedal H, Mints M, Bergström J, Wallin KL, Karlsen F, Johansson B, Andersson S. **E6/E7 mRNA expression analysis: a test for the objective assessment of cervical adenocarcinoma in clinical prognostic procedure**. Int J Oncol. 2010 Jun;36(6):1533-9.

II. Ramqvist T*, Mints M*, Tertipis N, Näsman A, Romanitan M, Munck-Wikland B, Dalianis T. **Studies on Human Papillomavirus (HPV) 16 E2, E5 and E7 mRNA in HPV-positive Tonsillar and Base of Tongue Cancer in Relation to Clinical Outcome and Immunological Parameters**. Oral Oncology (In press).

III. Mints M, Souchelnytskyi S. **Impact of combinations of EGF, TGFβ, 17β-oestradiol, and inhibitors of corresponding pathways on proliferation of breast cancer cell lines**. Exp Oncol. 2014 Jun;36(2):67-71.

IV. Mints M, Krantz D, Winerdal M, Rutishauser D, Zubarev R, Zirakzadeh AA, Hansson J, Sherif A, Winqvist O. **Individual proteomics on T-cell subsets in muscle-invasive urothelial bladder cancer patient lymph nodes reveals IL-16 as a central player in tumour-Treg cross-talk**. (Manuscript).

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LIST OF ABBREVIATIONS

APC	antigen-presenting cell
BCG	bacillus calmette-guerin
BOTSCC	base of tongue squamous cell carcinoma
CAR	chimeric antigen receptor
CIN	cervical intraepithelial neoplasia
CML	chronic myeloid leukaemia
CTL	cytotoxic T-cell
DC	dendritic cell
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ER	oestrogen receptor
FACS	fluorescence-assisted cell sorting
FFPE	formalin-fixed paraffin-embedded
HIF	hypoxia-inducible factor
HLA	human leukocyte antigen
HPV	human papillomavirus
hrHPV	high-risk human papillomavirus
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
LC	liquid chromatography
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MIBC	muscle-invasive bladder cancer
mRNA	messenger RNA
MS	mass spectrometry
nSN	non-sentinel node
NMIBC	non-muscle-invasive bladder cancer
OSCC	oropharyngeal squamous cell carcinoma
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
pRb	retinoblastoma protein
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-NASBA	real-time nucleic acid sequence based amplification
RT-PCR	real-time polymerase chain reaction
SN	sentinel node
SSCP	single strand conformational polymorphism

TAM	tumour-associated macrophage
TCR	T-cell receptor
Teff	effector T-cell
TGF-β	transforming growth factor beta
TIL	tumour-infiltrating lymphocyte
TKI	tyrosine kinase inhibitor
Treg	regulatory T-cell
TSCC	tonsillar squamous cell carcinoma
UBC	urinary bladder cancer
VEGF	vascular endothelial growth factor

1 INTRODUCTION

Despite more than a century of developments in cancer research, the global burden of cancer is still very high, with 14.1 million new cases and 8.2 million deaths worldwide in 2012 [1] and projected to increase, both because of an ageing population and more people adopting a Western lifestyle [2]. Current treatments, relying heavily on surgery, radio- and chemotherapy, have not been able to turn this trend. Surgery, while often beneficial in earlier stages, is not curative in advanced cancer and radiotherapy is seldom effective against large tumour bulks. These treatments are rarely curative in late-stage, metastatic disease, which causes the great majority of cancer deaths [3]. While some kind of pharmacological treatment is often necessary, chemotherapy comes with heavy side-effects and resistance development.

The cause of resistance development is the heterogeneous nature of tumours. Cancer is driven by genetic instability, allowing cells to acquire a wide range of random mutations, with clonal expansion of mutations providing a selective growth advantage [4]. Advanced cancers, consisting of many such clonal populations not only at sites of metastasis, but also within the primary tumour, are therefore resilient to external perturbations and may easily adapt to treatments which do not target this entire, genetically diverse, population of neoplastic sub-clones (Fig. 1) [5].

Recent advances in diagnostics, treatments and modelling have allowed a potential way to address the problem of heterogeneity in cancer. In diagnostics, omics-type approaches have allowed the characterisation of the global changes in gene and protein expression in single patients, tissues and even cells [6]. On the treatment side, the advent of small molecules specifically targeting defined receptors and pathways allows more effective treatments with fewer side effects for patients with tumours susceptible to these drugs [7]. The rapidly growing field of cancer immunotherapy is another approach to address heterogeneity and resistance development by harnessing the immune system to kill tumours. The immune system being a complex, adaptive system, has the ability to target a wide variety of cancer populations if immunosuppressive pathways induced by tumours can be repressed by drugs [8].

Systems biology approaches aim to make these advances useful in a clinical setting through integration of the growing amount of omics data with observed tumour phenotypes and clinical outcomes, modelling of perturbations in signalling pathways and predictions of drug effects. Integrating and modelling high-resolution patient data will allow the concept

of P4 - predictive, preventive, personalised and participatory – medicine, making sure that each single patient gets the right treatment targeting the right tumour at the right time [9].

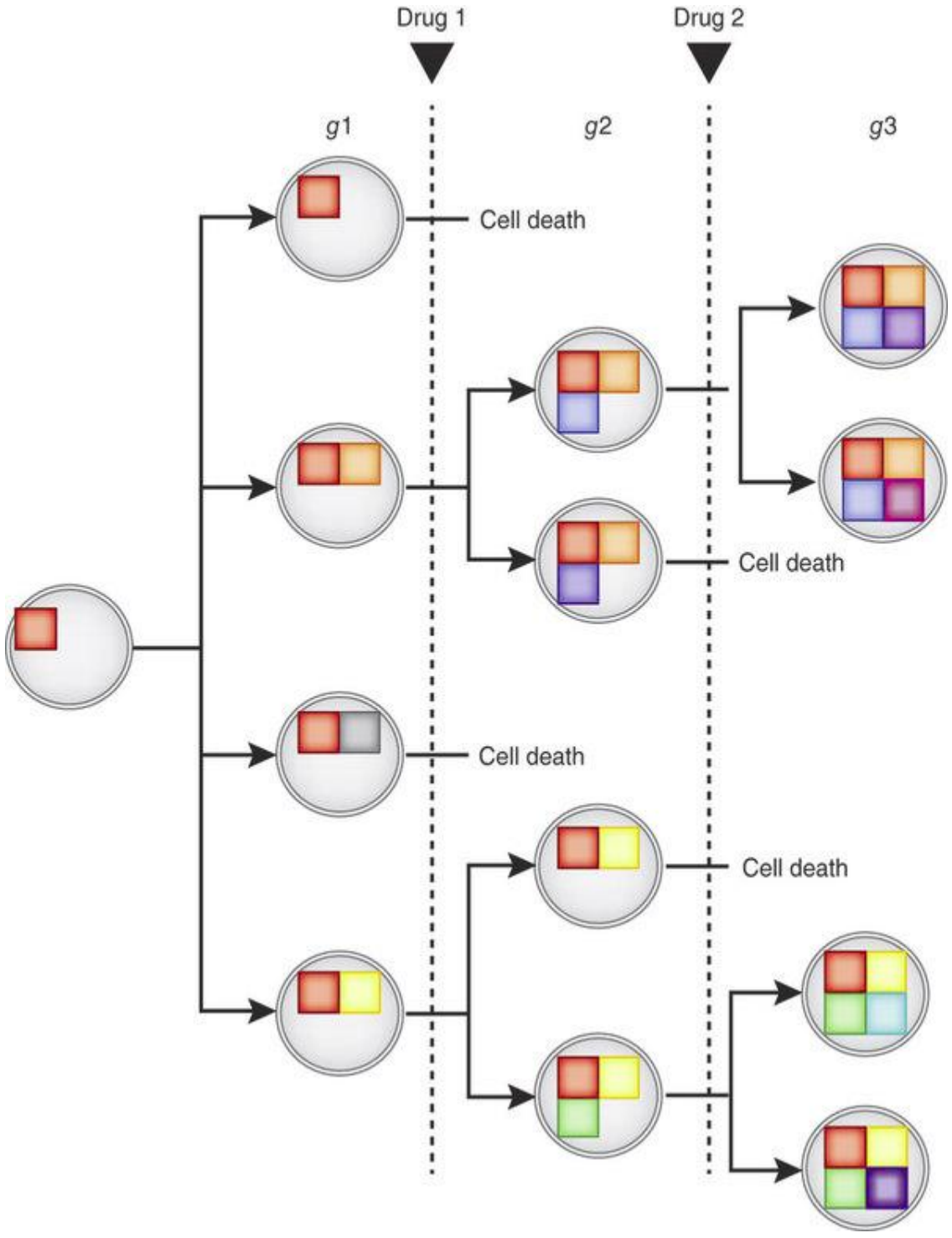


Figure 1. *Tumour heterogeneity provides resilience to selective pressure caused by anti-cancer drugs. Clonal expansion of resistant cells leads to multi-drug resistance. Reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology [10], copyright 2012.*

This thesis has laid forward several methods facilitating personalised medicine in a variety of cancer settings – more precise diagnostics (paper I) and prediction of treatment response (paper II) in HPV-driven cancer, replicating tumour heterogeneity to study combinations of targeted therapies in breast cancer (paper III) and studying the immunoproteome on lymph node level to predict proteins which may be targeted to overcome immunosuppression in urinary bladder cancer (paper IV).

1.1 CARCINOGENESIS

Cancer development is a multi-step process, where cells acquire capabilities allowing them to proliferate uncontrollably and spread throughout the body while avoiding host defence mechanisms. A model of initiation, promotion and progression has been established, which starts with a cancer-initiating mutation event. This initiation, through changing the function or activity of a critical regulatory gene, makes a cell or population of cells (in the case of heritable cancer syndromes) susceptible to hyper-proliferation upon further aberrations, but does in itself not result in marked phenotypical changes. Tumour promotion is a non-mutagenic, reversible event, causing clonal expansion of initiated cells, creating benign tumours. Progression introduces further genetic, irreversible changes into this expanded population, which becomes a malignant tumour consisting of aberrantly growing cells [11].

Fearon and Vogelstein developed the classic model of multi-step carcinogenesis in colon cancer, showing that subsequent acquisitions of mutations in at least five genes are necessary for cells to be malignant [12, 13]. These are either proto-oncogenes, which, if up-regulated through over-expression, mutation or translocation become oncogenes providing the tumour with capabilities of growth and metastasis, or tumour suppressors, which if down-regulated in the same manner remove intracellular checks against unlimited growth and dispersion. Figure 2 shows a general overview of the genetic changes during colon carcinogenesis.

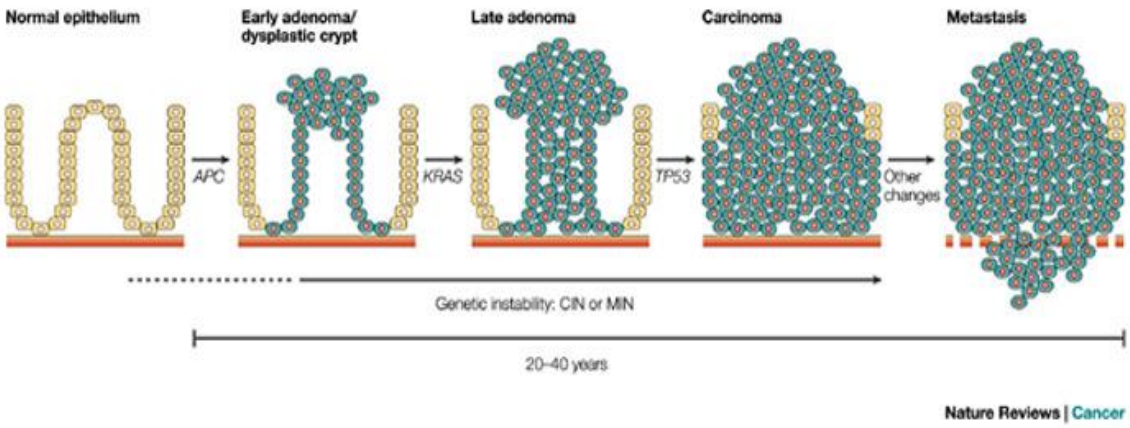


Figure 2. *Multi-step model of carcinogenesis in colon cancer. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer [4], copyright 2003.*

Genetic changes in themselves do not cause cancer unless the mutated cells successfully transfer the mutation to daughter cells and there is a selective clonal expansion of cells carrying the mutation. Since the probability of a carcinogenic mutation in a specific allele is very low, development of a tumour requires a large clonal expansion of the initiated cell to make it probable that at least one cell in the expanded population acquires another carcinogenic mutation, with repeated mutations followed by further expansion until a cancerous state is reached [14]. However, this does not mean that the final tumour will consist of identical clones. Rather, the population expanded from the first initiated cell will undergo numerous mutation events. During this process, cells that acquire mutations rendering them relatively genetically unstable, but still viable, will not have a growth advantage, but a fitness advantage over other cells, since they as a population are more likely to survive diverse selective forces. At each stage in carcinogenesis, this will produce multiple sub-clones with different mutations that may provide a growth advantage under the right selective pressure, producing expanded populations of several, genetically different clones (Fig. 3) [15].

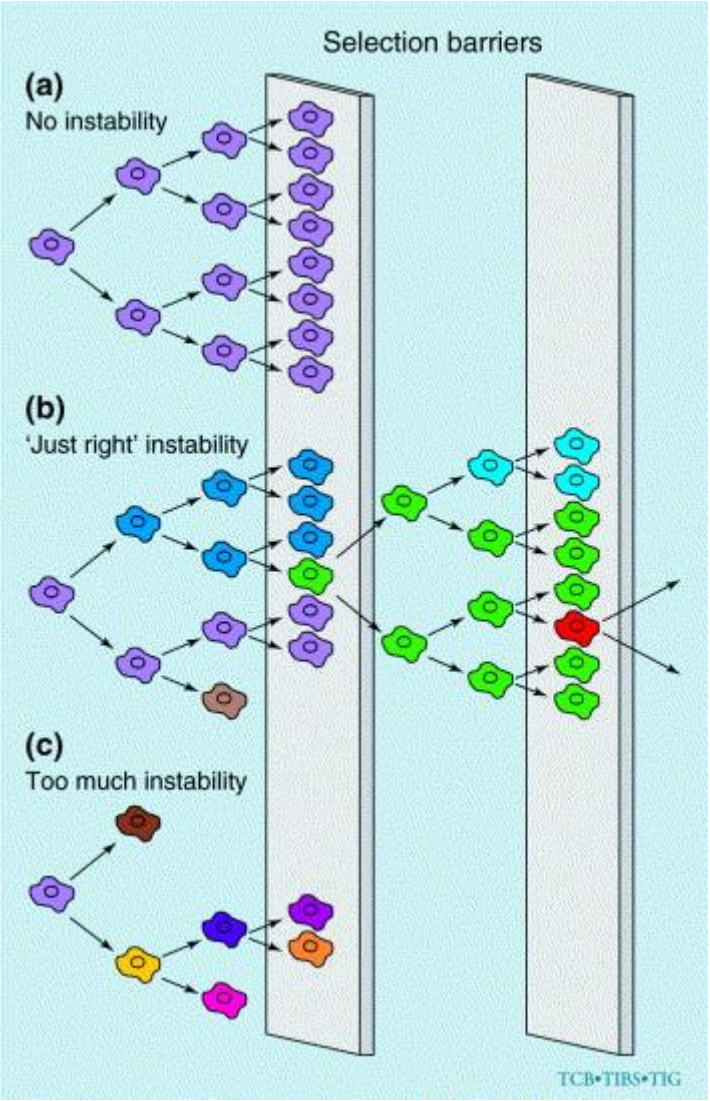


Figure 3. *Expansion of a genetically unstable population of pre-malignant cells creates different sub-clones, increasing the chance that the population as a whole can adapt to and bypass selection barriers. Reprinted from [15] with permission from Elsevier.*

1.2 CANCER SIGNALLING PATHWAYS

During the process of carcinogenesis from an initiated cell to a heterogeneous, invasive tumour, a multitude of pathways in the cancer cells themselves, as well as in surrounding tissues, are de-regulated. The general characteristics of these de-regulated pathways have been summarised by Hanahan and Weinberg as the Hallmarks of Cancer [16]. These hallmarks are: sustained proliferative signalling, evasion of growth suppressors, apoptosis resistance, immortality, angiogenesis and metastasis. The acquisition of these hallmarks is promoted by genetic instability and tumour-promoting inflammation, both of which create a multitude of mutations in somatic cells, of which a small number acquire mutations in pathways of the described hallmarks, giving them advantages in the areas of growth,

survival and invasion. The authors also describe two emerging hallmarks of cancer cells – changes in energy metabolism and evading elimination by the immune system (Fig. 4).

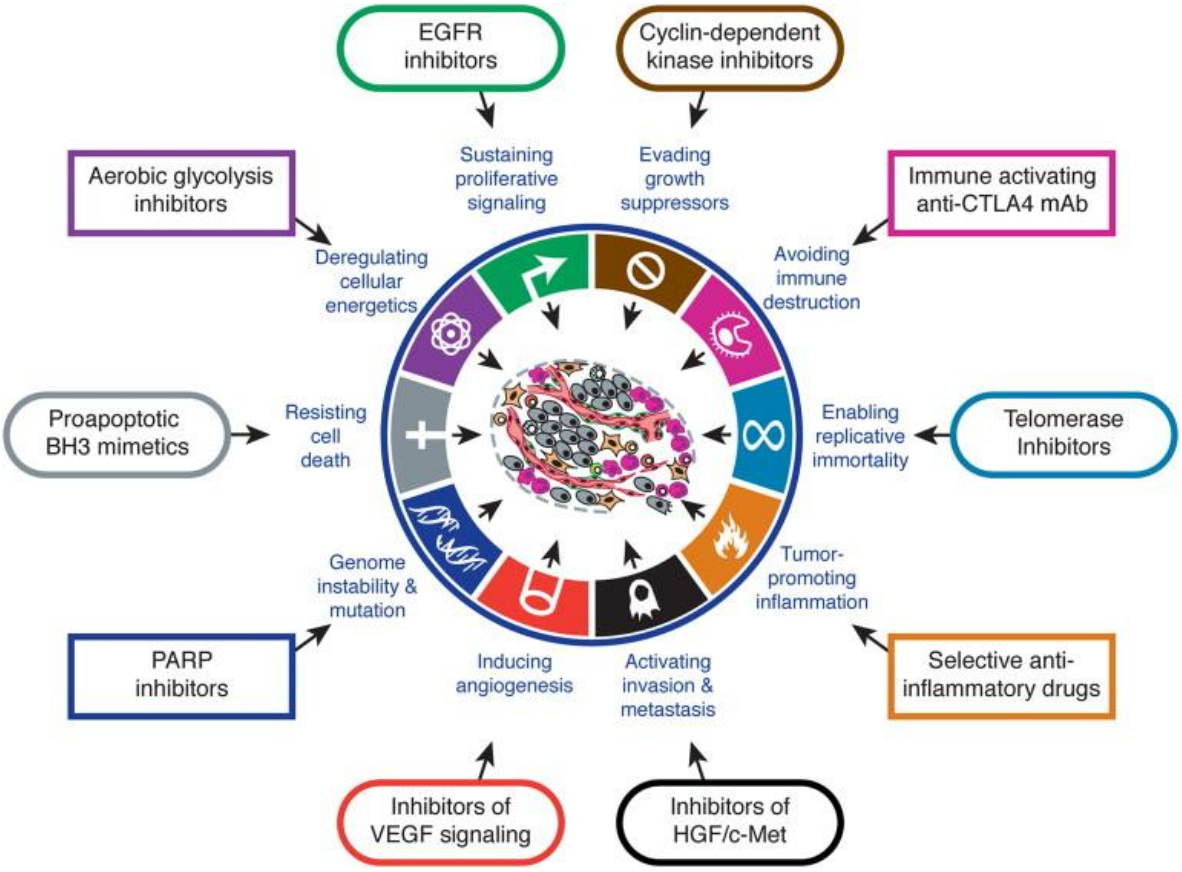


Figure 4. *The hallmarks of cancer, with inhibitors targeting hallmark pathways. Reprinted from [16] with permission from Elsevier.*

Normally, cells receive signals to proliferate through soluble ligands binding to cell surface growth receptors which, through intracellular signalling pathways, transfer the signal to the nucleus. Examples are the EGF, IGF and VEGF signalling pathways which all cross-talk through downstream signalling pathways such as the mTOR pathway [17]. In cancer cells, sustained proliferative signalling can be achieved by different modes of up-regulated proliferation pathway signalling such as increased production of growth factors, increased number of growth receptors, constitutively active receptors or downstream pathway components, disruption of negative feedback loops or through cross-talk with the surrounding stroma [18, 19].

Contact inhibition, cell cycle checkpoints and DNA damage sensors all contribute to tightly regulated cell proliferation. The numerous checks on proliferation, integrating signals from cell-cell contact inhibition, extracellular inhibitory factors, DNA damage and oxidative stress to avoid a hyper-proliferative phenotype, push cells either into cell cycle arrest,

apoptosis or senescence when growth conditions are deemed suboptimal. Cancer cells de-regulate these growth-regulatory pathways in a number of ways. The classic examples are mutation or (through MDM2 up-regulation) inactivation of the tumour suppressor p53 and inactivation of cell cycle inhibitor pRb through phosphorylation, allowing continued proliferation in the presence of DNA damage or extracellular inhibitory signals [20].

Apoptosis is likewise avoided either by inactivation of p53 or up-regulation of anti-apoptotic proteins [21], while the limit on the number of replicative cycles is broken through production of telomerase. This protein lengthens the telomeres, thus bypassing the Hayflick limit on replication [22].

Another hallmark is the capability to induce angiogenesis. Through production of angiogenic factors such as VEGF, FGF and PDGF, driven in part through the hypoxic tumour environment and HIF-1α signalling, formation of disorganised, leaky vessels with poor pericyte coverage and decreased perfusion in association to the tumour mass is induced [23].

Cancer tissues are also metabolically reprogrammed, with many cells switching to aerobic glycolysis for generation of ATP through fermentation of pyruvate to lactate even when oxygen is available. This is achieved through up-regulation of GLUT1 glucose transporters and enzymes in the glycolytic pathway while pyruvate dehydrogenase is inhibited, decreasing import of substrate for the citric acid cycle. HIF-1α is a central regulator of this reprogramming [24]. Although this is far less efficient than oxidative phosphorylation, aerobic glycolysis has several advantages. Apart from allowing survival in a hypoxic environment, aerobic glycolysis intermediates are shunted into the pentose phosphate pathway, creating nucleic acid precursors, necessary for sustained proliferation, and NADPH, necessary for cells to survive oxidative stress [25]. Excretion of lactate fuels neighbour cancer cells that use oxidative phosphorylation, reflecting a metabolic heterogeneity [26], while the excretion of protons, aided by up-regulation of ion exchange proteins, creates an acidic extracellular environment which increases invasiveness and hypoxic signalling while contributing to immunosuppression and drug resistance [27, 28].

Distant metastasis, the main cause of death in cancer patients, is another hallmark pathway. Functionally, cancer cells detach from their neighbours, break down extracellular matrix, become motile and migrate across the basal membrane, enter lymph and blood vessels, already remodelled through tumour-induced angiogenesis, and spread to distant organs where they intravasate and form new tumours [29]. To facilitate extravasation, successful

colonisation and growth of a metastasis from disseminated tumour cells, primary tumours secrete cytokines, growth factors and exosomes. These molecules signal a remodelling of distant tissues and recruit immune cells that are immunosuppressive and help remodel the tissue. This creates a pre-metastatic niche that is favourable to subsequent colonisation by disseminated tumour cells [30].

On a molecular level, the gain of invasive capabilities is associated with a transformation of epithelial cancer cells to a more mesenchymal-like state (EMT), driven by TGF-β, Wnt and Notch signalling [31]. Adhesion molecules such as E-cadherin are down-regulated while integrins, aiding motility, and matrix metalloproteinases, breaking down surrounding extracellular matrix, are up-regulated and cell polarity is lost through cytoskeletal rearrangements [32].

1.3 TUMOUR HETEROGENEITY

Driving these pathway changes is genetic instability, which is seen in a multitude of cancers and is associated with worsened prognosis. This instability is the effect of defects in DNA repair and cell cycle checkpoint proteins and contributes to an increased mutation rate. A mutator phenotype is often seen in hereditary cancer syndromes, with defects in DNA repair proteins such as BRCA1 in breast and ovarian cancer or MSH2 in Lynch syndrome, but sporadic cancers also show high rates of genetic instability in the form of microsatellite instability and aneuploidy [33].

Genetic instability creates a diverse population of tumour sub-clones, increasing the chances of overcoming barriers to proliferation and invasion through acquisition of mutations [34, 35] and of developing mechanisms of drug resistance [36, 37]. Although there is a degree of functional convergence between acquired genomic changes in different patients, relatively similar phenotypes can be achieved by point mutations at different sites, copy number alterations and epigenetic modifications of different genes, leading to the same cancer phenotype but with vastly different underlying genetic changes, making it difficult to apply one single treatment to large patient groups [38].

Heterogeneity is not only seen between, but also within patients, called intra-tumour heterogeneity. Genetically and functionally distinct sub-clones of cancer cells can be seen at different sites of metastasis, reflecting adaptations during the metastatic process and to the metastatic niche, but also at different sites within the same primary tumour. These sub-

clones are not only genetically, but also phenotypically different. Examples from glioblastoma [39] and renal cell carcinoma [40] show different transcriptomic profiles, suggesting different prognoses and drug responses in different biopsies from the same tumour. In addition to heterogeneity between the cancer cells, differences in tumour microenvironment add yet another layer of diversity [41]. Putting such a diverse population of clones under evolutionary pressure by applying treatments has a high chance of selecting resistant sub-clones for expansion, thus failing to eradicate the tumour [42].

1.4 THE IMMUNE SYSTEM AND CANCER

The immune system is a double-edged sword in the cancer setting, reviewed by Grivennikov et al. [43] (Fig. 5). Aspirin has been found to lower the lifetime risk of cancer [44], and chronic inflammation is associated with a range of tumours, from colorectal cancer in IBD patients or hepatocellular carcinoma in chronic hepatitis patients to gastric cancer in patients infected with H.pylori and pulmonary cancers linked to inflammation caused by smoking and asbestosis. Inflammation contributes to mutagenesis both through direct production of reactive oxygen species (ROS) and through induction, by cytokines, of ROS production in epithelial cells. Cytokine signalling also activates NF-κB and STAT3 signalling in tumour cells. These signalling pathways up-regulate a number of the cancer hallmarks, such as proliferation, angiogenesis and invasion (Fig. 6).

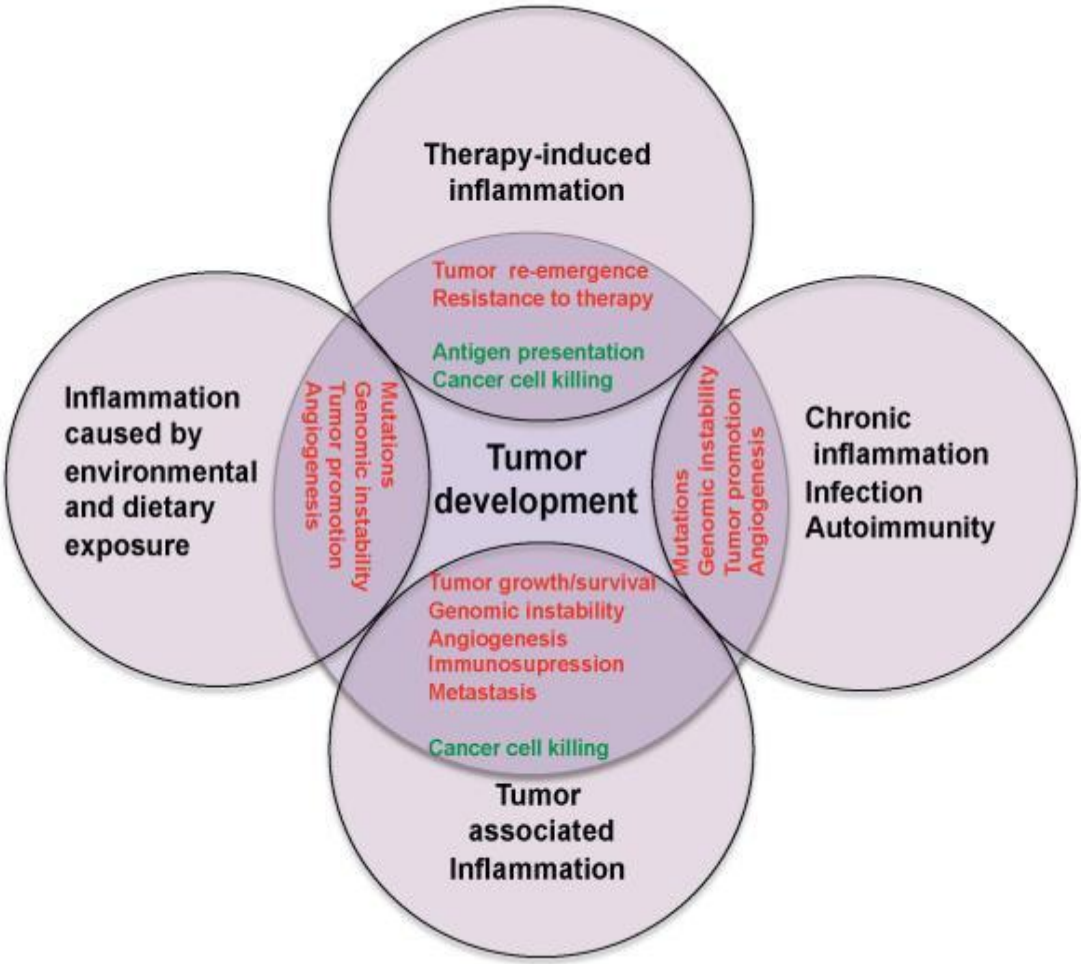


Figure 5. *Balance between pro- and anti-tumorigenic roles of the immune system. Reprinted from [43] with permission from Elsevier.*

In addition to this role, immune cells which contribute to tumour progression are actively recruited to the cancer site by tumour cells themselves through cytokine and TGF-β signalling and the attraction of immune cells to the hypoxic, necrotic tumour core. Tumour-associated macrophages (TAM) contribute to proliferation, angiogenesis and metastasis by production of growth factors and metalloproteases and tissue re-modelling. They have also been found to travel together with circulating tumour cells through the bloodstream. Myeloid-derived suppressor cells (MDSC) not only stimulate angiogenesis through VEGF production, but also limit elimination of the tumour by other immune cells such as NK cells and cytotoxic T-cells (CTL). Regulatory T-cells also have an immunosuppressive role, blocking CTL activation.

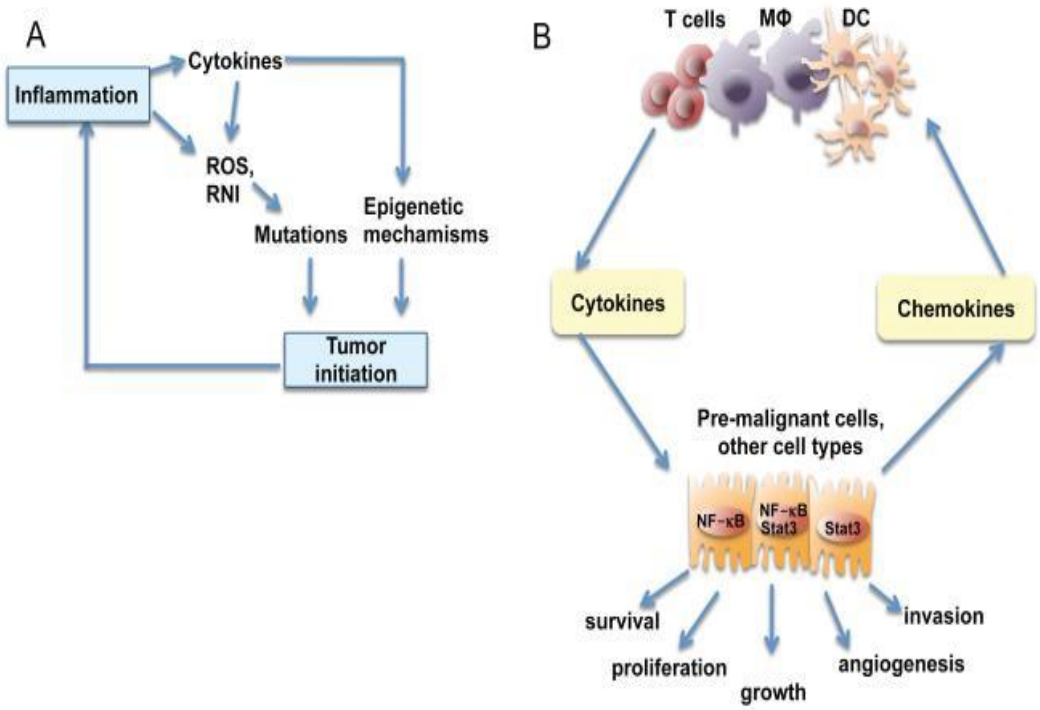


Figure 6. *Tumour initiation (A) and promotion (B) through immune signalling. Reprinted from [43] with permission from Elsevier.*

However, these pro-tumorigenic roles of the immune system are counterbalanced by two anti-tumorigenic roles. Firstly, activation of the immune system by dying tumour cells is critical to the efficacy of conventional chemo- and radiotherapy [45]. Secondly, there is an immunosurveillance process where the immune system eliminates cancer cells throughout the carcinogenic process. Studies on immune-deprived mice show an increased incidence of spontaneous tumours and transplant patients undergoing immunosuppressive therapies show increased rates of non-pathogen-related cancers, in sites such as bladder, kidney and liver [46]. Furthermore, high numbers of tumour-infiltrating CTLs have been associated with good prognosis in many cancer types, including melanoma [47], bladder [48] and head and neck cancer [49].

Mechanistically, early transformed cells are mainly eliminated by NK cells, recognising the early tumour as non-self, where after T-cells are primed by the dead cells and create a memory response against tumour antigens. However, the constant selective pressure of the immune system combined with the high rates of proliferation and mutation in cancer cells leads to a process of immunoediting, where cancer cells develop mechanisms to escape immune-mediated killing (Fig. 7) [50].

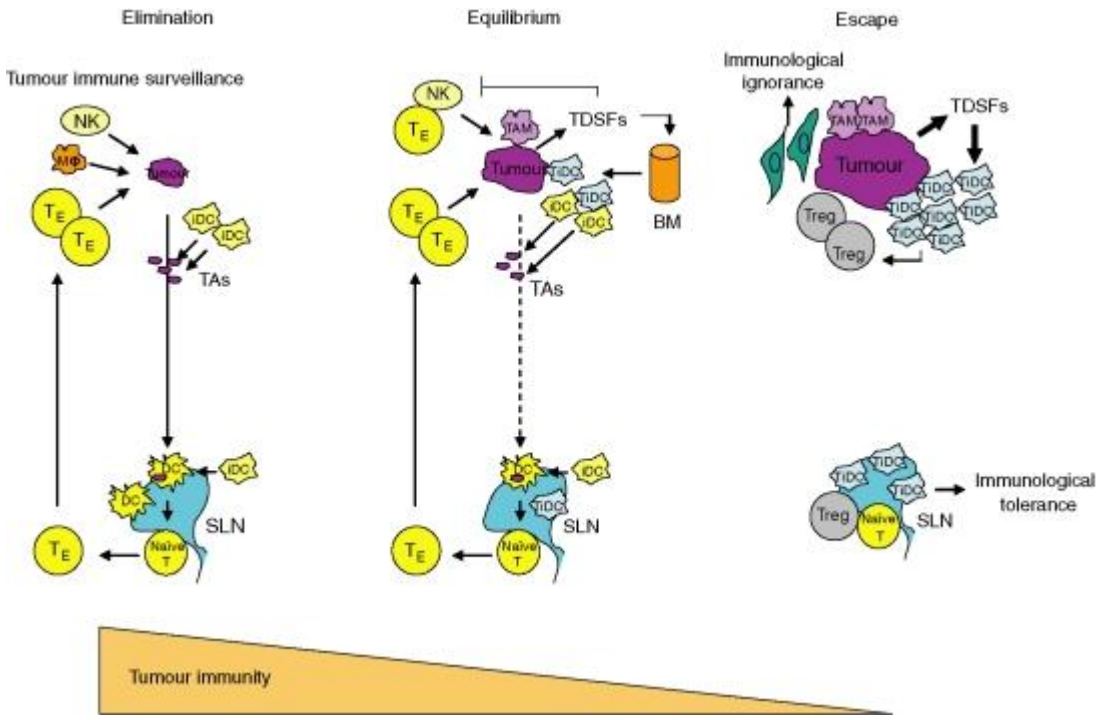


Figure 7. *The process of immunoediting, where cancer tissues step-wise acquire capabilities allowing them to escape immune-mediated killing [50]. Copyright © 2007, John Wiley and Sons.*

1.4.1 Immunosuppressive Mechanisms

Tumours can avoid immune destructions through two general mechanisms – either making themselves invisible to the immune system by down-regulating expression of tumour antigens and HLA class I molecules and up-regulating NK inhibitory ligands or by suppressing surrounding immune cells through recruitment of immunosuppressive cells and secretion of immunosuppressive molecules like TGF-β and IL-10. These mechanisms are reviewed by Vinay et al. [51]. Secretion of TGF-β by tumour cells and surrounding stroma directly inhibits CTLs and converts tumour-infiltrating $CD4^+$ T-cells into Tregs. These T-cells suppress the immune response through secretion of IL-10 and TGF-β, inhibition of Teff proliferation and cytokine release and inhibiting DC maturation [52]. Tumours also recruit, through chemoattractants, Tregs, MDSCs and TAMs. MDSCs suppress T-cells through down-regulation of the TCR ζ-chain, and TAMs produce TGF-β and IL-10, extending immunosuppression. These molecules inhibit DC maturation, producing immature, tolerogenic DCs.

Making themselves less visible and vulnerable to immune cells is achieved by cancer cells through down-regulating expression of MHC class I molecules and other components of the antigen-processing machinery, decreasing surface expression of antigens which may elicit an immune response. There is also death receptor down-regulation, making cancer cells less vulnerable to apoptosis by Fas-FasL interaction and up-regulation of ligands, such as PD-L1, which inhibit immune cells upon contact.

1.4.2 Immunotherapeutic Approaches

Cancer immunotherapy seeks to harness the anti-cancer effects of the immune system as a way to efficiently fight the disease and create a long-term immune memory to prevent relapse through down-regulating immunosuppressive tumour signalling and stimulating immune-mediated elimination of cancer cells.

To become activated, T-cells need two signals – binding of TCR to MHC class II peptide complex on APCs, guaranteeing a specific response against the antigen presented by the APC, and binding of CD28 to B7 on APCs, making sure that the antigen is presented by a dedicated APC. CTLA-4, expressed on T-cells, particularly Tregs, competitively binds B7, inhibiting the second signal and thus T-cell activation. Immune checkpoint blockade through antibodies targeting CTLA-4 restore T-cell activation and has been approved as melanoma treatment. Antibodies blocking PD-1 have also been approved as cancer treatment, while anti-PD-L1 antibodies are in clinical trials (Fig. 8) [53].

Another immunotherapeutic approach is vaccination. A vaccine against prostate cancer, consisting of DCs cultured with the tumour antigen prostatic acid phosphatase, succeeded in increasing survival and was approved for treatment of prostate cancer [54]. Yet another approach, in trials, is chimeric antigen receptors (CAR). T-cells are engineered to express an extracellular tumour-antigen specific antibody linked to intracellular kinase signalling domains, creating T-cells which are constitutively active against tumour cells expressing the antigen [55].

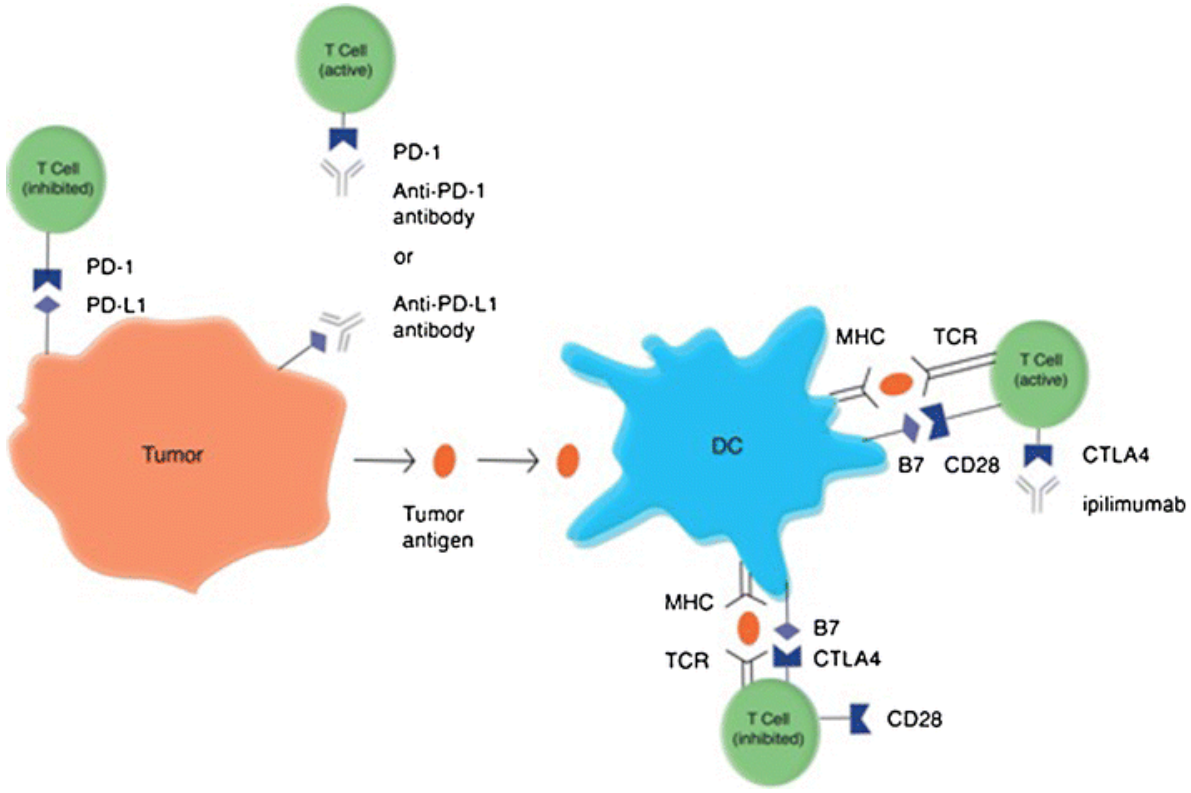


Figure 8. *Mechanisms of T-cell activation by immune checkpoint blockade antibodies. Reprinted from [56] with kind permission from Springer Science and Business Media.*

The graft-versus leukaemia effect, where donor bone marrow recognises host malignant host leukocytes as non-self, creating an immune reaction that eliminates the cancer is well established as a treatment for haematological malignancies [57]. Adoptive T-cell therapies use the same concept to target solid tumours [58]. TILs are separated from removed patient tumours, where after they are cultured with T-cell growth factors such as IL-2. T-cell clones shown to elicit a powerful immune response against tumour extract are then further expanded ex vivo and re-inserted into the patient as transfusion (Fig. 9).

An advantage of this approach is that it is possible to adapt to mutations and relapse through repeating the process against the relapsed tumour. Notably, it has been shown that it is necessary to deplete the patient of Tregs for the treatment to be effective [59], and for clinical usage this type of therapy should optimally be combined with targeted treatments removing tumour tissue immunosuppression, increasing apoptosis and cancer cell antigen presentation [58].

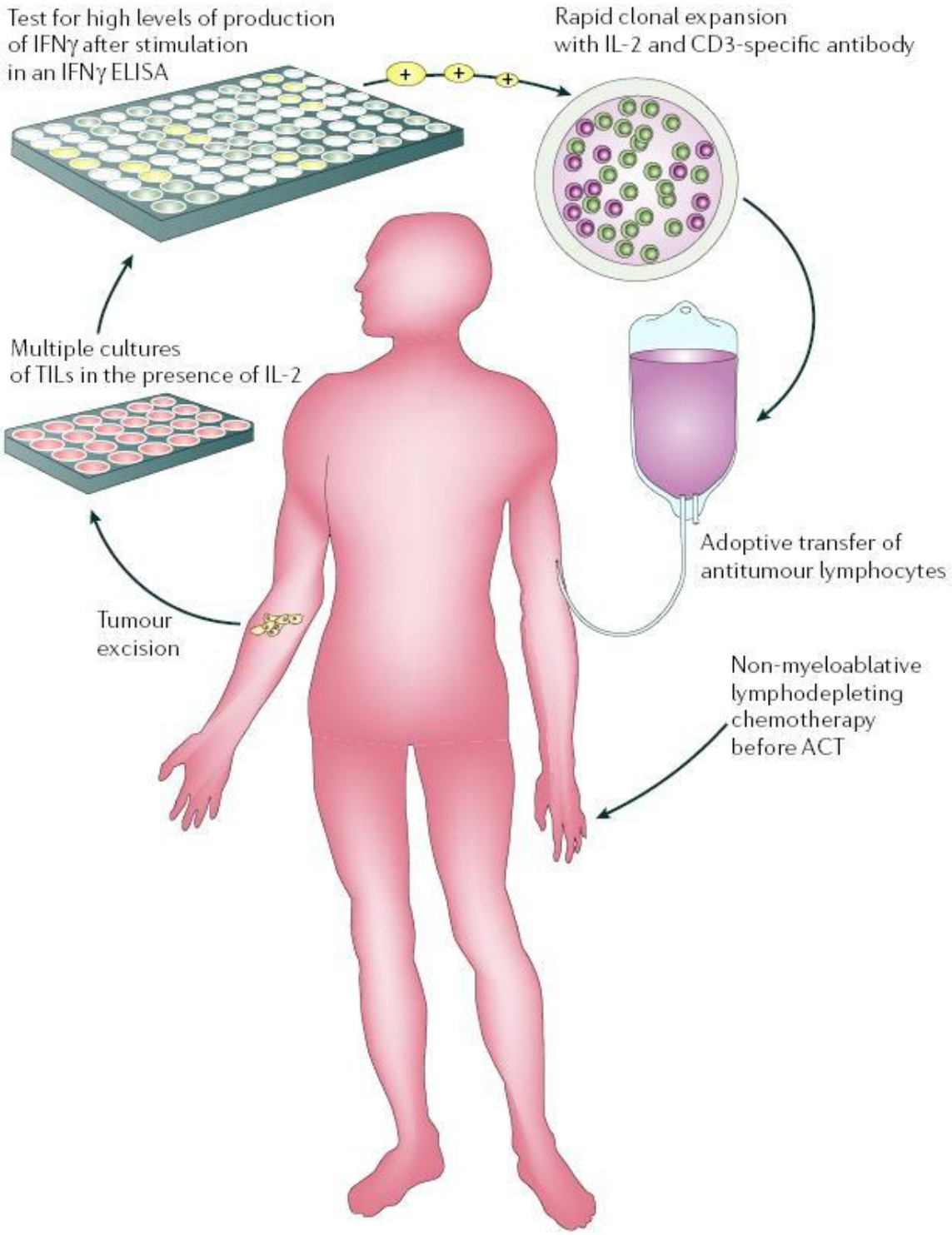


Figure 9. *Adoptive T-cell transfer for cancer immunotherapy Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology [60], copyright 2006.*

1.5 HUMAN PAPILLOMAVIRUS

A subset of cancers are transformed into malignant tumours through the action of HPV, which alone controls many of the pathways defined as hallmarks of cancer. HPV is an icosahedral double-stranded DNA virus in the Papillomaviridae family with tropism for squamous epithelia. In total, there are more than 170 different HPV types. Types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 are classified as high-risk (hrHPV) because of their carcinogenic potential. These are involved in the development of cervical, oropharyngeal and anogenital cancers. HPV16 and 18 are responsible for 50% and 20%, respectively, of cervical squamous cell carcinoma, while other hrHPV cause the remaining 30%. Oropharyngeal and anogenital squamous cell carcinomas, on the other hand, are mainly caused by HPV16 [61]. Apart from squamous cell carcinomas, HPV infection has also been linked to cervical adenocarcinoma [62].

The HPV genome is approximately 8 kb and encodes six early (E1, E2, E4, E5, E6, E7) and two late (L1, L2) proteins (Fig. 10). The genome also contains a long coding region (LCR), with elements regulating DNA replication and transcription [63].

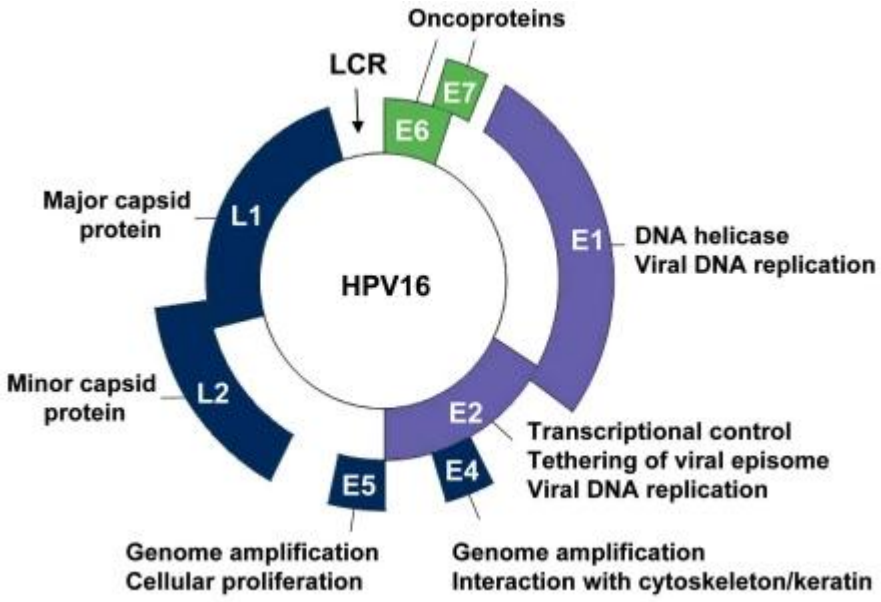


Figure 10. *Organisation of the HPV genome. Reprinted from [63].*

The HPV lifecycle starts with infection of proliferating cells in the basal layer of squamous epithelia. To reach the basal layer, breaks in the stratified epithelium are needed. Virions are internalised by clathrin-coated vesicles before unpacking inside the cell. In the basal layer, E1 and E2 keep episomal DNA separated from cellular DNA. As cells migrate to the supra-basal layer, expression of E6 and E7 pushes cells into S-phase, avoiding terminal

differentiation. These proliferating cells amplify the viral genome through the actions of E1, E2, E4 and E5. When the cells have reached the upper epithelial layers, L1 and L2 genes are expressed, forming a capsid protein where genomes are packed. This is followed by shedding and re-infection [64].

Through its lifecycle, HPV deregulates several hallmarks of cancer. Circa 80% of HPV infections are transient and cleared by the immune system [65], and in total only 1% of women infected with hrHPV will develop invasive cervical cancer [66]. Avoidance of immune clearance is achieved through several methods. HPV E5 causes down-regulation of MHC class I CTL ligands HLA-A and B, but not HLA-C and D, which inhibit the Natural Killer (NK) cell response [67]. Both E6 and E7 inhibit interferon signalling, with E7 also repressing expression of MHC class I and APM components on promoter level [68, 69].

The E6 and E7 proteins are most important for cell transformation. E6 causes ubiquitination of the tumour suppressor p53, leading to apoptosis resistance. It also induces expression of the telomerase hTERT, immortalising the infected cells. E7 removes the G1/S cell cycle checkpoint by binding pRB, releasing E2F to bind DNA and transcribe cell cycle genes, thus allowing continuous proliferation [70]. This has the side-effect of up-regulating cdk inhibitor p16, which is used as a surrogate marker of active HPV infection. E5, in addition to roles in immune escape and viral amplification, drives proliferation by increasing EGFR expression [71]. Chronic HPV infection may lead to integration of the viral genome into the host genome, giving the viral transcripts a longer half-life and disrupting E2 expression, which removes the suppressive effect of E2 on E6 and E7. In addition, genome integration causes genetic instability, favouring acquisition of the other hallmarks [72].

There are currently two approved prophylactic HPV vaccines, Gardasil directed against the L1-protein of HPV 6, 11, 16 and 18 and Cervarix against HPV16 and 18. However, vaccines targeting the L1 protein are not effective after cells have been infected by HPV. In addition, there is now also a nonavalent HPV vaccine, Gardasil 9, protecting against 6, 11, 16, 18, 31, 33, 45, 52 and 58, that was approved by FDA in December 2014 [73]. In Sweden, subsidised HPV vaccination was available for girls ages 13-17 years old 2006-2011. From 2012 onwards, HPV vaccination of girls ages 10-12 years was included as part of the national immunisation programme. Girls aged 13-18 years can also be vaccinated within a catch-up programme.

For cancerous or pre-cancerous lesions caused by HPV, immunotherapeutic strategies, targeting E6 and/or E7 are under development or in clinical trials, e.g. therapeutic peptide-based vaccines [74] and adoptive T-cell transfer [75].

1.5.1 Cervical Cancer

Worldwide, circa 500 000 women are diagnosed with cervical cancer annually, with the greater part being in developing countries, in many of which it is the leading cause of cancer death in women. 80% of cervical cancers are squamous cell carcinomas with the rest being adenocarcinomas; however, HPV is the causative agent in almost every case. Being HPV-driven, cervical cancer spreads through sexual activity, and risk factors include early sexual début, a high number of partners, immunosuppression and tobacco smoking [76].

The standard screening test has traditionally been cervical cytology, Pap smear, which has around 90% specificity but can reach as low as 37% sensitivity. Recently, HPV testing has been introduced as an alternative or complement. HPV testing is more sensitive, with a sensitivity of around 90%, and was lately recommended as standard screening by WHO [77].

Cervical cancer develops from in-situ dysplasia, which is ranked from CIN1 (least atypical cells) to CIN3 (most atypical). These may develop into invasive carcinomas which metastasise locally to adjacent pelvic organs and lymph nodes before distant metastasis. Discovery of atypical cells or hrHPV on screening is followed by cervical biopsy and staging based on histology and invasion, which establishes the diagnosis. Five-year survival varies from 100% survival in microinvasive stage IA to 5-15% in distantly metastasised stage IV. Treatment options consist of radical hysterectomy with lymphadenectomy and radiotherapy in earlier stages and cisplatin-containing chemoradiotherapy in stages II and above [76]. Recently, the anti-VEGF antibody bevacizumab has also been approved for treatment of advanced stages [78].

1.5.2 Oropharyngeal Cancer

Cancers of the oral cavity and oropharynx had an incidence of 400 000 with 223 000 deaths worldwide in 2008 [79]. Oropharyngeal squamous cell carcinomas (OSCC) can be divided into two clinically distinct subgroups – HPV-positive and HPV-negative. While risk factors

for the latter are mainly alcohol and smoking, the earlier is caused by HPV infection from sexual activity [80, 81]. In contrast to cervical cancer, where around 50% and 20% are attributed to HPV16 and HPV 18 respectively, close to 90% of HPV-positive OSCC are caused by HPV16, with HPV33 and 35 being the next most frequent types [82].

HPV-positive OSCC is mainly found in the tonsils (TSCC) and base of tongue (BOTSCC), with an HPV prevalence in the County of Stockholm of 79% and 64%, respectively, for these sites [82], while less than 20% of OSCC outside these sites contain HPV DNA [83]. In the Western world, rates of HPV-negative OSCC/TSCC are declining, probably due to a decrease in smoking, while HPV-positive OSCC/TSCC has been on the rise since the seventies [82, 84, 85]. There is a clear difference in prognosis between the two types, with five-year survival rates around 80% in HPV-positive vs 40% in HPV-negative cancers with conventional surgery and radiotherapy [86, 87].

HPV-positive OSCC are histologically less differentiated and show less keratinisation than HPV-negative OSCC. They also show much lower rates of p53 mutations, and have p16 up- instead of down-regulated, when compared with HPV-negative OSCC. These differences are due to the impact of HPV on p53 degradation and pRb inactivation [88].

OSCC is most often discovered when patients get regional lymph node metastases. Before 2008 treatment consisted mainly of conventional therapy and/or surgery. Thereafter, treatment has been intensified and now includes hyperfractionated radiotherapy, induction/concomitant chemotherapy and EGFR antibodies. In HPV-positive OSCC, several biomarkers have been found to be correlated to increased treatment response. E.g. patients with high numbers of $CD8^+$ TILs or absence of MHC class I expression have survival rates of 95-100% with conventional radiotherapy alone [49, 89-91]. Many are thus over-treated, contributing to side-effects such as jawbone necrosis, difficulties swallowing and mouth dryness [87, 92]. By combining several biomarkers together with knowledge of tumour HPV status, OSCC patients can potentially be selected for less intensive treatment.

1.6 BREAST CANCER

Breast cancer is the most commonly diagnosed and leading cause of cancer death in women worldwide, with circa 1 700 000 cases and 500 000 deaths in 2012 [93]. Hormone replacement therapy, oral contraceptives, smoking, hysterectomy and diabetes mellitus increase breast cancer risk, while breastfeeding decreases it [94, 95]. There are also

hereditary risk factors, notably hereditary breast and ovarian cancer syndrome, caused by BRCA gene germline mutations [96].

Breast cancer is diagnosed using multiple modalities. MRI is recommended for younger women with a high risk of developing breast cancer. In the majority of cases, a finding on screening mammography (in Sweden starting at 40 years of age) is followed by diagnostic mammography, ultrasound and core needle biopsy. The biopsy is stained for oestrogen receptor (ER), progesterone receptor (PR) and HER2/neu expression and analysed by a pathologist.

Tumours are classed based on histological grade, receptor status and TNM staging. Being a very heterogeneous disease, the prognosis depends on these parameters. Five-year survival for all women diagnosed is around 90%, but survival depends on stage and receptor status, with stage IV patients having below 20% five-year survival. Negative hormone receptor status and young age of onset (< 40 years old) are further negative prognostic factors [97, 98].

The most common subtypes of breast cancer are invasive ductal carcinoma (80% of cases) and invasive lobular carcinoma (10%) [99]. At least 18 different histological types exist. Based on gene expression data, these can be grouped into seven molecular subtypes; luminal, HER2, basal-like, normal-breast-like, apocrine, interferon-related and claudin-low types [100]. Microarray studies have created gene expression signatures predictive of prognosis and treatment response, which have identified proliferation and hormone receptor genes to have the most impact on prediction [101].

Depending on pathology, receptor expression, age and tumour dissemination at time of diagnosis, different treatment options are available. In locally advanced and inflammatory cancers, neoadjuvant chemotherapy is administered before surgery [102]. When surgery is performed, partial or complete mastectomy is performed together with SN biopsy and axillary dissection when the SN is positive. This is followed by chemotherapy (if indicated) and irradiation. Standard treatment for ER and/or PR positive patients is ER antagonist Tamoxifen for five years following surgery [99]. Lately, targeted agents have also begun to be used in breast cancer treatment. Antibodies against HER2 (trastuzumab) have increased survival [103], VEGF antibodies (bevacizumab) are approved and increase progression-free but not overall survival [104], while antibodies and drugs targeting other proteins such as tyrosine kinases and PARP are in trials [105].

1.7 URINARY BLADDER CANCER

Urinary bladder cancer is the fifth most common cancer in men worldwide, with a global yearly incidence of 380 000 new cases and 150 000 deaths in both genders [79]. The incidence in men is three times higher than in women. Smoking is the main environmental risk factor, thought to cause about half of all cases. Other environmental risk factors include polycyclic hydrocarbons, arsenic and chronic schisostomosis. In the Western world, >90% of bladder cancers are urothelial in origin [106].

Urothelial bladder cancer is divided into two clinically and molecularly distinct subtypes, muscle-invasive (MIBC) and non-muscle-invasive (NMIBC) based on invasion into the detrusor musculature. NMIBC is treated with cystoscopic resection, with BCG upon relapse. Recurrence is common, but progression into invasive carcinoma occurs in only 20-30%, and five-year survival is 90% [107]. MIBC, which makes up 20-25% of all newly-discovered bladder malignancies, has a poor prognosis, with 5-year survival for stages T2-T4 being around 50% with best treatment, consisting of radical cystectomy, lymphadenectomy and neoadjuvant chemotherapy [106, 108].

MIBC is highly genetically unstable, showing high rates of aneuploidy and heterogeneity [109]. Mutations in DNA maintenance and repair genes including MCM4, ERCC2, ATM and FANCA have been associated with MIBC, and chromosome 9 loss is common in both NMIBC and MIBC. Activating mutations in FGFR3 and overexpression of EGFR, as well as p53 mutations, are also common in MIBC [106].

MIBC is a relatively quickly metastasising malignancy. One of few larger autopsy studies found 69% of MIBC patients to have metastases, mainly to the liver and local lymph nodes. In the patient subset metastases, 90% had tumour cells in the lymph nodes, showing that MIBC spreads mainly through local lymph nodes. Metastasis frequency follows tumour stage, with 80% of T4 patients and 36% of T2 patients showing either regional or distant metastasis. [110].

1.8 PERSONALISED CANCER MEDICINE

Starting with Paul Ehrlich's concept of the “magic bullet”, selectively targeting disease-causing micro-organisms or cells with specific receptors while sparing other cells, personalised medicine as a theory has a long history. The development and subsequent clinical use of monoclonal antibodies towards growth factor receptors achieved the magic

bullet in oncology. However, the great redundancy in cell signalling pathways along with the heterogeneity of tumours means that targeted therapy alone is rarely curative. However, with recent advances in omics technologies, providing complete genomes, transcriptomes and proteomes for each patient, truly personalised cancer medicine is within reach. Through integrating deep knowledge of individual tumour signalling with known effects of the available array of targeted treatments, one can predict optimal treatment combinations for each patient [111].

Personalised medicine aims to treat the right patient with the right combination of drugs at the right time to achieve maximum efficacy while minimising side-effects and resistance development. However, for this trivial definition to transfer to actual patient benefit a number of conditions must be fulfilled. Diagnostic modalities are necessary that have the capability to correctly predict prognosis, stratify patients to different treatments and monitor treatment response by re-applying tests between and after treatments to find surviving resistant clones. In addition, drugs need to specifically target the different pathways affected in cancer patients in such combinations that clones resistant to all drugs in the treatment combination have a very low risk of arising. Naturally, both diagnostics and treatments must be able to account for the massive inter- and intra-tumour heterogeneity [9].

1.8.1 Omics

Omics refers to a number of technologies that are able to quantify and study the global expression patterns of biomolecules as opposed to single genes or proteins [112]. Omics approaches exist on all levels of cell signalling. Genomics and transcriptomics, greatly boosted through the arrival of next-generation sequencing, study of whole genomes and transcriptomes, as well as patterns of epigenetic modifications and non-coding RNAs. Several tumour types have had their entire genome sequenced, revealing previously unknown driver genes [113]. Mass-spectrometry (MS) based proteomics have reached a coverage of over 10 000 proteins, identifying new protein-coding loci through integration with known DNA sequences [114], and are also able to identigy post-translational modifications and protein-protein interactions. Metabolomics, studying small metabolic molecules through mass spectrometry and nuclear magnetic resonance spectroscopy, also has wide applications in cancer, such as exploring metabolic changes, drug mechanisms and toxicities [115].

These technologies have been applied in many cancer settings, successfully creating expression signatures classifying cancers by sub-type and found hundreds of putative prognostic and treatment biomarkers on different biological scales. However, much fewer treatments targeting these genes and proteins have been approved and successfully used in the clinic. The main limitations lie in the validation pipeline. Model systems used to identify biomarker signatures often fail to accurately represent the cancer ecosystem; thus, experimental findings may not be transferrable to clinical use. Furthermore, biomarkers identified in a certain patient subset may not be applicable in those patients selected for clinical trials, typically suffering from advanced disease. The genetic instability and heterogeneity of these patients, coupled with the redundancy of cancer signalling pathways, means that drugs targeted against single biomarkers may not have any clinical effect because of the high resilience to selective pressures in advanced tumours displaying a wide range of sub-clones [116].

1.8.2 Targeted Treatments

Small molecules targeting proteins specific to or overexpressed in cancer cells are a necessity if the biomarkers identified by omics technologies are to be exploited. Cancer-specific proteins are better targets since drugs specific against these targets will not affect normal cells. There have been certain successes, including the tyrosine kinase inhibitor (TKI) imatinib which targets the BCR-ABL fusion protein in chronic myeloid leukaemia (CML) and has successfully increased survival in CML patients [117] and PARP inhibitors in BRCA-mutated cancers, which exploit the fact that only BRCA-mutated cells are sensitive to PARP inhibition because of their already defective DNA repair [118]. Approved targeted therapies against proteins amplified in cancer include trastuzumab in Her2-positive breast cancer [119] and both antibodies and kinase inhibitors against EGFR antibodies in a range of cancers, including lung, pancreas and head and neck [120, 121].

However, resistance development is a problem, especially when only single targeted drugs are administered. Both in the case of TKIs [122] and antibodies [123], tumour heterogeneity greatly increases the risk that a subset of cells survive the treatment and expand, forming resistant clones. Resistance can either be intrinsic or acquired, and can be achieved through numerous mechanisms, including mutations in the target itself, activation of bypass pathways either through up-regulation of non-targeted kinases or re-wiring of downstream signalling, and disabling of apoptosis pathways. To overcome this problem,

combining multiple targeted treatments is an attractive option, which accounts for a larger part of tumour heterogeneity and diminishes the possibility of pathway re-wiring [124]. Examples include combining RAF inhibitors with MEK and PI3K inhibitors in melanoma, which limits the number of possible resistant growth signalling pathways [125].

1.8.3 Systems Medicine

The availability of wide-spanning, high-resolution mapping of cancers from genome to metabolome has underlined the fact that cancer is a complex disease of disrupted signalling networks, that, due to intra-tumour heterogeneity, is highly resilient to perturbations. The limited success of targeted therapies underlines the fact that a reductionistic approach focusing on single oncogenic molecules is sub-optimal. Systems biology in cancer addresses this problem by studying cancer as a big signalling network, constructing mathematical models that can predict, in an unbiased manner, the properties of the entire network based on biomolecular data, and how external perturbations such as addition of drugs will impact this network [126].

The application of systems biology through integration of omics data with in silico modelling has already shown promise. Examples include the prediction and validation of PTEN levels as predictive of response to trastuzumab [127], modelling of glioma dynamics to improve radiotherapy scheduling [128] and integration of sequencing data with cellular responses to ligand stimulation in order to predict the impact of 23 targeted drugs on pathway signalling and outcome [129].

Systems biology is a necessary component for personalised medicine. Modelling integrated data on clinical outcomes, tumour characteristics and drug structure and mechanisms and applying machine learning algorithms, one arrives, in an unbiased manner, at predicted optimal treatment combinations for patients with defined signalling characteristics and dynamics [10]. This leads precisely to the goal of personalised medicine – the right treatment for the right patient at the right time (Fig. 11).

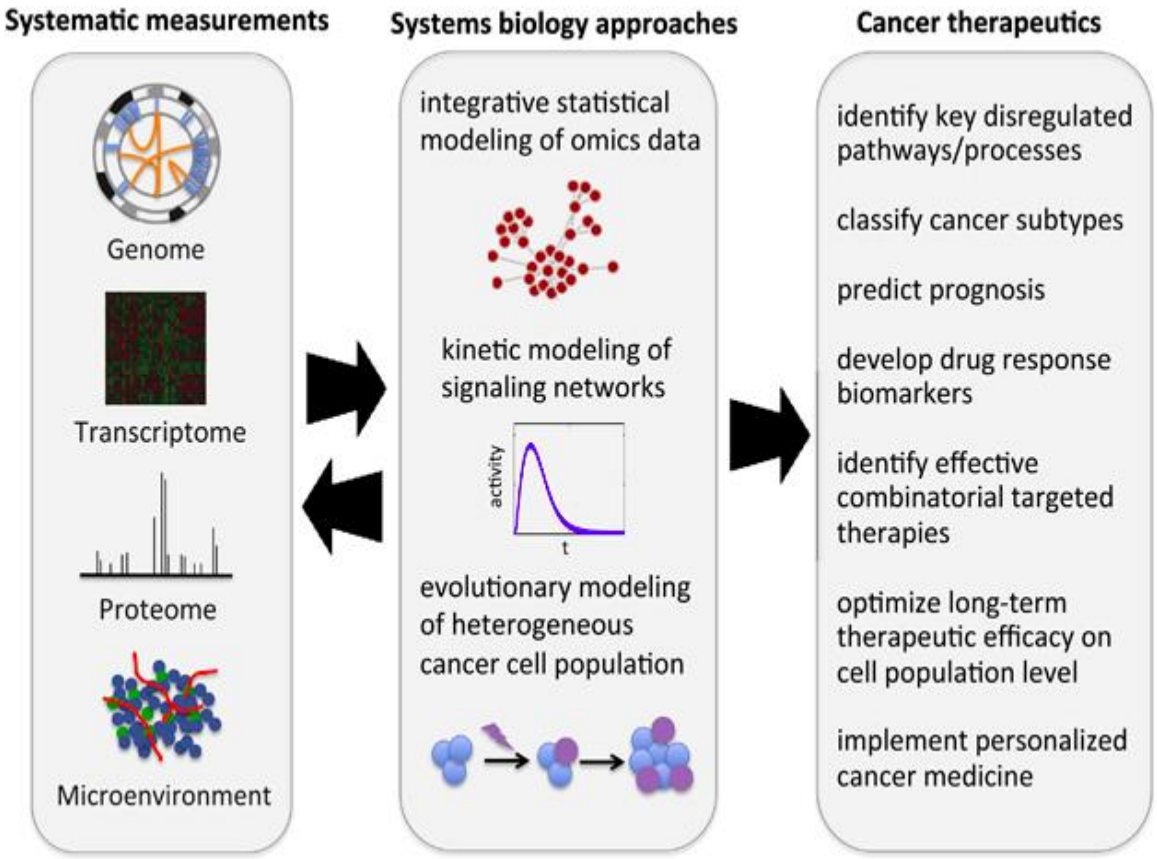


Figure 11. *The systems biology approach integrates patient data down to genome level with known drug effects and clinical data, creating models which adapt diagnosis and treatment to individual patients. Reprinted by permission from Macmillan Publishers Ltd: Oncogene [126], copyright 2014.*

2 AIMS

The general aim of this thesis was to evaluate and integrate different methods of analysing tumours, and apply them to personalisation of cancer medicine, focusing on predicting optimal treatment. The specific aims for each paper were:

Paper I. Compare DNA and mRNA analysis for HPV sub-typing of cervical carcinoma in order to accurately differentiate between high- and low-risk patients.

Paper II. Study HPV16 E2, E5 and E7 mRNA expression in relation to patient survival, HLA class I expression and $CD8^+$ TIL infiltration in TSCC and BOTSCC to improve stratification of patients.

Paper III. Identify optimal combinations of targeted drugs for different breast cancer signalling states through screening breast cancer cell lines with combinations of TGF-β, oestrogen, EGF and targeted drugs against these pathways.

Paper IV. Evaluate whether proteomics on Tregs and Teffs in lymph nodes in individual MIBC patients combined with interactome modelling can predict targets for optimisation of immunotherapy.

3 MATERIALS AND METHODS

3.1 PATIENTS AND SAMPLES (PAPER I, II, IV)

Paper I included formalin-fixed paraffin-embedded (FFPE) tumour samples from 98 primary cervical adenocarcinomas from patients who were diagnosed and underwent surgery at Karolinska University Hospital between 1992 and 2000. They were followed up until January 2007.

Paper II included FFPE biopsies from 112 TSCC and 21 BOTSCC patients treated curatively between 2000 and 2011 at Karolinska University Hospital with follow-up data for at least three years.

Paper IV included four MIBC patients who underwent radical cystectomy with lymphadenectomy at Umeå University Hospital, Gävle and Västerås hospitals. Prior to surgery, radioactive tracer (80 MBq technetium) was injected peritumorally. At surgery, sentinel nodes, defined by uptake of tracer measured by a hand-held Geiger counter, and control non-sentinel nodes were removed and put in RPMI on ice for transport to the laboratory. In addition, venous blood was taken and transported in heparin-containing tubes at room temperature.

3.2 HPV GENOTYPING (PAPER I-II)

In paper I, presence of HPV DNA was assessed by PCR with GP5+/6+ probes (targeting the L1 region). HPV typing was performed using single-strand conformational polymorphism (SSCP) and/or by direct DNA sequencing of the PCR products from the L1 region with previously developed assays [130]. Samples showing inconsistent results were analysed by reverse line blot [131].

For tumours included in paper II, presence of HPV DNA had for most samples been determined by PCR with GP5+/6+ and CPI/IIG probes, and genotyping for HPV16 by HPV16 E6-specific PCR, as described earlier [132]. Alternatively, mainly for samples after 2008, the presence of HPV and genotyping was performed with a bead-based multiplex assay on a MagPix instrument, assaying for 24-27 HPV types [82]

3.3 RNA ANALYSIS (PAPER I, II, IV)

In paper I, samples were analysed for E6/E7 mRNA from HPV types 16, 18, 31, 33 and 45 through RT-NASBA (PreTect HPV-Proofer, NorChip AS). GAPDH was used as control for presence of mRNA in the samples.

In paper II, a multiplex bead-based assay was developed to simultaneously analyse samples for several different HPV16 mRNA transcripts. After RNA extraction and treatment with DNAse, cDNA was synthesised using random primers. PCR was performed using primers for HPV16 E2, E5, E7, β-globin (control for DNA contamination) and U1A (control for cDNA synthesis from mRNA). PCR products were hybridised to sequence-specific probes coupled to fluorescent magnetic FlexMap beads. After incubation and washing, fluorescent streptavidin detection molecules were conjugated to the probe-bound amplicons. Presence of different amplicons was quantified in a MagPix instrument (Luminex Inc.) through illuminating the beads with a red laser for bead detection and a green laser for streptavidin detection.

In paper IV, after RNA extraction and cDNA synthesis, RT-PCR was performed using probes against IL-16, with GAPDH used as housekeeping gene to normalise values.

3.4 CELL CULTURE EXPERIMENTS (PAPER II-IV)

In paper II, for validation of the multiplex HPV16 cDNA assay, the cervical cancer cell line Siha, tongue cancer cell lines UM-SCC-47 and UPCI-SCC-154 (all positive for HPV16) and the HPV-negative oral cancer cell line UM-SCC-14 were used.

In paper III, MDA-MB-231 and MCF7 breast cancer cell lines were used. MDA-MB-231 is an oestrogen-negative, aggressive, mesenchymal-like cell line which forms metastases when injected into animals, while MCF7 is oestrogen-positive, slowly growing and forms tumours but not metastases in animals.

The cells were seeded at 10 000 cells/well in a 96-well plate, cultured overnight where after they were treated with a total of 90 combinations of drugs and growth factors at different concentrations per cell line. 18 combinations of growth factors were used, with TGF-β1 at concentrations of 1 and 10 ng/ml, EGF at 50 and 100 ng/ml and 17-β-oestradiol at 10 μM. They were combined with either no drugs, 10 μM of the TGF-β kinase inhibitor SB431542,

1 μM of oestrogen antagonist Tamoxifen, 10 μM of EGFR TKI Iressa or all three drugs together. Impact on proliferation was determined by MTT assay after 48 hours of treatment.

In paper IV, the urinary bladder cancer cell line 5637 was cultured for 36 hours, where after culture medium was aspirated and frozen at -80^{o}C. For stimulation experiments, cells harvested from the patients were seeded at 300 000 cells/well in 200 μl total volume. Cells were either cultured in 150 μl tumour-derived supernatant and 50 μl basal culture medium or basal culture medium alone. Cells were harvested after 72 h for RNA isolation and PCR as described above.

3.5 FLOW CYTOMETRY (PAPER IV)

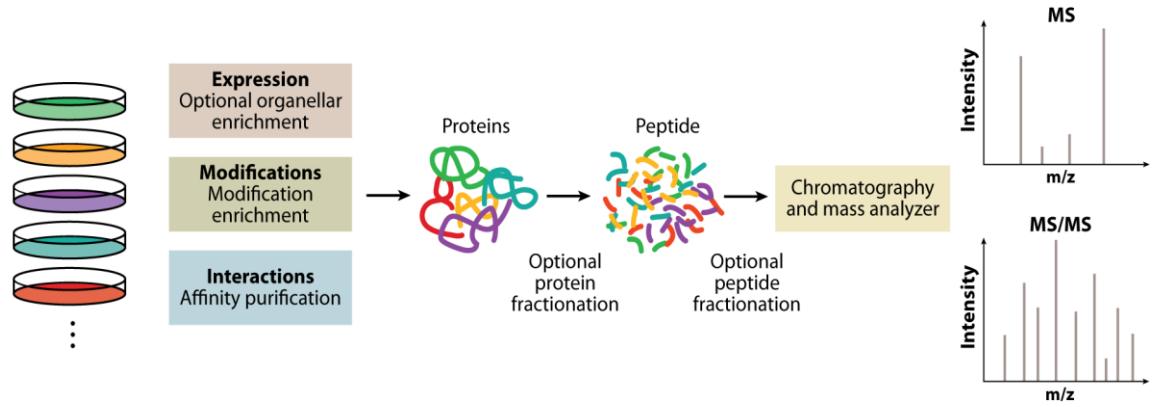
PBMC were isolated using Ficoll-Paque PLUS (Amersham Biosciences) density centrifugation gradient. From lymph nodes, leukocytes were extracted by gentle homogenization through a 40μm cell strainer. After extraction, $CD4^{+}$ cells were isolated using EasySep Human CD4 positive selection kit (Stemcell Technologies).

For flow cytometry, 500 000 cells/sample were first stained with LIVE/DEAD Fixable blue dead cell stain kit (Life technologies) followed by surface staining with fluorochrome-conjugated antibodies. For intracellular antigens, surface staining was followed by fixation, permeabilisation and staining. Data was acquired on an LSRFortessa II (BD Biosciences). For sorting experiments, cells were surface stained, as described above, and sorted using the FACSAria flow cytometer (BD Biosystems). Sorted cells were immediately frozen in -80^{o}C. Data were analysed using FlowJO X 10.0.7r2 software (Tree Star, Ashland OR). Post sorting Treg and Teff purity was >90%.

For intracellular cytokine staining, cells were suspended at a density of 2*10^6 cells/ml in Falcon 14 mL Polystyrene Round-Bottom Tubes (Becton Dickinson) in the prescence or absence of Phorbol Myristate Acetate (50 ng/ml) and Ionomycin (1 ug/ml). To inhibit cytokine secretion, Brefeldin A (GolgiPlug) was added 1 h after stimulation. Cells were harvested for FACS staining at 6 h after stimulation.

3.6 PROTEOMICS (PAPER IV)

Frozen cell pellets were thawed on ice, sonicated and centrifuged to remove debris. 5 µg of protein/sample underwent denaturation, tryptic digestion and clean-up, as described in detail in the paper. The resulting mixture of tryptic peptides (1 µg/sample) was loaded onto a nano-Ultimate HPLC system with an acetonitrile gradient (Thermo Scientific) in-line coupled to a QExactive orbitrap mass spectrometer (Thermo Scientific). Mass spectra were searched against the SwissProt database using Mascot software (Matrix Science Ltd.). The false-discovery was limited at 1% over the entire peptide population. Quantitation, using Quanti software [133], was done through quantifying the ion current for each MS/MS spectrum. Only proteins with at least two identified peptides were quantified. A general overview of the workflow is found in figure 12.



Cox J, Mann M. 2011.
Annu. Rev. Biochem. 80:273–99

Figure 12. *Overview of LC-MS/MS-based proteomics workflow. Reprinted from [134].*

3.7 NETWORK ANALYSIS (PAPER IV)

Identified proteins and their interaction partners, taken from validated human protein-protein interactions in the SPIKE database [135], were used to construct protein interaction networks in Cytoscape [136]. Hierarchical clustering was performed with the clusterMaker plugin [137], network centralities (betweenness and node degree) were calculated with Centiscape [138] and the JEPETTO plugin [139] was used to identify enriched signalling pathways.

3.8 STATISTICS (PAPER I-IV)

Paper I used standard measures of diagnostic accuracy such as sensitivity and specificity to compare the differences in performance between DNA- and RNA-based tests and Cox regression analysis to identify differences in survival between HPV mRNA-positive and negative patients.

Paper II used the log-rank test to compare survival differences between groups. For progression-free survival, patients who died of causes other than cancer were censored. Patients were considered progression-free until relapse or death due to cancer. For relapse-free survival, patients who were never disease-free, or for whom no relapse had been diagnosed at time of death, were censored in addition to those who died of other causes. Fisher's exact test was used to compare HLA class I expression between groups, as this had been coded as a binary variable. The numbers of $CD8^+$ TILs were not normally distributed. For analysis, TIL numbers were therefore divided into four quartiles and group differences analysed using ordinal logistic regression from the MASS package in R.

In paper III, Student's t-test was used for comparisons of proliferation between treatment conditions and for comparisons of tumour characteristics in the analysis of clinical data.

In paper IV, the JEPETTO Cytoscape plugin [139] was used to test for enrichment of signalling pathways. The XD-score shows how close the input set of proteins is to a specific signalling pathway in the STRING molecular interaction network [140], and the q-value is the significance value of Fisher's exact test corrected for multiple testing.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Background and Aims

In the last decades, there has been an increase in the incidence of cervical adenocarcinoma in several countries [141]. In the US, the incidence increased by 32% between 1973 and 2007. Cervical adenocarcinoma has worse survival and a higher rate of metastasis than squamous cell carcinoma [142]. Arising high up in the endometrial canal, it is less likely to be detected with pap smear screening [143]. As the great majority of cervical adenocarcinomas are caused by hrHPV infection, hrHPV testing should be introduced in screening programs to identify these patients [144]. Since expression of E6 and E7 mRNA indicate an active HPV infection, the aim of this study was to evaluate whether detection of hrHPV E6/E7 mRNA through RT-NASBA was feasible in FFPE adenocarcinoma biopsies, and to compare the diagnostic performance of mRNA analysis with genotyping.

Materials and Methods

FFPE biopsies from 98 cervical adenocarcinomas were sectioned and underwent nucleic acid extraction. Presence of HPV DNA was assessed by PCR with GP5+/6+ probes targeting the L1 region. HPV genotyping was performed using SSCP and/or by direct DNA sequencing. RNA was analysed for E6/E7 mRNA from HPV types 16, 18, 31, 33 and 45 through RT-NASBA (PreTect HPV-Proofer, NorChip AS). Samples showing inconsistent results between DNA and RNA analysis were analysed by reverse line blot [131].

Main Results

- HPV DNA and RNA analysis agreed in 77% of cases for detection of HPV of any type.

- All type mismatches that were re-analysed confirmed the HPV type as identified by RT-NASBA.

- The DNA assay reached a maximum sensitivity of 76.9% with a panel of four HPV types, while the RNA assay reached 80.8% sensitivity using three HPV types.

- E6/E7 mRNA analysis through RT-NASBA is feasible in FFPE and more accurate than DNA genotyping for HPV classification.

Discussion

Correct HPV classification is important not only for identification of cervical adenocarcinoma patients in a screening setting, but also for research. Since HPV DNA integration is seen during development of invasive cancer, HPV infections may be missed when analysing samples with probes for episomal DNA [145]. This study proved the feasibility of analysing HPV mRNA in stored FFPE biopsies. The PreTect RT-NASBA assay used in the present study has since then also been evaluated in other studies, showing higher specificity than DNA-based tests [146, 147].

Weaknesses in this study were the small number of patients, the fact that not all samples yielded enough DNA for re-analysis and the absence of an uninfected control group, so that specificity values could be obtained.

It should be noted that while presence of HPV DNA by itself is not proof of an HPV-driven carcinoma or pre-stages of cancer, the expression of HPV E6/E7 is a much stronger indication. Thus, when possible, E6/E7 RNA analysis of potentially HPV-driven tumours is preferable. Validation of an E6/E7 mRNA assay for cervical adenocarcinoma is important, since it helps to accurately identify patients in need of diagnostic biopsies, while also letting low-risk patients avoid invasive tests.

4.2 PAPER II

Background and Aims

In the Western world, HPV-positive oropharyngeal cancer, where BOTSCC and TSCC account for the majority of cases, has been steadily increasing during the last decades. The prognosis is much better than for HPV-negative OSCC – 80% vs 40% 3-year disease-free survival. OSCC treatment has recently become more intensive, with chemoradiotherapy and anti-EGFR antibodies, leading to more side-effects. Since HPV-positive OSCC patients with absent HLA class I expression or a high number of $CD8^+$ TILs have 95-100% survival with conventional radiotherapy alone, they can probably receive milder treatment, limiting side-effects [87]. The aim of this study was to see whether E2, the absence of which is associated with decreased survival in cervical cancer [148] and E5 expression, which has been shown to down-regulate MHC class I in cell lines [67] are associated with survival, $CD8^+$ TIL counts or MHC class I expression in HPV-positive BOTSCC and TSCC, with

the goal of further improving identification of patients with good prognosis who can be selected for milder treatment.

Materials and Methods

FFPE biopsies from 112 TSCC and 21 BOTSCC patients, curatively treated with three years of follow-up, were analysed. All patients had previously been considered positive for HPV16 DNA, and stained for MHC class I (HC-10) expression and $CD8^+$ TIL infiltration. RNA extraction with DNAse treatment was followed by cDNA synthesis and multiplex PCR with E2, E5, E7, U1A and β-globin probes – the latter two for quality control. RNA expression could successfully be analysed in 127 patients. Patients were defined as HPV16-positive if E7 mRNA was expressed.

Main Results

- 10 HPV16 DNA-positive tumours did not express E7. Patients with E7-negative tumours had significantly worse survival and fewer $CD8^+$ TILs than those with E7-expressing tumours, but E7 was not associated with HLA class I expression.

- Neither E2 nor E5 mRNA expression were significantly associated with HLA class I expression or $CD8^+$ TILs.

- Absence of E2 expression was associated with significantly decreased disease- and progression-free survival.

Discussion

Absence of HPV E2 expression has previously been shown to be a poor prognostic factor for cervical cancer, but this study is the first to show that it is true in head and neck cancer as well. Disruption of the E2 gene is considered as an indication of HPV integration into the host genome, and has been show to increase transcription of E6 and E7 by removal of E2-mediated inhibition of these genes [72]. The role of E5 in down-regulating MHC class I, seen in cell lines, was however not confirmed in our study. MHC class I down-regulation has been associated with poor prognosis in cervical cancer [149], while the opposite is true in HPV positive OSCC [91], and we showed that this paradoxical effect in OSCC is not dependent on E2, E5 or E7 expression.

The fact that neither E2 nor E5 were associated with MHC class I expression or $CD8^+$ TIL counts, both indicating good prognosis, while absence of E2 was associated with decreased disease-free survival means that E2 expression is an independent predictor of survival,

which can be combined with MHC class I expression and $CD8^+$ TILs to increase the accuracy in stratifying patients with good prognosis to milder treatment.

The correlation between E2 and clinical outcome was evaluated for 117 E7-expressing TSCC and BOTSCC patients. To be able to use HPV16 E2-expression as a prognostic marker in a clinical setting there is a need to confirm the data obtained in this study on a separate validation set. In addition, as mentioned above, E2 expression as often been regarded as an indication that the E2 is disrupted due to integration of the HPV genome into the cellular genome [150]. However, this is not necessarily so, as there may be deletions also in episomal HPV genomes [151] and E2 expression is not only repressed due to deletions also be repressed by methylation [152]. Notably, E2 methylation has been found to be highest in OSCC with integrated HPV genomes where the E2 gene is intact, intermediate in OSCC with episomal HPV and low when the HPV genome is integrated and E2 disrupted [152]. Further studies are needed to evaluate if the variation in E2 expression observed in TSCC/BOTSCC in the present study is related to integration and/or methylation. Also, the mechanisms behind TIL infiltration and down-regulation of HLA class I were not explained by our study, and remain to be explored.

4.3 PAPER III

Background and Aims

Tumour heterogeneity contributes to resistance development, which is why targeted treatments are rarely curative. Combinatorial treatments of several targeted drugs address this problem by simultaneously targeting multiple signalling pathways, decreasing the risk of resistance development through mutational re-wiring of signalling networks [10]. Treatment combinations may however interact unpredictably, producing unexpected toxicities [153] or having antagonistic effects [154]. Taking the complexity of tumour signalling networks, heterogeneity and cross-talk with the microenvironment into account also means that the effect of a combination will vary even within the same patient, depending on the signalling state of individual tumour sites.

Oestrogen, EGF and TGF-β signalling are all driver pathways in breast carcinogenesis which cross-talk with each other [155-157]. ER and TGF-β signalling are mutually inhibitory. ER is mitogenic in early breast cancer, while TGF-β, growth-inhibitory in early

cancer, drives EMT and immunosuppression in later stages [158]. EGF contributes to hormone-independent growth, angiogenesis and invasion [159].

The aim of this study was to screen breast cancer cells in different states of tumour development with different combinations of oestrogen, EGF and TGF-β and targeted treatments against these pathways in order to find response patterns predicting which combinations to use based on the tumour signalling state.

Materials and Methods

The ER-negative, metastatic, mesenchymal like MDA-MB-231 and ER-positive, epithelial, non-metastatic MCF7 breast cancer cell lines were treated with a total of 90 combinations of drugs and growth factors (EGF, 17-β-oestradiol, TGF-β1, SB431542, Tamoxifen, Iressa) for 48 hours. Proliferation was measured by MTT assay. Published transcriptome studies were re-analysed to validate cell line observations.

Main Results

- TGF-β1 inhibited proliferation of MDA-MB-231 cells treated with EGF and oestrogen.

- Tamoxifen increased MDA-MB-231 proliferation, but addition of EGF and TGF-β to Tamoxifen decreased proliferation.

- In MCF7 cells, combinations of Tamoxifen, EGF and oestradiol inhibited proliferation regardless of TGF-β.

- EGF together with Iressa had a stimulatory effect, blocked by TGFβ, in MDA-MB-231 cells.

- Combining all three drugs yielded the same patterns as not adding drugs, suggesting antagonistic interactions.

Discussion

The purpose of the study was to try out combinations of targeted drugs in an environment replicating tumour heterogeneity through usage of different breast cancer cell lines treated with growth factor combinations. Certain of our findings, notably the inhibitory combination of Tamoxifen, EGF and TGFβ, was in line with reports from earlier studies [160], confirming the validity of our screening approach.

The cross-talk between the TGFβ and ER signalling pathways was further investigated in a follow-up study, by proteome profiling of cells treated with SB431542 and Tamoxifen, and it was established that the pathways interact through BMP signalling (unpublished results).

A notable weakness in this study is the absence of taking into account dynamic effects, with proliferation only studied at one time-point. Earlier experiments optimising the assay had showed that 48 h was a good compromise treatment time, especially since oestrogen signalling takes a long time to affect proliferation. Without automation, screenings such as in the present study have utility in generating hypotheses to be tested in-depth, but primary screening results are less useful.

In the future, such screening approaches, creating a two-dimensional search space of drugs against signalling states with functional characteristics, such as proliferation or invasiveness as outputs, can be combined with in silico models in an iterative manner, with the model predicting useful combinations to be screened, and screening results used to improve the model [161].

4.4 PAPER IV

Background and Aims

MIBC is a disease with five-year survival around 50%, with somewhat better prognosis in responders to neoadjuvant cisplatin-containing chemotherapy, which, however, is associated with heavy side-effects. Targeted treatments have thus far been unsuccessful. The high rate of mutations [162], decreasing the efficacy of targeted treatments but increasing the chance of there being tumour-specific antigens, makes adoptive immunotherapy an attractive treatment option. However, immunosuppressive mechanisms must be overcome for this to be effective. MIBC is known to spread through the lymph nodes [110], where an immunosuppressive environment is established in a pre-metastatic before actual metastasis. The aim of this study was to perform proteome profiling of Tregs and Teffs in SN and nSN in order to see whether this method can be used to identify individual differences in T-cell signalling between patients and lymph nodes that can shed light on immunosuppressive mechanisms in the lymph nodes and be exploited to develop treatments that optimise the effects of adoptive T-cell transfer.

Materials and Methods

Tregs and Teffs from peripheral blood, SN and nSN of two patients were sorted by FACS and analysed by LC-MS/MS-based proteomics. Network analysis was performed on protein interaction networks formed by the identified proteins. Validation experiments using FACS and RT-PCR were performed using cells from two other patients.

Main Results

- Tregs in SN displayed up-regulated growth and immune signalling pathways.

- IL-16 is a central protein in SN-Treg signalling.

- Secreted factors from the tumour down-regulate IL-16 mRNA expression.

- Immunoproteomics is a useful tool to reveal important differences in signalling between patients and between lymph nodes in the same patient.

Discussion

The safety and feasibility of adoptive T-cell immunotherapy using expanded lymphocytes from sentinel nodes has been demonstrated in urinary bladder cancer, with a pilot study showing objective responses according to RECIST criteria followed by improved overall survival [163]. It has already been shown that the presence of Tregs in blood decreases the efficacy of adoptive T-cell transfer as cancer treatment [164], and the presence of Tregs among TILs in urinary bladder cancer has been associated with poor prognosis [165]. Considering that we propose to use TILs from the sentinel nodes for adoptive transfer, it is important to map the function of Tregs in these sites in order to find approaches to diminish their suppressive effects.

This is, to our knowledge, the first study to profile the proteome of Tregs in lymph nodes. Considering that only two patients were profiled, the study is rather a proof of concept. Another weakness is the fact that the suppressive capabilities of the extracted Tregs could not be assessed, since all sorted cells were needed for proteomics and validation experiments.

However, we did find SN-Treg growth and immune signalling to be up-regulated in both patients. Also, IL-16, which has previously been shown to be elevated in sera of cancer patients [166], was predicted to be central to SN-Treg signalling. We performed validation experiments in two other patients and showed that IL-16 is indeed expressed in Tregs,

something that has not been shown before. We also showed that it is more highly expressed in lymph node Tregs than in peripheral blood, and that co-culturing with tumour supernatant down-regulates IL-16 expression.

This is an especially interesting finding, considering that IL-16 has a dual role. The secreted C-terminal part of the protein acts as a chemoattractant, inducing T-cell migration, while the N-terminal part is internalised and works as a cell cycle inhibitor in the nucleus [167].

Further studies will focus on the mechanisms and impact of IL-16 down-regulation and on applying this immunoproteomic approach in a larger group of patients, trying to find and validate biomarkers which can be used as prognostic markers of immunotherapy success or as targets to decrease immunosuppression.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In Paper I, we showed that mRNA detection through RT-NASBA is more accurate and sensitive than DNA genotyping for detecting and properly classifying HPV infection in cervical adenocarcinoma. This method has since been used successfully in other studies on cervical carcinoma. Seeing as RT-NASBA can be multiplexed, an obvious future direction is development of an assay which adds probes for other biomarkers of prognosis and treatment [168], improving diagnostic accuracy and treatment selection.

Paper II applied HPV mRNA analysis to head and neck cancer, showing that absence of E2 expression is an independent predictor of poor survival. This will be combined with data on HLA class I expression and $CD8^+$ TIL counts, other independent predictors of survival, to select patients for milder treatment. The accuracy of such a three-variable model should be validated in a larger dataset. Other directions are continued studies on the role of E5 in relation to other biomarkers and clinical outcome in oropharyngeal cancer, which was not clarified by our study and the mechanisms behind HLA class I down-regulation.

Paper III studied breast cancer through a systems medicine approach, where a search space of drugs and growth factors was created with proliferation as output. This project generated hypotheses, which are being followed up, that mechanistic studies of TGF-β-oestrogen cross-talk could provide new targets for treatment of breast cancer, and also showed how a simple screening approach can identify unexpectedly successful treatment combinations.

Paper IV combined proteomics, systems biology and immunology for a clinical study of urinary bladder cancer. The identification of up-regulated growth and immune signalling in SN-Tregs, with IL-16 as a cytokine central to SN-Treg signalling was interesting, and further studies will focus on the mechanistic nature of IL-16 in Tregs, as well as on expanding this pilot study to a larger cohort, hopefully arriving at biomarkers helping to individually tailor adoptive immunotherapy.

In conclusion, this thesis has applied a wide range of methodologies contributing to efficiently stratify patients and predict treatment combinations and target biomarkers in different cancer settings. Integration of data generated by these approaches with clinical outcomes and modelling will greatly advance the road towards personalised cancer medicine.

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7 REFERENCES

[1] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, *et al.*, "Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012," *Int J Cancer,* vol. 136, pp. E359-86, Mar 2015.

[2] F. Bray, A. Jemal, N. Grey, J. Ferlay, and D. Forman, "Global cancer transitions according to the Human Development Index (2008-2030): a population-based study," *Lancet Oncol,* vol. 13, pp. 790-801, Aug 2012.

[3] C. Coghlin and G. I. Murray, "Current and emerging concepts in tumour metastasis," *J Pathol,* vol. 222, pp. 1-15, Sep 2010.

[4] H. Rajagopalan, M. A. Nowak, B. Vogelstein, and C. Lengauer, "The significance of unstable chromosomes in colorectal cancer," *Nat Rev Cancer,* vol. 3, pp. 695-701, Sep 2003.

[5] C. E. Meacham and S. J. Morrison, "Tumour heterogeneity and cancer cell plasticity," *Nature,* vol. 501, pp. 328-37, Sep 2013.

[6] J. Wang, Y. Zuo, Y. G. Man, I. Avital, A. Stojadinovic, M. Liu, *et al.*, "Pathway and network approaches for identification of cancer signature markers from omics data," *J Cancer,* vol. 6, pp. 54-65, 2015.

[7] I. A. Cree, "Designing personalised cancer treatments," *J Control Release,* Jul 16 2013.

[8] J. N. Blattman and P. D. Greenberg, "Cancer immunotherapy: a treatment for the masses," *Science,* vol. 305, pp. 200-5, Jul 2004.

[9] Q. Tian, N. D. Price, and L. Hood, "Systems cancer medicine: towards realization of predictive, preventive, personalized and participatory (P4) medicine," *J Intern Med,* vol. 271, pp. 111-21, Feb 2012.

[10] B. Al-Lazikani, U. Banerji, and P. Workman, "Combinatorial drug therapy for cancer in the post-genomic era," *Nat Biotechnol,* vol. 30, pp. 679-92, Jul 2012.

[11] H. Hennings, A. B. Glick, D. A. Greenhalgh, D. L. Morgan, J. E. Strickland, T. Tennenbaum, *et al.*, "Critical aspects of initiation, promotion, and progression in multistage epidermal carcinogenesis," *Proc Soc Exp Biol Med,* vol. 202, pp. 1-8, Jan 1993.

[12] E. R. Fearon and B. Vogelstein, "A genetic model for colorectal tumorigenesis," *Cell,* vol. 61, pp. 759-67, Jun 1990.

[13] K. R. Cho and B. Vogelstein, "Genetic alterations in the adenoma--carcinoma sequence," *Cancer,* vol. 70, pp. 1727-31, Sep 1992.

[14] J. S. Bertram, "The molecular biology of cancer," *Mol Aspects Med,* vol. 21, pp. 167-223, Dec 2000.

[15] D. P. Cahill, K. W. Kinzler, B. Vogelstein, and C. Lengauer, "Genetic instability and darwinian selection in tumours," *Trends Cell Biol,* vol. 9, pp. M57-60, Dec 1999.

[16] D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: the next generation," *Cell,* vol. 144, pp. 646-74, Mar 4 2011.

[17] D. Mahalingam, K. Sankhala, A. Mita, F. J. Giles, and M. M. Mita, "Targeting the mTOR pathway using deforolimus in cancer therapy," *Future Oncol,* vol. 5, pp. 291-303, Apr 2009.

[18] E. Witsch, M. Sela, and Y. Yarden, "Roles for growth factors in cancer progression," *Physiology (Bethesda),* vol. 25, pp. 85-101, Apr 2010.

[19] N. A. Bhowmick, E. G. Neilson, and H. L. Moses, "Stromal fibroblasts in cancer initiation and progression," *Nature,* vol. 432, pp. 332-7, Nov 2004.

[20] C. J. Sherr and F. McCormick, "The RB and p53 pathways in cancer," *Cancer Cell,* vol. 2, pp. 103-12, Aug 2002.

[21] J. M. Adams and S. Cory, "The Bcl-2 apoptotic switch in cancer development and therapy," *Oncogene,* vol. 26, pp. 1324-37, Feb 2007.

[22] J. W. Shay and W. E. Wright, "Hayflick, his limit, and cellular ageing," *Nat Rev Mol Cell Biol,* vol. 1, pp. 72-6, Oct 2000.

[23] Z. Wang, C. Dabrosin, X. Yin, M. M. Fuster, A. Arreola, W. K. Rathmell, *et al.*, "Broad targeting of angiogenesis for cancer prevention and therapy," *Semin Cancer Biol,* Jan 2015.

[24] R. A. Cairns, I. S. Harris, and T. W. Mak, "Regulation of cancer cell metabolism," *Nat Rev Cancer,* vol. 11, pp. 85-95, Feb 2011.

[25] W. Du, P. Jiang, A. Mancuso, A. Stonestrom, M. D. Brewer, A. J. Minn, *et al.*, "TAp73 enhances the pentose phosphate pathway and supports cell proliferation," *Nat Cell Biol,* vol. 15, pp. 991-1000, Aug 2013.

[26] P. Sonveaux, F. Végran, T. Schroeder, M. C. Wergin, J. Verrax, Z. N. Rabbani, *et al.*, "Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice," *J Clin Invest,* vol. 118, pp. 3930-42, Dec 2008.

[27] E. P. Spugnini, P. Sonveaux, C. Stock, M. Perez-Sayans, A. De Milito, S. Avnet, *et al.*, "Proton channels and exchangers in cancer," *Biochim Biophys Acta,* vol. 1848, pp. 2715-26, Oct 2015.

[28] A. De Milito and S. Fais, "Tumor acidity, chemoresistance and proton pump inhibitors," *Future Oncol,* vol. 1, pp. 779-86, Dec 2005.

[29] J. E. Talmadge and I. J. Fidler, "AACR centennial series: the biology of cancer metastasis: historical perspective," *Cancer Res,* vol. 70, pp. 5649-69, Jul 2010.

[30] J. Sceneay, M. J. Smyth, and A. Möller, "The pre-metastatic niche: finding common ground," *Cancer Metastasis Rev,* vol. 32, pp. 449-64, Dec 2013.

[31] H. Son and A. Moon, "Epithelial-mesenchymal Transition and Cell Invasion," *Toxicol Res,* vol. 26, pp. 245-52, Dec 2010.

[32] J. T. Price and E. W. Thompson, "Mechanisms of tumour invasion and metastasis: emerging targets for therapy," *Expert Opin Ther Targets,* vol. 6, pp. 217-33, Apr 2002.

[33] S. A. Martin, M. Hewish, C. J. Lord, and A. Ashworth, "Genomic instability and the selection of treatments for cancer," *J Pathol,* vol. 220, pp. 281-9, Jan 2010.

[34] S. L. Thompson, S. F. Bakhoum, and D. A. Compton, "Mechanisms of chromosomal instability," *Curr Biol,* vol. 20, pp. R285-95, Mar 2010.

[35] D. J. Gordon, B. Resio, and D. Pellman, "Causes and consequences of aneuploidy in cancer," *Nat Rev Genet,* vol. 13, pp. 189-203, Mar 2012.

[36] R. A. Burrell, N. McGranahan, J. Bartek, and C. Swanton, "The causes and consequences of genetic heterogeneity in cancer evolution," *Nature,* vol. 501, pp. 338-45, Sep 2013.

[37] D. B. Longley and P. G. Johnston, "Molecular mechanisms of drug resistance," *J Pathol,* vol. 205, pp. 275-92, Jan 2005.

[38] B. Vogelstein, N. Papadopoulos, V. E. Velculescu, S. Zhou, L. A. Diaz, and K. W. Kinzler, "Cancer genome landscapes," *Science,* vol. 339, pp. 1546-58, Mar 2013.

[39] A. Sottoriva, I. Spiteri, S. G. Piccirillo, A. Touloumis, V. P. Collins, J. C. Marioni, *et al.,* "Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics," *Proc Natl Acad Sci U S A,* vol. 110, pp. 4009-14, Mar 2013.

[40] M. Gerlinger, A. J. Rowan, S. Horswell, J. Larkin, D. Endesfelder, E. Gronroos, *et al.,* "Intratumor heterogeneity and branched evolution revealed by multiregion sequencing," *N Engl J Med,* vol. 366, pp. 883-92, Mar 2012.

[41] M. R. Junttila and F. J. de Sauvage, "Influence of tumour micro-environment heterogeneity on therapeutic response," *Nature,* vol. 501, pp. 346-54, Sep 2013.

[42] L. Ding, T. J. Ley, D. E. Larson, C. A. Miller, D. C. Koboldt, J. S. Welch, *et al.,* "Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing," *Nature,* vol. 481, pp. 506-10, Jan 2012.

[43] S. I. Grivennikov, F. R. Greten, and M. Karin, "Immunity, inflammation, and cancer," *Cell,* vol. 140, pp. 883-99, Mar 2010.

[44] S. M. Crusz and F. R. Balkwill, "Inflammation and cancer: advances and new agents," *Nat Rev Clin Oncol,* Jun 2015.

[45] L. Apetoh, A. Tesniere, F. Ghiringhelli, G. Kroemer, and L. Zitvogel, "Molecular interactions between dying tumor cells and the innate immune system determine the efficacy of conventional anticancer therapies," *Cancer Res,* vol. 68, pp. 4026-30, Jun 2008.

[46] A. Gutierrez-Dalmau and J. M. Campistol, "Immunosuppressive therapy and malignancy in organ transplant recipients: a systematic review," *Drugs,* vol. 67, pp. 1167-98, 2007.

[47] F. Piras, R. Colombari, L. Minerba, D. Murtas, C. Floris, C. Maxia, *et al.,* "The predictive value of CD8, CD4, CD68, and human leukocyte antigen-D-related cells in the prognosis of cutaneous malignant melanoma with vertical growth phase," *Cancer,* vol. 104, pp. 1246-54, Sep 2005.

[48] P. Sharma, Y. Shen, S. Wen, S. Yamada, A. A. Jungbluth, S. Gnjatic, *et al.,* "CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma," *Proc Natl Acad Sci U S A,* vol. 104, pp. 3967-72, Mar 2007.

[49] C. Nordfors, N. Grün, N. Tertipis, A. Ährlund-Richter, L. Haeggblom, L. Sivars, *et al.,* "CD8+ and CD4+ tumour infiltrating lymphocytes in relation to human papillomavirus status and clinical outcome in tonsillar and base of tongue squamous cell carcinoma," *Eur J Cancer,* vol. 49, pp. 2522-30, Jul 2013.

[50] R. Kim, M. Emi, and K. Tanabe, "Cancer immunoediting from immune surveillance to immune escape," *Immunology,* vol. 121, pp. 1-14, May 2007.

[51] D. S. Vinay, E. P. Ryan, G. Pawelec, W. H. Talib, J. Stagg, E. Elkord, *et al.*, "Immune evasion in cancer: Mechanistic basis and therapeutic strategies," *Semin Cancer Biol,* Mar 2015.

[52] D. O. Adeegbe and H. Nishikawa, "Regulatory T cells in cancer; can they be controlled?," *Immunotherapy,* vol. 7, pp. 843-6, Aug 2015.

[53] P. Sharma and J. P. Allison, "The future of immune checkpoint therapy," *Science,* vol. 348, pp. 56-61, Apr 2015.

[54] P. W. Kantoff, C. S. Higano, N. D. Shore, E. R. Berger, E. J. Small, D. F. Penson, *et al.*, "Sipuleucel-T immunotherapy for castration-resistant prostate cancer," *N Engl J Med,* vol. 363, pp. 411-22, Jul 2010.

[55] P. Kebriaei, S. S. Kelly, P. Manuri, B. Jena, R. Jackson, E. Shpall, *et al.*, "Chimeric antibody receptors (CARs): driving T-cell specificity to enhance anti-tumor immunity," *Front Biosci (Schol Ed),* vol. 4, pp. 520-31, 2012.

[56] M. L. Cheng and L. Fong, "Beyond sipuleucel-T: immune approaches to treating prostate cancer," *Curr Treat Options Oncol,* vol. 15, pp. 115-26, Mar 2014.

[57] D. Mavroudis and J. Barrett, "The graft-versus-leukemia effect," *Curr Opin Hematol,* vol. 3, pp. 423-9, Nov 1996.

[58] N. P. Restifo, M. E. Dudley, and S. A. Rosenberg, "Adoptive immunotherapy for cancer: harnessing the T cell response," *Nat Rev Immunol,* vol. 12, pp. 269-81, Apr 2012.

[59] P. A. Antony, C. A. Piccirillo, A. Akpinarli, S. E. Finkelstein, P. J. Speiss, D. R. Surman, *et al.*, "CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells," *J Immunol,* vol. 174, pp. 2591-601, Mar 2005.

[60] L. Gattinoni, D. J. Powell, S. A. Rosenberg, and N. P. Restifo, "Adoptive immunotherapy for cancer: building on success," *Nat Rev Immunol,* vol. 6, pp. 383-93, May 2006.

[61] R. Ghittoni, R. Accardi, S. Chiocca, and M. Tommasino, "Role of human papillomaviruses in carcinogenesis," *Ecancermedicalscience,* vol. 9, p. 526, 2015.

[62] G. K. Chew, M. E. Cruickshank, P. H. Rooney, I. D. Miller, D. E. Parkin, and G. I. Murray, "Human papillomavirus 16 infection in adenocarcinoma of the cervix," *Br J Cancer,* vol. 93, pp. 1301-4, Nov 2005.

[63] C. M. D'Abramo and J. Archambault, "Small molecule inhibitors of human papillomavirus protein - protein interactions," *Open Virol J,* vol. 5, pp. 80-95, 2011.

[64] J. Doorbar, "The papillomavirus life cycle," *J Clin Virol,* vol. 32 Suppl 1, pp. S7-15, Mar 2005.

[65] C. J. Meijer, P. J. Snijders, and A. J. van den Brule, "Screening for cervical cancer: should we test for infection with high-risk HPV?," *CMAJ,* vol. 163, pp. 535-8, Sep 2000.

[66] A. G. Ostör, "Natural history of cervical intraepithelial neoplasia: a critical review," *Int J Gynecol Pathol,* vol. 12, pp. 186-92, Apr 1993.

[67] G. H. Ashrafi, M. R. Haghshenas, B. Marchetti, P. M. O'Brien, and M. S. Campo, "E5 protein of human papillomavirus type 16 selectively downregulates surface HLA class I," *Int J Cancer,* vol. 113, pp. 276-83, Jan 2005.

[68] P. M. O'Brien and M. S. Campo, "Papillomaviruses: a correlation between immune evasion and oncogenicity?," *Trends Microbiol,* vol. 11, pp. 300-5, Jul 2003.

[69] P. Barnard, E. Payne, and N. A. McMillan, "The human papillomavirus E7 protein is able to inhibit the antiviral and anti-growth functions of interferon-alpha," *Virology,* vol. 277, pp. 411-9, Nov 2000.

[70] K. Münger, A. Baldwin, K. M. Edwards, H. Hayakawa, C. L. Nguyen, M. Owens, *et al.*, "Mechanisms of human papillomavirus-induced oncogenesis," *J Virol,* vol. 78, pp. 11451-60, Nov 2004.

[71] S. H. Um, N. Mundi, J. Yoo, D. A. Palma, K. Fung, D. MacNeil, *et al.*, "Variable expression of the forgotten oncogene E5 in HPV-positive oropharyngeal cancer," *J Clin Virol,* vol. 61, pp. 94-100, Sep 2014.

[72] K. Akagi, J. Li, T. R. Broutian, H. Padilla-Nash, W. Xiao, B. Jiang, *et al.*, "Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability," *Genome Res,* vol. 24, pp. 185-99, Feb 2014.

[73] N. Kash, M. A. Lee, R. Kollipara, C. Downing, J. Guidry, and S. K. Tyring, "Safety and Efficacy Data on Vaccines and Immunization to Human Papillomavirus," *J Clin Med,* vol. 4, pp. 614-33, 2015.

[74] M. J. Welters, G. G. Kenter, P. J. de Vos van Steenwijk, M. J. Löwik, D. M. Berends-van der Meer, F. Essahsah, *et al.*, "Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses," *Proc Natl Acad Sci U S A,* vol. 107, pp. 11895-9, Jun 2010.

[75] J. I. Sin, J. M. Kim, S. H. Bae, I. H. Lee, J. S. Park, and H. M. Ryoo, "Adoptive transfer of human papillomavirus E7-specific CTL enhances tumor chemoresponse through the perforin/granzyme-mediated pathway," *Mol Ther,* vol. 17, pp. 906-13, May 2009.

[76] S. E. Waggoner, "Cervical cancer," *Lancet,* vol. 361, pp. 2217-25, Jun 2003.

[77] A. Goodman, "HPV testing as a screen for cervical cancer," *BMJ,* vol. 350, p. h2372, 2015.

[78] L. S. Krill and K. S. Tewari, "Integration of bevacizumab with chemotherapy doublets for advanced cervical cancer," *Expert Opin Pharmacother,* vol. 16, pp. 675-83, Apr 2015.

[79] J. Ferlay, H. R. Shin, F. Bray, D. Forman, C. Mathers, and D. M. Parkin, "Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008," *Int J Cancer,* vol. 127, pp. 2893-917, Dec 2010.

[80] G. D'Souza, K. Cullen, J. Bowie, R. Thorpe, and C. Fakhry, "Differences in oral sexual behaviors by gender, age, and race explain observed differences in prevalence of oral human papillomavirus infection," *PLoS One,* vol. 9, p. e86023, 2014.

[81] C. H. Chung, A. Bagheri, and G. D'Souza, "Epidemiology of oral human papillomavirus infection," *Oral Oncol,* vol. 50, pp. 364-9, May 2014.

[82] A. Näsman, C. Nordfors, S. Holzhauser, A. Vlastos, N. Tertipis, U. Hammar, *et al.*, "Incidence of human papillomavirus positive tonsillar and base of tongue carcinoma:

a stabilisation of an epidemic of viral induced carcinoma?," *Eur J Cancer,* vol. 51, pp. 55-61, Jan 2015.

[83] L. Marklund, A. Näsman, T. Ramqvist, T. Dalianis, E. Munck-Wikland, and L. Hammarstedt, "Prevalence of human papillomavirus and survival in oropharyngeal cancer other than tonsil or base of tongue cancer," *Cancer Med,* vol. 1, pp. 82-8, Aug 2012.

[84] A. K. Chaturvedi, W. F. Anderson, J. Lortet-Tieulent, M. P. Curado, J. Ferlay, S. Franceschi, *et al.*, "Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers," *J Clin Oncol,* vol. 31, pp. 4550-9, Dec 2013.

[85] A. Näsman, P. Attner, L. Hammarstedt, J. Du, M. Eriksson, G. Giraud, *et al.*, "Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma?," *Int J Cancer,* vol. 125, pp. 362-6, Jul 2009.

[86] D. Lindquist, M. Romanitan, L. Hammarstedt, A. Näsman, H. Dahlstrand, J. Lindholm, *et al.*, "Human papillomavirus is a favourable prognostic factor in tonsillar cancer and its oncogenic role is supported by the expression of E6 and E7," *Mol Oncol,* vol. 1, pp. 350-5, Dec 2007.

[87] T. Dalianis, "Human papillomavirus and oropharyngeal cancer, the epidemics, and significance of additional clinical biomarkers for prediction of response to therapy (Review)," *Int J Oncol,* vol. 44, pp. 1799-805, Jun 2014.

[88] D. L. Miller, M. D. Puricelli, and M. S. Stack, "Virology and molecular pathogenesis of HPV (human papillomavirus)-associated oropharyngeal squamous cell carcinoma," *Biochem J,* vol. 443, pp. 339-53, Apr 2012.

[89] A. Näsman, M. Romanitan, C. Nordfors, N. Grün, H. Johansson, L. Hammarstedt, *et al.*, "Tumor infiltrating CD8+ and Foxp3+ lymphocytes correlate to clinical outcome and human papillomavirus (HPV) status in tonsillar cancer," *PLoS One,* vol. 7, p. e38711, 2012.

[90] A. Näsman, E. Andersson, C. Nordfors, N. Grün, H. Johansson, E. Munck-Wikland, *et al.*, "MHC class I expression in HPV positive and negative tonsillar squamous cell carcinoma in correlation to clinical outcome," *Int J Cancer,* vol. 132, pp. 72-81, Jan 2013.

[91] A. Näsman, E. Andersson, L. Marklund, N. Tertipis, L. Hammarstedt-Nordenvall, P. Attner, *et al.*, "HLA class I and II expression in oropharyngeal squamous cell carcinoma in relation to tumor HPV status and clinical outcome," *PLoS One,* vol. 8, p. e77025, 2013.

[92] Y. Monnier and C. Simon, "Surgery Versus Radiotherapy for Early Oropharyngeal Tumors: a Never-Ending Debate," *Curr Treat Options Oncol,* vol. 16, p. 362, Sep 2015.

[93] C. E. DeSantis, F. Bray, J. Ferlay, J. Lortet-Tieulent, B. O. Anderson, and A. Jemal, "International Variation in Female Breast Cancer Incidence and Mortality Rates," *Cancer Epidemiol Biomarkers Prev,* Sep 2015.

[94] I. T. Croghan, S. Pruthi, J. T. Hays, S. Cha, R. E. Johnson, M. Kosel, *et al.*, "The role of smoking in breast cancer development: an analysis of a Mayo Clinic cohort," *Breast J,* vol. 15, pp. 489-95, 2009 Sep-Oct 2009.

[95] T. Anothaisintawee, C. Wiratkapun, P. Lerdsitthichai, V. Kasamesup, S. Wongwaisayawan, J. Srinakarin, *et al.*, "Risk factors of breast cancer: a systematic review and meta-analysis," *Asia Pac J Public Health,* vol. 25, pp. 368-87, Sep 2013.

[96] T. Pal and S. T. Vadaparampil, "Genetic risk assessments in individuals at high risk for inherited breast cancer in the breast oncology care setting," *Cancer Control,* vol. 19, pp. 255-66, Oct 2012.

[97] L. Chen, H. M. Linden, B. O. Anderson, and C. I. Li, "Trends in 5-year survival rates among breast cancer patients by hormone receptor status and stage," *Breast Cancer Res Treat,* vol. 147, pp. 609-16, Oct 2014.

[98] J. Peppercorn, "Breast cancer in women under 40," *Oncology (Williston Park),* vol. 23, pp. 465-74, May 2009.

[99] C. B. Matsen and L. A. Neumayer, "Breast Cancer: A Review for the General Surgeon," *JAMA Surg,* Aug 28 2013.

[100] B. Weigelt, F. C. Geyer, and J. S. Reis-Filho, "Histological types of breast cancer: how special are they?," *Mol Oncol,* vol. 4, pp. 192-208, Jun 2010.

[101] P. Wirapati, C. Sotiriou, S. Kunkel, P. Farmer, S. Pradervand, B. Haibe-Kains, *et al.*, "Meta-analysis of gene expression profiles in breast cancer: toward a unified understanding of breast cancer subtyping and prognosis signatures," *Breast Cancer Res,* vol. 10, p. R65, 2008.

[102] J. Specht and J. R. Gralow, "Neoadjuvant chemotherapy for locally advanced breast cancer," *Semin Radiat Oncol,* vol. 19, pp. 222-8, Oct 2009.

[103] E. A. Perez, E. H. Romond, V. J. Suman, J. H. Jeong, G. Sledge, C. E. Geyer, *et al.*, "Trastuzumab plus adjuvant chemotherapy for human epidermal growth factor receptor 2-positive breast cancer: planned joint analysis of overall survival from NSABP B-31 and NCCTG N9831," *J Clin Oncol,* vol. 32, pp. 3744-52, Nov 2014.

[104] A. D. Wagner, C. Thomssen, J. Haerting, and S. Unverzagt, "Vascular-endothelial-growth-factor (VEGF) targeting therapies for endocrine refractory or resistant metastatic breast cancer," *Cochrane Database Syst Rev,* vol. 7, p. CD008941, 2012.

[105] S. Di Cosimo and J. Baselga, "Targeted therapies in breast cancer: where are we now?," *Eur J Cancer,* vol. 44, pp. 2781-90, Dec 2008.

[106] M. A. Knowles and C. D. Hurst, "Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity," *Nat Rev Cancer,* vol. 15, pp. 25-41, Jan 2015.

[107] G. Dalbagni, "The management of superficial bladder cancer," *Nat Clin Pract Urol,* vol. 4, pp. 254-60, May 2007.

[108] A. Stenzl, N. C. Cowan, M. De Santis, M. A. Kuczyk, A. S. Merseburger, M. J. Ribal, *et al.*, "Treatment of muscle-invasive and metastatic bladder cancer: update of the EAU guidelines," *Eur Urol,* vol. 59, pp. 1009-18, Jun 2011.

[109] C. D. Morrison, P. Liu, A. Woloszynska-Read, J. Zhang, W. Luo, M. Qin, *et al.*, "Whole-genome sequencing identifies genomic heterogeneity at a nucleotide and chromosomal level in bladder cancer," *Proc Natl Acad Sci U S A,* vol. 111, pp. E672-81, Feb 2014.

[110] A. Wallmeroth, U. Wagner, H. Moch, T. C. Gasser, G. Sauter, and M. J. Mihatsch, "Patterns of metastasis in muscle-invasive bladder cancer (pT2-4): An autopsy study on 367 patients," *Urol Int,* vol. 62, pp. 69-75, 1999.

[111] J. Mendelsohn, "Personalizing oncology: perspectives and prospects," *J Clin Oncol,* vol. 31, pp. 1904-11, May 2013.

[112] J. Casado-Vela, A. Cebrián, M. T. Gómez del Pulgar, and J. C. Lacal, "Approaches for the study of cancer: towards the integration of genomics, proteomics and metabolomics," *Clin Transl Oncol,* vol. 13, pp. 617-28, Sep 2011.

[113] L. Sastre, "New DNA sequencing technologies open a promising era for cancer research and treatment," *Clin Transl Oncol,* vol. 13, pp. 301-6, May 2011.

[114] R. M. Branca, L. M. Orre, H. J. Johansson, V. Granholm, M. Huss, Å. Pérez-Bercoff, *et al.*, "HiRIEF LC-MS enables deep proteome coverage and unbiased proteogenomics," *Nat Methods,* vol. 11, pp. 59-62, Jan 2014.

[115] K. A. Vermeersch and M. P. Styczynski, "Applications of metabolomics in cancer research," *J Carcinog,* vol. 12, p. 9, 2013.

[116] J. P. Garay and J. W. Gray, "Omics and therapy - a basis for precision medicine," *Mol Oncol,* vol. 6, pp. 128-39, Apr 2012.

[117] S. G. O'Brien, F. Guilhot, R. A. Larson, I. Gathmann, M. Baccarani, F. Cervantes, *et al.*, "Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia," *N Engl J Med,* vol. 348, pp. 994-1004, Mar 2003.

[118] P. C. Fong, D. S. Boss, T. A. Yap, A. Tutt, P. Wu, M. Mergui-Roelvink, *et al.*, "Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers," *N Engl J Med,* vol. 361, pp. 123-34, Jul 2009.

[119] C. A. Hudis, "Trastuzumab--mechanism of action and use in clinical practice," *N Engl J Med,* vol. 357, pp. 39-51, Jul 2007.

[120] R. Iyer and A. Bharthuar, "A review of erlotinib--an oral, selective epidermal growth factor receptor tyrosine kinase inhibitor," *Expert Opin Pharmacother,* vol. 11, pp. 311-20, Feb 2010.

[121] A. G. Sacco and E. E. Cohen, "Current Treatment Options for Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma," *J Clin Oncol,* Sep 2015.

[122] S. Bagrodia, T. Smeal, and R. T. Abraham, "Mechanisms of intrinsic and acquired resistance to kinase-targeted therapies," *Pigment Cell Melanoma Res,* vol. 25, pp. 819-31, Nov 2012.

[123] B. N. Rexer and C. L. Arteaga, "Intrinsic and acquired resistance to HER2-targeted therapies in HER2 gene-amplified breast cancer: mechanisms and clinical implications," *Crit Rev Oncog,* vol. 17, pp. 1-16, 2012.

[124] G. Tortora, R. Bianco, G. Daniele, F. Ciardiello, J. A. McCubrey, M. R. Ricciardi, *et al.*, "Overcoming resistance to molecularly targeted anticancer therapies: Rational drug combinations based on EGFR and MAPK inhibition for solid tumours and haematologic malignancies," *Drug Resist Updat,* vol. 10, pp. 81-100, Jun 2007.

[125] J. G. Greger, S. D. Eastman, V. Zhang, M. R. Bleam, A. M. Hughes, K. N. Smitheman, *et al.*, "Combinations of BRAF, MEK, and PI3K/mTOR inhibitors overcome acquired resistance to the BRAF inhibitor GSK2118436 dabrafenib,

mediated by NRAS or MEK mutations," *Mol Cancer Ther*, vol. 11, pp. 909-20, Apr 2012.

[126] W. Du and O. Elemento, "Cancer systems biology: embracing complexity to develop better anticancer therapeutic strategies," *Oncogene*, vol. 34, pp. 3215-25, Jun 2015.

[127] D. Faratian, A. Goltsov, G. Lebedeva, A. Sorokin, S. Moodie, P. Mullen, *et al.*, "Systems biology reveals new strategies for personalizing cancer medicine and confirms the role of PTEN in resistance to trastuzumab," *Cancer Res*, vol. 69, pp. 6713-20, Aug 2009.

[128] K. Leder, K. Pitter, Q. Laplant, D. Hambardzumyan, B. D. Ross, T. A. Chan, *et al.*, "Mathematical modeling of PDGF-driven glioblastoma reveals optimized radiation dosing schedules," *Cell*, vol. 156, pp. 603-16, Jan 2014.

[129] M. Niepel, M. Hafner, E. A. Pace, M. Chung, D. H. Chai, L. Zhou, *et al.*, "Profiles of Basal and stimulated receptor signaling networks predict drug response in breast cancer lines," *Sci Signal*, vol. 6, p. ra84, Sep 2013.

[130] S. Andersson, B. Larson, A. Hjerpe, C. Silfverswärd, J. Sällström, E. Wilander, *et al.*, "Adenocarcinoma of the uterine cervix: the presence of human papillomavirus and the method of detection," *Acta Obstet Gynecol Scand*, vol. 82, pp. 960-5, Oct 2003.

[131] A. J. van den Brule, R. Pol, N. Fransen-Daalmeijer, L. M. Schouls, C. J. Meijer, and P. J. Snijders, "GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes," *J Clin Microbiol*, vol. 40, pp. 779-87, Mar 2002.

[132] L. Hammarstedt, D. Lindquist, H. Dahlstrand, M. Romanitan, L. O. Dahlgren, J. Joneberg, *et al.*, "Human papillomavirus as a risk factor for the increase in incidence of tonsillar cancer," *Int J Cancer*, vol. 119, pp. 2620-3, Dec 2006.

[133] Y. Lyutvinskiy, H. Yang, D. Rutishauser, and R. A. Zubarev, "In silico instrumental response correction improves precision of label-free proteomics and accuracy of proteomics-based predictive models," *Mol Cell Proteomics*, vol. 12, pp. 2324-31, Aug 2013.

[134] J. Cox and M. Mann, "Quantitative, high-resolution proteomics for data-driven systems biology," *Annu Rev Biochem*, vol. 80, pp. 273-99, 2011.

[135] A. Paz, Z. Brownstein, Y. Ber, S. Bialik, E. David, D. Sagir, *et al.*, "SPIKE: a database of highly curated human signaling pathways," *Nucleic Acids Res*, vol. 39, pp. D793-9, Jan 2011.

[136] G. Su, J. H. Morris, B. Demchak, and G. D. Bader, "Biological network exploration with cytoscape 3," *Curr Protoc Bioinformatics*, vol. 47, pp. 8.13.1-8.13.24, 2014.

[137] J. H. Morris, L. Apeltsin, A. M. Newman, J. Baumbach, T. Wittkop, G. Su, *et al.*, "clusterMaker: a multi-algorithm clustering plugin for Cytoscape," *BMC Bioinformatics*, vol. 12, p. 436, 2011.

[138] G. Scardoni, M. Petterlini, and C. Laudanna, "Analyzing biological network parameters with CentiScaPe," *Bioinformatics*, vol. 25, pp. 2857-9, Nov 1 2009.

[139] C. Winterhalter, P. Widera, and N. Krasnogor, "JEPETTO: a Cytoscape plugin for gene set enrichment and topological analysis based on interaction networks," *Bioinformatics*, vol. 30, pp. 1029-30, Apr 2014.

[140] A. Franceschini, D. Szklarczyk, S. Frankild, M. Kuhn, M. Simonovic, A. Roth, *et al.*, "STRING v9.1: protein-protein interaction networks, with increased coverage and integration," *Nucleic Acids Res*, vol. 41, pp. D808-15, Jan 2013.

[141] F. Bray, B. Carstensen, H. Møller, M. Zappa, M. P. Zakelj, G. Lawrence, *et al.*, "Incidence trends of adenocarcinoma of the cervix in 13 European countries," *Cancer Epidemiol Biomarkers Prev*, vol. 14, pp. 2191-9, Sep 2005.

[142] N. L. Williams, T. L. Werner, E. A. Jarboe, and D. K. Gaffney, "Adenocarcinoma of the cervix: should we treat it differently?," *Curr Oncol Rep*, vol. 17, p. 17, Apr 2015.

[143] A. Berrington de González, J. Green, and I. C. o. E. S. o. C. Cancer, "Comparison of risk factors for invasive squamous cell carcinoma and adenocarcinoma of the cervix: collaborative reanalysis of individual data on 8,097 women with squamous cell carcinoma and 1,374 women with adenocarcinoma from 12 epidemiological studies," *Int J Cancer*, vol. 120, pp. 885-91, Feb 2007.

[144] X. Castellsagué, M. Díaz, S. de Sanjosé, N. Muñoz, R. Herrero, S. Franceschi, *et al.*, "Worldwide human papillomavirus etiology of cervical adenocarcinoma and its cofactors: implications for screening and prevention," *J Natl Cancer Inst*, vol. 98, pp. 303-15, Mar 2006.

[145] J. S. Park, E. S. Hwang, S. N. Park, H. K. Ahn, S. J. Um, C. J. Kim, *et al.*, "Physical status and expression of HPV genes in cervical cancers," *Gynecol Oncol*, vol. 65, pp. 121-9, Apr 1997.

[146] S. W. Sørbye, S. Fismen, T. J. Gutteberg, E. S. Mortensen, and F. E. Skjeldestad, "HPV mRNA is more specific than HPV DNA in triage of women with minor cervical lesions," *PLoS One*, vol. 9, p. e112934, 2014.

[147] H. Keegan, J. Mc Inerney, L. Pilkington, P. Grønn, I. Silva, F. Karlsen, *et al.*, "Comparison of HPV detection technologies: Hybrid capture 2, PreTect HPV-Proofer and analysis of HPV DNA viral load in HPV16, HPV18 and HPV33 E6/E7 mRNA positive specimens," *J Virol Methods*, vol. 155, pp. 61-6, Jan 2009.

[148] S. D. Vernon, E. R. Unger, D. L. Miller, D. R. Lee, and W. C. Reeves, "Association of human papillomavirus type 16 integration in the E2 gene with poor disease-free survival from cervical cancer," *Int J Cancer*, vol. 74, pp. 50-6, Feb 1997.

[149] E. S. Jordanova, A. Gorter, O. Ayachi, F. Prins, L. G. Durrant, G. G. Kenter, *et al.*, "Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients?," *Clin Cancer Res*, vol. 14, pp. 2028-35, Apr 2008.

[150] P. Peitsaro, B. Johansson, and S. Syrjänen, "Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique," *J Clin Microbiol*, vol. 40, pp. 886-91, Mar 2002.

[151] H. Mellin, L. Dahlgren, E. Munck-Wikland, J. Lindholm, H. Rabbani, M. Kalantari, *et al.*, "Human papillomavirus type 16 is episomal and a high viral load may be correlated to better prognosis in tonsillar cancer," *Int J Cancer*, vol. 102, pp. 152-8, Nov 2002.

[152] M. Reuschenbach, C. U. Huebbers, E. S. Prigge, J. L. Bermejo, M. S. Kalteis, S. F. Preuss, *et al.*, "Methylation status of HPV16 E2-binding sites classifies subtypes of HPV-associated oropharyngeal cancers," *Cancer*, vol. 121, pp. 1966-76, Jun 2015.

[153] S. R. Park, M. Davis, J. H. Doroshow, and S. Kummar, "Safety and feasibility of targeted agent combinations in solid tumours," *Nat Rev Clin Oncol,* vol. 10, pp. 154-68, Mar 2013.

[154] J. Rodon, J. Perez, and R. Kurzrock, "Combining targeted therapies: practical issues to consider at the bench and bedside," *Oncologist,* vol. 15, pp. 37-50, 2010.

[155] C. K. Osborne, J. Shou, S. Massarweh, and R. Schiff, "Crosstalk between estrogen receptor and growth factor receptor pathways as a cause for endocrine therapy resistance in breast cancer," *Clin Cancer Res,* vol. 11, pp. 865s-70s, Jan 15 2005.

[156] Y. Ren, L. Wu, A. R. Frost, W. Grizzle, X. Cao, and M. Wan, "Dual effects of TGF-beta on ERalpha-mediated estrogenic transcriptional activity in breast cancer," *Mol Cancer,* vol. 8, p. 111, 2009.

[157] M. Kang, S. Choi, S. J. Jeong, S. A. Lee, T. K. Kwak, H. Kim, *et al.*, "Cross-talk between TGFbeta1 and EGFR signalling pathways induces TM4SF5 expression and epithelial-mesenchymal transition," *Biochem J,* vol. 443, pp. 691-700, May 1 2012.

[158] A. M. Band and M. Laiho, "Crosstalk of TGF-β and estrogen receptor signaling in breast cancer," *J Mammary Gland Biol Neoplasia,* vol. 16, pp. 109-15, Jun 2011.

[159] H. Masuda, D. Zhang, C. Bartholomeusz, H. Doihara, G. N. Hortobagyi, and N. T. Ueno, "Role of epidermal growth factor receptor in breast cancer," *Breast Cancer Res Treat,* vol. 136, pp. 331-45, Nov 2012.

[160] M. B. Lyng, A. V. Laenkholm, Q. Tan, W. Vach, K. H. Gravgaard, A. Knoop, *et al.*, "Gene expression signatures that predict outcome of tamoxifen-treated estrogen receptor-positive, high-risk, primary breast cancer patients: a DBCG study," *PLoS One,* vol. 8, p. e54078, 2013.

[161] X. M. Zhao, M. Iskar, G. Zeller, M. Kuhn, V. van Noort, and P. Bork, "Prediction of drug combinations by integrating molecular and pharmacological data," *PLoS Comput Biol,* vol. 7, p. e1002323, Dec 2011.

[162] C. G. A. R. Network, "Comprehensive molecular characterization of urothelial bladder carcinoma," *Nature,* vol. 507, pp. 315-22, Mar 2014.

[163] A. Sherif, M. N. Hasan, E. Radecka, A. L. Rodriguez, S. Shabo, M. Karlsson, *et al.*, "Pilot study of adoptive immunotherapy with sentinel node-derived T cells in muscle-invasive urinary bladder cancer," *Scand J Urol,* pp. 1-10, Jul 2015.

[164] X. Yao, M. Ahmadzadeh, Y. C. Lu, D. J. Liewehr, M. E. Dudley, F. Liu, *et al.*, "Levels of peripheral CD4(+)FoxP3(+) regulatory T cells are negatively associated with clinical response to adoptive immunotherapy of human cancer," *Blood,* vol. 119, pp. 5688-96, Jun 2012.

[165] M. E. Winerdal, P. Marits, M. Winerdal, M. Hasan, R. Rosenblatt, A. Tolf, *et al.*, "FOXP3 and survival in urinary bladder cancer," *BJU Int,* vol. 108, pp. 1672-8, Nov 2011.

[166] E. Kovacs, "The serum levels of IL-12 and IL-16 in cancer patients. Relation to the tumour stage and previous therapy," *Biomed Pharmacother,* vol. 55, pp. 111-6, Mar 2001.

[167] J. Richmond, M. Tuzova, W. Cruikshank, and D. Center, "Regulation of cellular processes by interleukin-16 in homeostasis and cancer," *J Cell Physiol,* vol. 229, pp. 139-47, Feb 2014.

[168] S. Dasari, R. Wudayagiri, and L. Valluru, "Cervical cancer: Biomarkers for diagnosis and treatment," *Clin Chim Acta*, vol. 445, pp. 7-11, May 2015.