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Molecular biomarkers related to DNA damage and repair:

their role in carcinogenesis, patients' treatment and monitoring

Molekulární biomarkery související s poškozením a opravou DNA:

jejich role v procesu karcinogeneze, léčbě a monitorování pacientů

Dissertation Thesis

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Identifikační záznam

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List of Abbreviations

5-FU	5-fluorouracil
AC(s)	Aberrant cell(s)
AFP	Alpha-fetoprotein
aOR(s)	Adjusted OR(s)
BER	Base excision repair
CA (125 or 15-3)	Cancer antigen (125 or 15-3)
CA(s)	Chromosomal aberration(s)
CART	Classification & regression tree
CAtot	Total chromosomal aberrations
CEA	Carcinoembryonic antigen
CI(s)	Confidence interval(s)
CSA(s)	Chromosome-type aberration(s)
CTA(s)	Chromatid-type aberration(s)
DDA	DNA damaging agents
DDR	DNA damage response
DRC	DNA repair capacity
DSB(s)	Double-strand break(s)
EFS	Event-free survival
FA	Fanconi anaemia
GWAS	Genome-wide association study
HCG	Human chorionic gonadotropin
HR	Hazard ratio
HR*	Homologous recombination
IF	Impact factor
IR	Ionizing radiation
MMR	Mismatch repair
MSA	Mutagen sensitivity assay
MSI	Microsatellite instability
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
OR(s)	Odds ratio(s)

OS	Overall survival
PBL(s)	Peripheral blood lymphocyte(s)
PSA	Prostate-specific antigen
RFS	Recurrence-free survival
r_s	Spearman's rho
RTL	Relative telomere length
SB(s)	Strand break(s)
SNP(s)	Single nucleotide polymorphism(s)
SSB(s)	Single-strand break(s)
TL	Telomere length
TNM	Tumour-node-metastasis
TRP	Transient receptor potential
UV	Ultraviolet

Summary

Genome instability represents one of the leading forces driving the onset and development of cancer. It arises as a consequence of the combined effect of DNA damage and errors made by the DNA repair system. In many cancers, DNA damage tolerance and DNA repair pathways are disrupted or deregulated, thereby promoting cancer progression. DNA repair also appears to play a substantial role in cancer therapy response. This Dissertation Thesis was performed in response to several unclear and unresolved issues of the role of DNA damage and DNA repair in cancer pathogenesis.

The aim of the Thesis was to search for potential novel biomarkers and confirmation of the validity of already existing biomarkers related to DNA damage and DNA repair, which may be associated with cancer susceptibility and patient's clinical outcome. We also explored the biological basis of different biomarkers and their associations.

The major outcomes of this Thesis are: **1)** The elevated chromosomal aberrations (CAs) in peripheral blood lymphocytes (PBLs) may serve as a biomarker of cancer susceptibility and partially affects patients' clinical outcome. While telomere shortening contributes to the formation of CAs in PBLs only in healthy individuals, less efficient DNA double-strand break repair in PBLs is associated with telomere shortening only in cancer patients. **2)** Several genetic variants in DNA repair genes and their gene-gene interactions have been discovered that modulated the levels of CAs in PBLs. In genome-wide associations studies, several new genetic variants associated with CA frequency in PBLs were also indicated. **3)** The associations of several genetic variants in DNA repair genes with cancer susceptibility and patient's clinical outcome have been identified. The importance of studying DNA repair at a functional level, directly in tumour and non-malignant tissue, has been pointed out to reveal its potential predictive and prognostic value.

In conclusion, this Dissertation Thesis suggested and/or verified several potential candidate biomarkers associated with cancer susceptibility and patients' clinical outcome for further use in population monitoring and clinical use. However, additional studies on larger independent populations and performing functional tests are needed to replicate our findings and unravel the biological mechanisms behind.

Shrnutí

Nestabilita genomu představuje jednu z předních sil, která řídí vznik a rozvoj nádorového onemocnění. Vzniká v důsledku kombinovaného účinku poškození DNA a chyb způsobených opravným systémem DNA. V mnoha nádorech jsou tolerance k poškození DNA a opravné dráhy DNA narušeny nebo deregulovány, což podporuje jejich progresi. Oprava DNA také hraje významnou roli v odpovědi na léčbu nádorových onemocnění. Tato disertační práce vznikla v reakci na několik nejasných a nevyřešených otázek úlohy poškození DNA a opravy DNA v patogenezi nádorových onemocnění.

Cílem práce bylo hledání potenciálních nových biomarkerů a potvrzení platnosti již existujících biomarkerů souvisejících s poškozením DNA a opravou DNA, které mohou být spojeny s náchylností ke vzniku nádorových onemocnění a klinickým výsledkem pacienta. Také byl zkoumán biologický základ různých biomarkerů a jejich vzájemné vztahy.

Hlavní výstupy této práce jsou: **1)** Zvýšené hladiny chromozomálních aberací (CA) v lymfocytech periferní krve (PBL) mohou sloužit jako biomarker náchylnosti ke vzniku nádorových onemocnění a částečně ovlivňují klinický výsledek pacientů. Zatímco zkracování telomer přispívá k tvorbě CA v PBL pouze u zdravých jedinců, méně účinná oprava dvouřetězcových zlomů DNA v PBL je spojena se zkrácením telomer pouze u pacientů s nádorovým onemocněním. **2)** Bylo objeveno několik genetických variant v genech zapojených do opravy DNA a jejich vzájemné interakce, které ovlivňovaly hladiny CA v PBL. Celogenomové asociační studie také naznačily několik nových variant spojených s frekvencí CA v PBL. **3)** U několika genetických variant v genech pro opravu DNA byl identifikován jejich vztah s náchylností ke vzniku nádorových onemocnění a klinickým výsledkem pacienta. Zároveň byla zdůrazněna důležitost studia opravy DNA na funkční úrovni, a to přímo v nádorové a přilehlé nenádorové tkáni za účelem odhalení její potenciální prediktivní a prognostické hodnoty.

Závěrem, tato disertační práce navrhla a/nebo ověřila několik potenciálních kandidátních biomarkerů spojených náchylností ke vzniku nádorových onemocnění a klinickým výsledkem pacientů pro jejich další použití při monitorování populace a v klinické praxi. Pro potvrzení našich výsledků a k odhalení biologických mechanismů je však zapotřebí provést další studie na větších nezávislých populacích a potvrdit funkčními testy.

1. Introduction

This Dissertation Thesis consists of an overview of the research I have been involved in during the time of my PhD studies and which were published between 2015 and 2020. These include eight original research articles (Publications I–VII) and four review articles (Publications IX–XII) summarizing the problematics published in the original research articles. All publications, either related or unrelated to this Thesis are shown in detail in section "Publication activity". Publications I–XII *in extenso* are presented in Annexes of this Thesis.

1.1 Cancer: An overview

1.1.1 Causes of and risk factors for cancer and its development

Cancer is a general term for a large group of diseases, whose causes, characteristics and occurrence can vary. It develops as a consequence of the complex interactions between various factors, and therefore, it is commonly called as a complex (previously multifactorial) disease. These factors include lifestyle factors (alcohol consumption, tobacco smoking, poor diet, lack of physical activity, or being overweight and obese), ageing, environmental and occupational exposure (chemicals and other substances), food contaminants, exposure to ultraviolet (UV) and ionizing radiation (IR), infectious agents (some viruses and bacteria), certain hormones, chronic inflammation, immunosuppression, genetic susceptibility, and family history of cancer [1, 2] (Figure 1).

Malignant tumours are triggered and developed through the multistep process of carcinogenesis. The nature of this process is the genetic (e.g. point mutations and chromosomal rearrangements) and/or epigenetic (e.g. DNA methylation, microRNA) changes and their accumulation over time leading to malignant transformation of a normal cell into a tumour cell [3]. The primary cause of carcinogenesis is the change in crucial genes, also called as "driver" genes. These include proto-oncogenes, tumour suppressor genes, and DNA repair (mutator) genes [4].

Apart from this long-lasting dogma of gradual tumour evolution by the acquisition of genetic and/or epigenetic changes over time, a new concept of tumour formation called "chromothripsis" was formulated. This phenomenon challenged this dogma and was first

described by Stephens *et al.* in 2011 [5]. Chromothripsis is characterized by massive chromosomal rearrangements arising all at once in localised and confined genomic regions in one or a few chromosomes. If the occurrence of such an event is on the upper limit of what a cell can tolerate, the cell can withstand and survive such a destructive event [6]. Today, other unanticipated catastrophic events leading to the same or similar consequences, i.e. to sudden multiple changes in the genome, are known and the most recent reviews confer on details [7, 8].

In 2000 and a decade later, Hanahan and Weinberg published their reviews summarizing the hallmarks of cancer [9, 10]. These reviews have managed to persist at the core of literature about cancer biology, serving as blueprints for understanding the core traits of cancer. However, Lazebnik in his review article from 2010 argued that "cancer" is often used to refer to malignant tumours and a "hallmark" is a distinguishing feature [11]. He subsequently pointed out that the only true hallmark of cancer is the invasion and metastasis since other original hallmarks are characteristic for both benign and malignant growths. The revisiting the hallmarks of cancer published in 2017 concluded to organize the dense complexities of cancer biology into seven major hallmarks: i) selective growth and proliferative advantage, ii) altered stress response favouring overall survival (including DNA repair, apoptosis, autophagy, and senescence), iii) vascularization, iv) invasion and metastasis, v) metabolic rewiring, iv) abetting microenvironment, and vii) immune modulation [12] (Figure 1). Thus, it is obvious that the field of cancer biology is still evolving and despite the rapid progress in its understanding, there are still questions that should be answered in the future.
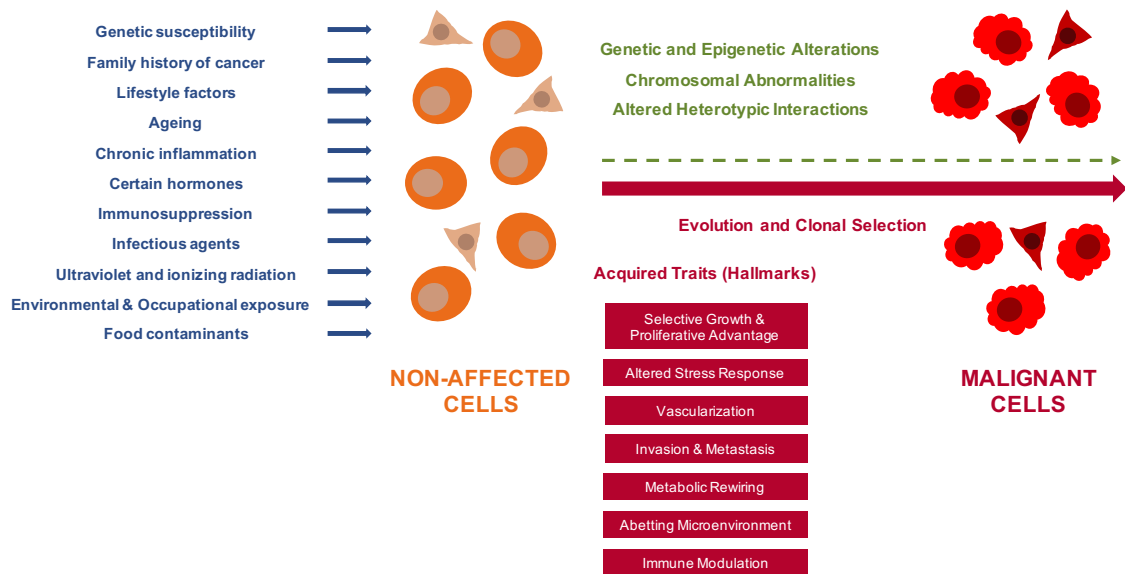
Figure 1. Causes of and risk factors for cancer and its development along with seven revisited hallmarks of cancer (modified according to [12]).

Different factors (in blue) continuously interact with cells (in orange) leading to transformative alterations in genetics/epigenetics, alterations in chromosomal numbers and structure, and heterotypic interactions (in green) which, along the pathways towards malignancy, undergo cycles of evolution and clonal selection leading to the acquisition of cancer-competent traits, so-called the hallmarks of cancer (in red).

1.1.2 Cancer incidence and mortality

Affecting almost all types of tissues in the human body, cancer represents one of the most severe health burdens in the world. According to the World Health Organization estimates, cancer causes more than 8.9 million deaths every year. With the increase in incidence by 28 % between 2006 and 2016, there were 17.2 million new cancer cases worldwide in 2016 [13, 14]. The International Agency for Research on Cancer released the New Global Cancer Data about estimates of cancer incidence and mortality, which have predicted 18.1 million new cancer cases (17.0 million excluding non-melanoma skin cancer) and 9.6 million cancer deaths (9.5 million excluding non-melanoma skin cancer) in 2018. In both sexes combined, lung cancer is the most commonly diagnosed cancer (11.6 % of the total cases) and the leading cause of cancer-related deaths (18.4 % of the total cancer deaths), closely followed by breast, colorectal, prostate, and stomach cancer for incidence, and colorectal, stomach, liver, and breast cancer for mortality [15] (Figure 2).
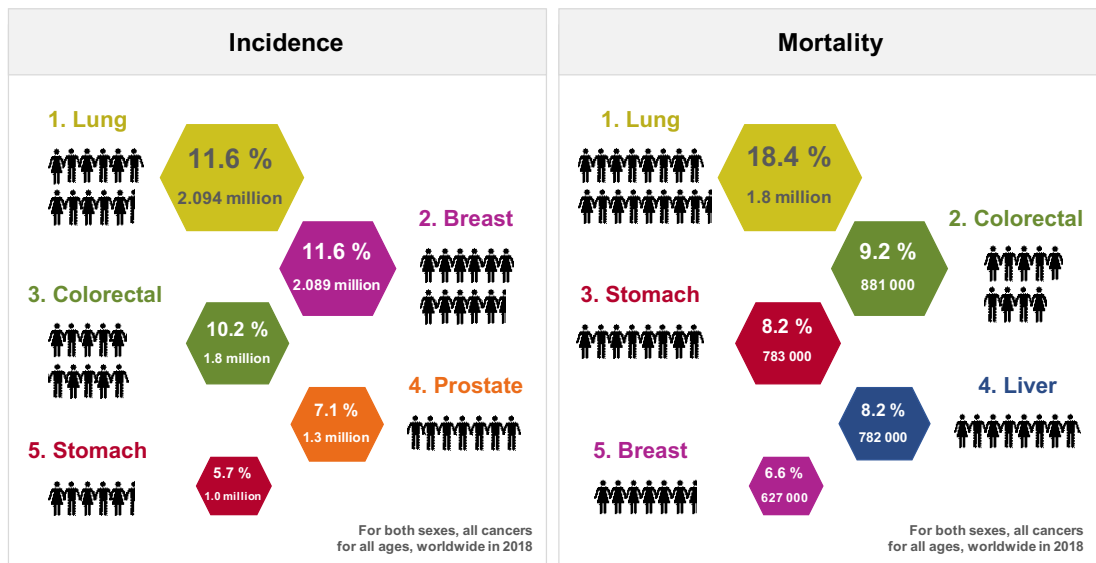
Figure 2. The most common cancer types worldwide in 2018 (modified according to [16]).

Percentages mean the proportions of individual cancer type incidence/mortality of all new cancer cases/cancer deaths.

The Czech Republic is ranking in the top 20 countries for the cumulative risk of incidence worldwide. On the contrary, due to the high quality of health care, the cumulative risk of cancer mortality decreases. As a consequence, the Czech Republic is 41st in terms of cancer mortality compared to other countries around the world [15] (Figure 3). According to the latest data published by the Institute of Health Information and Statistics of the Czech Republic on web portal www.svod.cz [17], both incidence and mortality of the three most common cancers worldwide decrease in the Czech Republic over time, except for the incidence of breast cancer (Figure 4:A1-3). When looking at the age structure of the patient populations, lung and breast cancers are most often diagnosed in individuals in the category of age between 65 and 69, while colorectal cancer incidence is highest in the age category between 70 and 74 (Figure 4:B1-3). Moreover, breast cancer is also commonly diagnosed in younger individuals aged 35 to 50 years. However, the tumour-node-metastasis (TNM) stage at which the tumour is diagnosed is critical for increasing the success rate of the treatment of cancer patients. Lung cancer is mainly diagnosed at the TNM stage IV, while the vast majority of breast cancer patients are diagnosed at early TNM stages (I+II). More than half of colorectal cancer patients are diagnosed at late TNM stages (III+IV) [17].

Incidence
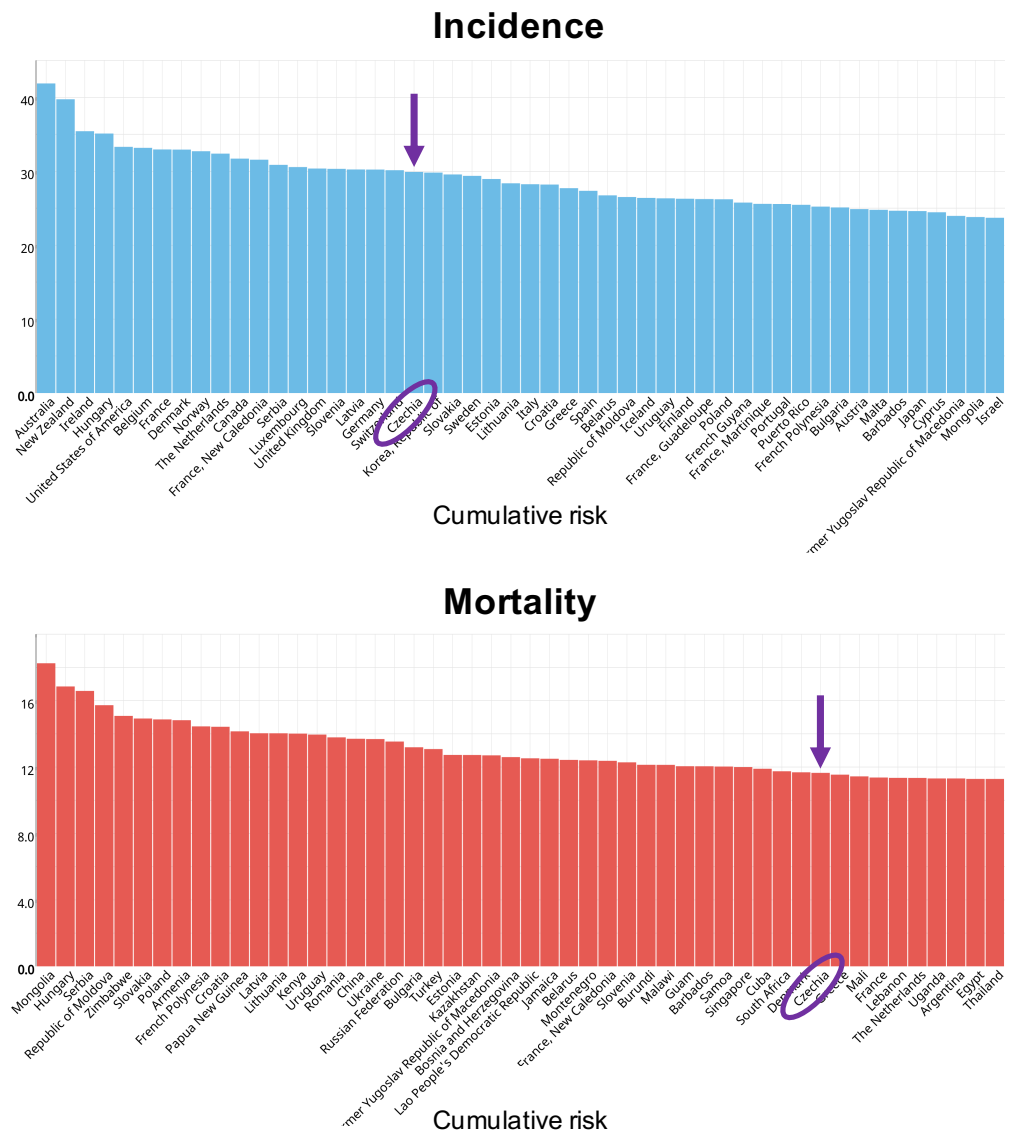
Mortality

Figure 3. The estimated cumulative risk of incidence and mortality for all cancers, both sexes, and ages 0-74 for different countries in 2018 (adopted from [18]).

Cumulative incidence/mortality is defined as the probability or risk of individuals getting/dying from the disease over a specified age-span. Cumulative risk is expressed as the number of cases/deaths per 1000 person-years that are expected to occur in a given population between the specified age limits (e.g. between birth and the age 74 years) if the cancer rates were as those observed in the specified time period in the absence of competing causes [19].
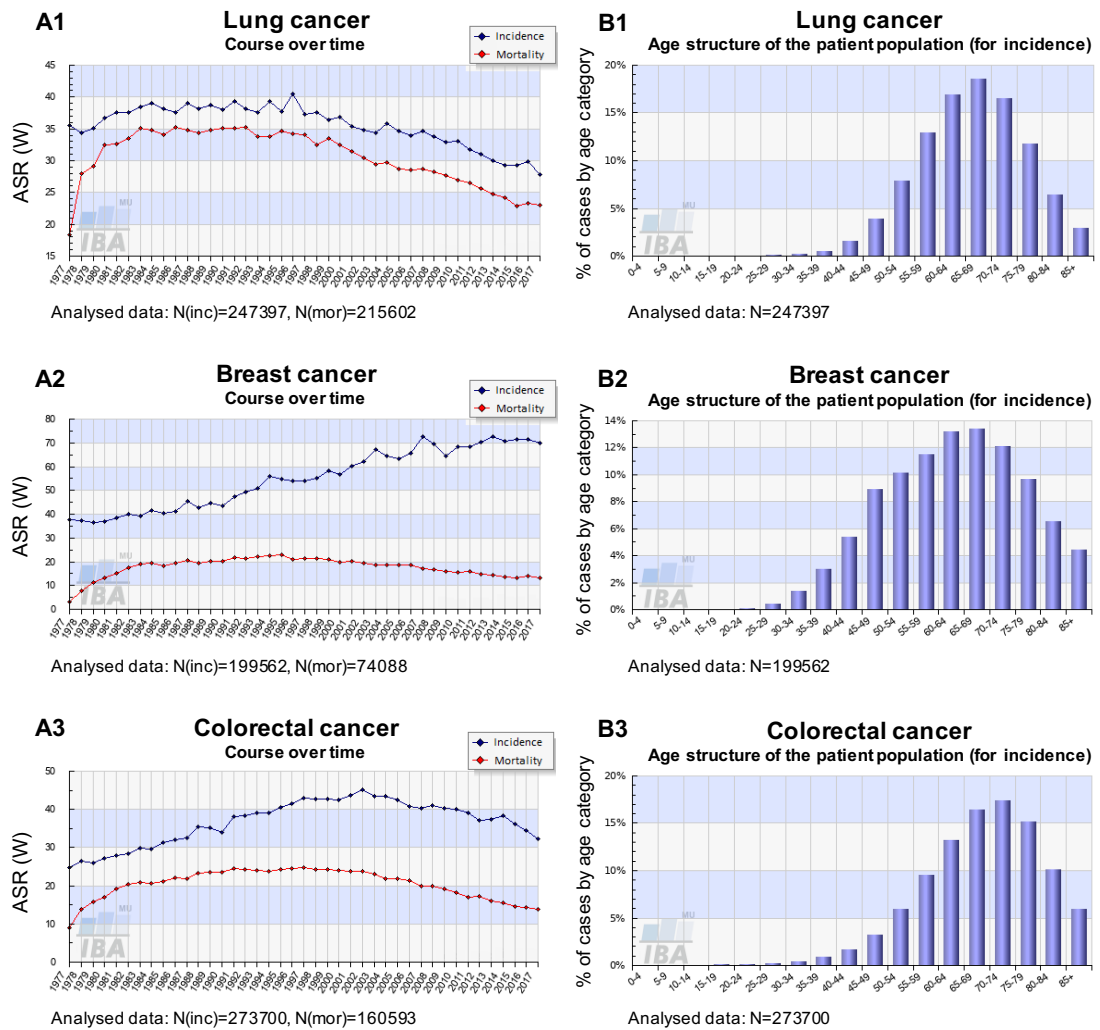
Figure 4. The incidence and mortality rates over time and the age structure of the patient populations for the three most commonly diagnosed cancers – lung (A-B1), breast (A-B2), and colorectal (A-B3) for the Czech Republic (adopted from [17]).

Figures A1-3 represent the course of incidence (blue line) and mortality (red line) over time. Figures B1-3 represent the age structure of the patient populations for incidence for individual cancer types. All data are for the Czech Republic. The ASR is a weighted mean of the age-specific rates where the weights are taken from the population distribution of a standard population; the ASR is expressed per 100,000. Comparison of rates referring to different time periods or different geographical areas is only possible after considering the differences in the age structure of the underlying populations. The age-standardisation allows the comparison of the rates that are arithmetically adjusted to have the same age structure of the standard population [19]. Abbreviations: ASR-W – age-standardised rate (conversion to world standard).

1.1.3 Cancer prevention strategies

Apart from the primary prevention which purpose is to prevent a disease from ever occurring (via limitation of risk exposure or increasing the immunity of individuals at risk of the disease), the secondary (the screening programs) and tertiary (the therapy strategies) prevention are essential as well [20].

Secondary prevention (the screening programs): An essential criterion for the treatment efficacy of all cancer types is their early diagnosis, preferably still in the pre-cancerous stages. Therefore, there is an increasing tendency across developed countries to implement screening programs in standard healthcare [21]. Unfortunately, general lung cancer screening is currently not available in the Czech Republic. However, the Czech standard healthcare provides breast cancer screening for a non-risk female population aged over 45 at two-year intervals since 2002. There are also special dispensary programs for women at very high risk of breast cancer development [22]. In 2008 and 2009, the Ministry of Health of the Czech Republic launched a nationwide screening program aimed at the early detection of cervical and colorectal cancer, respectively. Cervical cancer screening is designed for all adult women in one-year intervals [23]. Colorectal cancer screening is available for all people aged between 50 and 54 once per year (the faecal occult blood test), and for all people aged over 55 every ten years (the colonoscopy) [24].

Tertiary prevention (the therapy strategies): Once a malignant disease is diagnosed, current practice to choose and implement the therapy for cancer patients is primarily based on the tumour location, TNM stage, and results from tumour histopathological examination (tumour type). Other factors, such as patient's age and general condition, associated diseases, etc. are also taken into consideration. The treatment regimens substantially differ for lung, breast, and colorectal cancer patients as well as for other cancer types. In all cancer types, surgical removal of the tumour represents the fundamental treatment method. Therefore, if possible, surgeons always try to remove all tumour tissue mass. Other main treatment strategies include chemotherapy, radiation therapy, targeted therapy, and hormone therapy [21]. The recent success of immunotherapy strategies such as immune checkpoint blockade in several malignancies has established the role of immunotherapy in the treatment of cancer as well [25]. In

leukaemia, lymphoma and myeloma, also stem cell or bone marrow transplants are applied as a cancer treatment [26] (Figure 5).

Figure 5. Cancer treatment options (adopted from [27]).

Although all the levels of cancer prevention are significantly developing, the tools for their implementation are often less specific and less sensitive, usually invasive, and inconvenient for patients. As a consequence, tumours are often diagnosed at advanced stages of the disease, and not all patients benefit from cancer treatment and often suffer from severe adverse effects caused by therapy. Searching for new tools in the form of molecular biomarkers that can be easily collected (for instance, from blood) thus represents the most attractive approach in the era of personalized medicine. To bring a more in-depth insight into this issue, the next two chapters will focus on biomarkers and their role in cancer research and clinical practice.

1.2 Introduction to biomarkers

1.2.1 Definition of biomarkers

Biological markers (biomarkers), also called molecular markers and signature molecules [28], have been defined by Hulka *et al.* in 1990 as *"cellular, biochemical or molecular alterations that are measurable in biological media, such as human tissues, cells, or fluids"* [29]. Indeed, there are plenty of more accurate definitions of biomarkers in the literature which substantially overlap [30-32]. Nevertheless, the World Health

Organization has stated that a true definition of biomarkers includes *"almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction"* [33].

1.2.2 Study of biomarkers

Biomarkers have been used by generations of epidemiologists, physicians, and scientists to study human diseases. Until the 1990s, available technologies only allowed analysing individual proteins or genes, alternatively their very finite groups. Traditionally, biomedical research has been hypothesis-driven; investigators put forth hypotheses and design experiments to test them. Advances in laboratory techniques have given rise to more technology-driven research. Rather than putting forth a hypothesis, investigators apply high-throughput methods to biological systems and look for exciting results that could lead to hypothesis generation for further testing. Both hypothesis-driven and technology-driven approaches are applicable to biomarker discovery [34]. Despite numerous published studies on biomarkers every year, the relatively low number of those is actually used in clinical practice. There are several causes; however, they mainly reside in the biomarker's own development process [35, 36]. Specifically, consideration of methodological issues regarding the design, conduct, analysis, and interpretation of the results is fundamental to address a research question appropriately [37]. The main reasons for biomarker failures are summarized in the review by Pavlou *et al.* [38].

Because of the critical role of biomarkers at all stages of the disease, their development involves multiple processes. From initial discovery, they must undergo rigorous evaluation, including analytical validation, clinical validation, and assessment of clinical utility prior to incorporation into routine clinical care [39-41]. Concerning the properties of all mentioned types of biomarkers, an ideal biomarker should meet the following criteria: it should be reliable, highly sensitive and specific, robust, accurate, reproducible, cheap, and the biological sample for its evaluation should be easy to collect. Moreover, its metabolism should be clearly understood, it should be chemically stable, and no circadian or day to day variation should not occur for its validation [38].

Biomarkers can be measured in several human biological specimens, typically with the use of biochemical, molecular, and cytogenetic techniques. They can be detected in the circulation (whole blood, serum, or plasma) or excretions or secretions (urine, stool, saliva, ascites, cerebrospinal fluid, sputum, or nipple discharge), and thus easily assessed non-invasively and serially. Alternatively, they can be tissue-derived and require either biopsy or specialized imaging for evaluation [42, 43]. Genetic/Germline biomarkers can be inherited and detected as sequence variations in germline DNA isolated from whole blood, sputum, or buccal cells, or can be somatic, and identified as mutations in DNA derived from tumour tissue [39].

1.2.3 Types of biomarkers

Depending on the ***property***, biomarkers can be divided into two main groups: molecular and classical biomarkers. *Molecular biomarkers* are substances and biomolecules that form a genome, epigenome, transcriptome, proteome or metabolome (Figure 6). They can be further classified as nucleic acids (e.g. genes, genetic variations, mRNAs, microRNAs, or other non-coding RNAs), proteins (e.g. enzymes, receptors, and antibodies), hormones, peptides, metabolites, etc. A molecular biomarker can also represent a collection of alterations (altered structure or function), such as gene expression, and proteomic and metabolomic signatures. *Classical biomarkers* are the morphological and functional characteristics of the phenome (e.g. size, histology and grading of the tumour, presence of invasion, mitosis, metastasis, results of functional imaging examinations, age, sex, patient's comorbidity, etc.) [36, 44, 45]. From classical biomarkers, a typical example of a complex biomarker is the TNM classification [46].

In terms of ***origin***, we can divide biomarkers according to the biological levels at which the measurements were performed: cell ("cellome"), tissue ("tissueome"), organism ("organome"), and population ("populome") (Figure 6). In oncology, it is further distinguished whether the biomarker originates from the tumour (biomarkers associated with cancer/tumour, cancer/tumour biomarkers) or from its host (host-associated biomarkers) [34, 44, 47].

The most frequently used classification of biomarkers in clinical practice is based on their ***function***. These types of biomarkers describe their association with the patient and his/her disease (in detail in chapter 1.3).
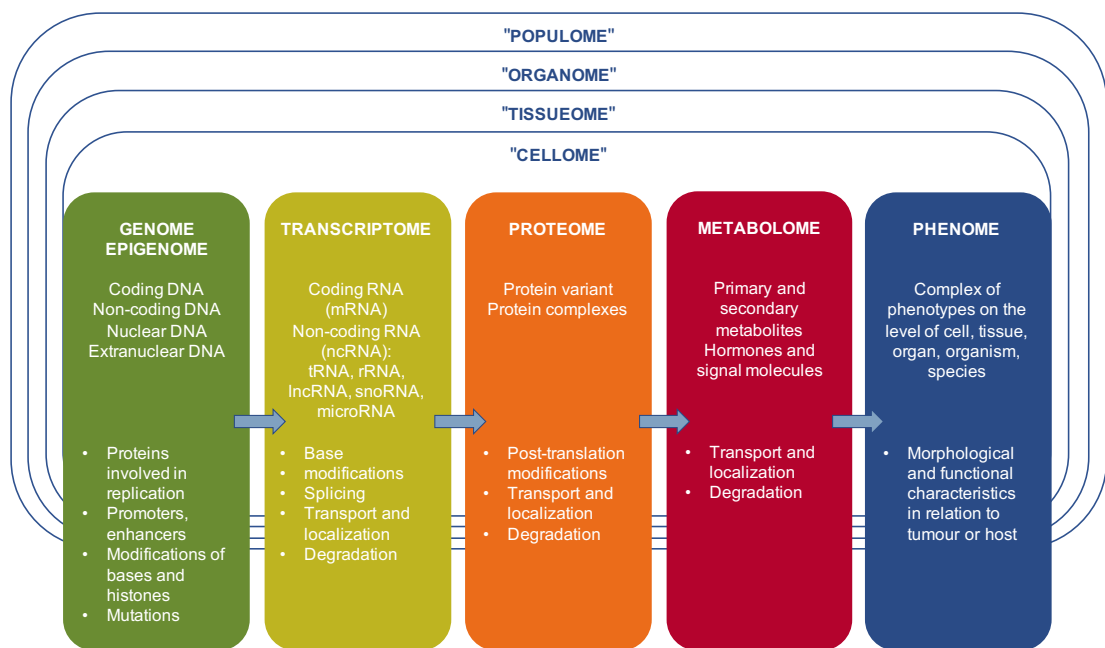
Figure 6. Biogenesis of biomarkers (modified according to [34, 47]).

1.3 Molecular cancer epidemiology

1.3.1 Implementation of molecular cancer epidemiology

High-throughput technologies have significantly led to a rapidly increasing use of molecular biomarkers as such and in epidemiological studies, i.e. in a field known as "molecular epidemiology" [48-51]. In 1982, Perera and Weinstein proposed "molecular cancer epidemiology" as a new paradigm for cancer research [52]. Based on the concept that there is a continuum between exposure to an external agent(s), its/their metabolism within the human body, and the onset of time-delayed disease (such as cancer), molecular cancer epidemiology is heading toward uncovering the "black box" of traditional epidemiology by searching for molecular biomarkers. Biomarkers can be focused on different stages of the onset and/or the development of the disease. We can distinguish three main types of biomarkers that are able to address the internal process of interaction between the external agent and the human body. These include 1) *Biomarkers of exposure/internal dose*, 2) *Biomarkers of early biological effect*, and 3) *Biomarkers of susceptibility* [53, 54] (Figure 7).

Biomarkers of susceptibility are unique since they may include all the mentioned types of biomarkers. They cover inherited and acquired genetic susceptibility, epigenetic modifications as well as alterations in physiological structures and functions induced by age, pathological conditions, and lifestyle factors, and may lead to different phenotypic manifestation [55]. Mainly inherited genetic susceptibility may play a role in influencing the individual response to exogenous exposures in a complex gene-environment interaction [56]. Therefore, understanding of which genetic variants, genotoxic changes, epigenetic profiles, and host factors may affect the susceptibility to cancer onset, progression and/or response to therapy appear to be essential to get insights into the still not well-understood exposure-disease continuum.

Biomarkers also have many valuable applications in disease detection and monitoring of the patient's health status. Therefore, the three types of biomarkers mentioned above could be supplemented with the fourth group, 4) *Biomarkers of the disease (Cancer biomarkers)*. This group is usually used, among others, in oncology in terms of clinical utility; however, it is not commonly classified as one of the main groups of biomarkers. It can be further divided into five subgroups: 4A) *Screening biomarkers*, 4B) *Diagnostic biomarkers*, 4C) *Prognostic biomarkers*, 4D) *Predictive biomarkers*, and 4E) *Biomarkers for monitoring* [43] (described in detail in the following paragraphs). All the mentioned types of biomarkers with their specific examples are summarized in Figure 7.
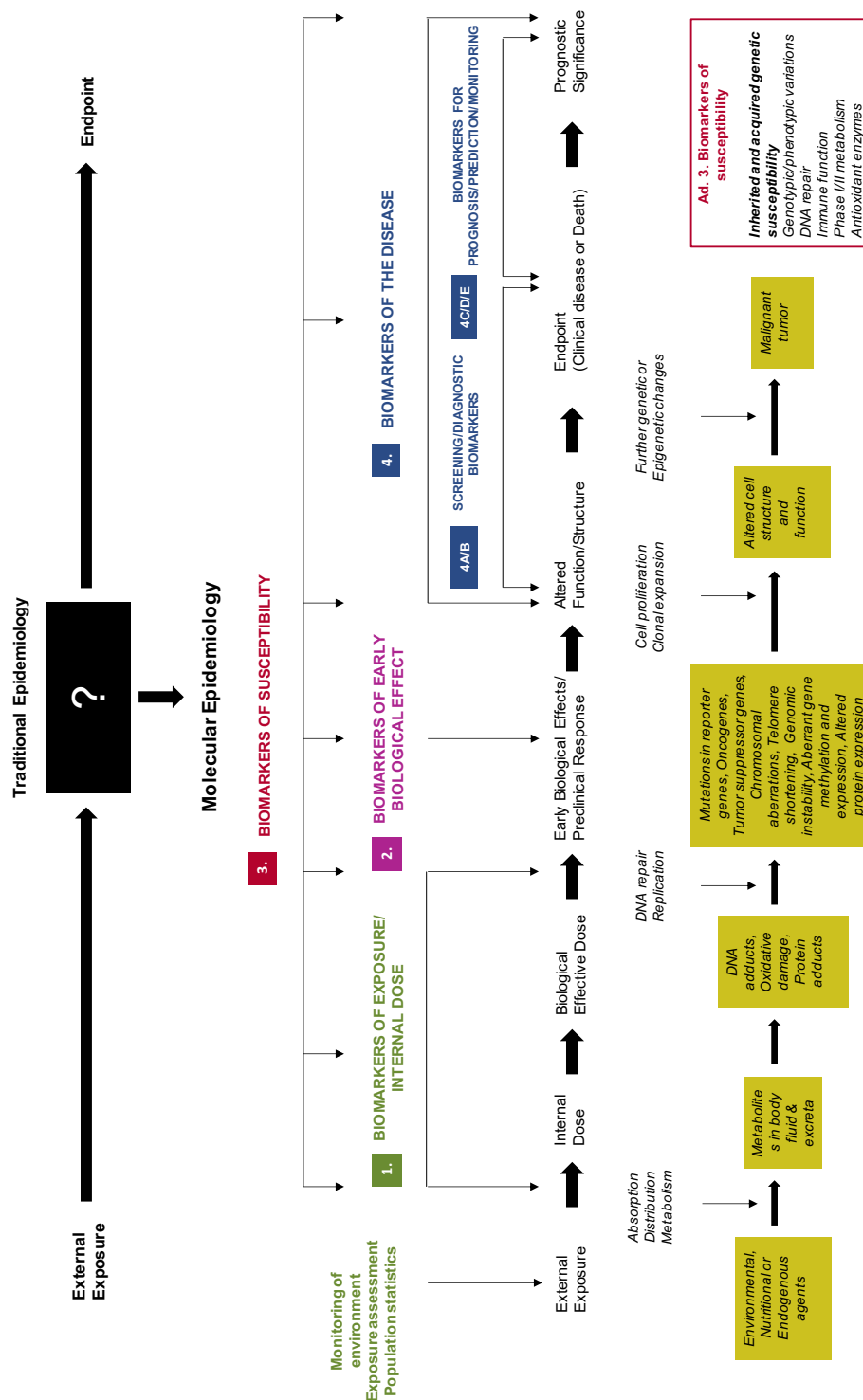
Figure 7. An overview of different types of biomarkers used in molecular cancer epidemiology (updated according to [43, 53, 57]).

Figure summarizes three main classes of biomarkers used in molecular cancer epidemiology, supplemented by the fourth group commonly used in oncology. The specific examples are described in boxes at the bottom.

1.3.2 Cancer biomarkers

Cancer (tumour) biomarkers refer to substances that are defined as *"molecules which indicate the presence of cancer or provides information about the likely future behaviour of cancer, i.e. likelihood of progression or response to therapy"* [58]. A challenge of cancer biomarker study is to translate the molecular-biological information into advances in patient's care [59]. Biomarkers can be used for cancer patient assessment in various clinical settings, including estimating risk of developing cancer (*Biomarkers of susceptibility*, also called as *Biomarkers of risk assessment*), screening for primary occult cancers (*Screening biomarkers*, also called as *Detection biomarkers*), distinguishing benign from malignant tumours or one type of malignancy from another (*Diagnostic biomarkers*), determining the prognosis of patients (*Prognostic biomarkers*), prediction of response to treatment (*Predictive biomarkers*), and monitoring status of the disease, either to detect recurrence or progression of the disease (*Biomarkers for monitoring*) [39] (Figure 8).

The use of biomarkers in oncology has been much more extensive than in other diseases for more than 30 years [60]. Over the past ten years, there have been almost 34,400 publications indexed in PubMed with the joint headings of "cancer" and "biomarker" in Title/Abstract [61]. Before the genomic era, most biomarkers used in clinical practice represented protein-based biomarkers which are still widely used. Although these biomarkers are cheap and easy to measure, they do not evince sufficient specificity and sensitivity and should be supplemented or even replaced by nucleic acid-based biomarkers in the future. In the following paragraph, examples of individual cancer biomarkers (mainly for solid cancers) currently used in the clinical practice are described.

Susceptibility	Screening	Diagnosis	Prognosis	Prediction	Monitoring
Identify factors to assess disease susceptibility	Indicate the presence of disease; early detection	Definitive diagnosis and general typing	Assess disease aggressiveness & likelihood of recurrence	Predict efficacy or response to different treatments	Monitor disease recurrence & therapeutic response

Figure 8. An overview of different types of cancer biomarkers (modified according to [62]).

1.3.3 Cancer biomarkers currently available in clinic

Biomarkers of susceptibility: Identification of individuals who are at an increased risk of developing cancer is the goal of the risk assessment. This is currently possible with genetic testing for known cancer-related syndromes. The identification of many of the genes predisposing to hereditary cancer syndromes has been established within the past 15 years [63, 64]. Examples of predisposing genes for hereditary cancer syndromes include *BRCA1/2* (hereditary breast and ovarian cancer), *APC* (familial adenomatous polyposis), *MLH1, MSH2, MSH6, PMS1, and PMS2* (hereditary non-polyposis colorectal cancer), *MUTYH* (colorectal cancer associated with familial adenomatous polyposis), *MEN1* (multiple endocrine neoplasia type 1), *RET* (multiple endocrine neoplasia type 2), *RB* (retinoblastoma), and *CDK4* and *CDKN2A* (familiar melanoma) [65]. Consequently, genetic testing for cancer susceptibility can be carried out within high-risk families.

Screening biomarkers: One of the greatest challenges in oncology nowadays is the detection of cancer at an early stage, which means at a potentially treatable stage. Indeed, the development of screening (or early detection) biomarkers is currently one of the highest priorities in cancer research. Unfortunately, these types of biomarkers possess insufficient sensitivity for small tumours or premalignant lesions and lack of specificity in general. These features limit the use of the most available biomarkers in population-based screening for early malignancy [66, 67]. Despite these issues, several biomarkers have undergone evaluation for cancer screening, since they are usually cheap and easy to measure. Indeed, a few biomarkers are currently widely used in asymptomatic people for a screening of early cancer, i.e. prostate-specific antigen (PSA) for prostate cancer [68, 69], the faecal occult blood test for colorectal cancer [70], and Human Papilloma Virus testing (Pap smear) for cervical cancer [71].

Diagnostic biomarkers: Diagnosis, contrary to screening, involves patients with specific symptoms, which may or may not be due to cancer. In general, serum protein biomarkers contribute little to the early diagnosis of cancer, mainly due to the lack of both sensitivity and specificity. However, serum biomarkers may aid in the differential diagnosis of benign and malignant diseases in a small number of cases. Specifically, serum cancer antigen (CA) 125 is as an adjunct in differentiating between benign and malignant pelvic masses (ovarian cancer) in postmenopausal women [72-74]. Serum human epididymis protein 4 has superior specificity to CA 125, especially in premenopausal women, and

may be used similarly as CA 125 as a diagnostic aid in women with pelvic masses [74]. Another biomarker that can aid cancer diagnosis, in this case, hepatocellular cancer detection, is alpha-fetoprotein (AFP) [75]. Besides, AFP is also used to screen patients at high-risk of hepatocellular cancer [76].

Prognostic biomarkers: Following diagnosis and surgical removal of the primary tumour, the essential questions to be addressed for patient management are i) how aggressive is the tumour, and ii) is necessary to administrate the adjuvant (i.e. post-operative) systemic therapy. Thus, if a tumour is deemed to be indolent (i.e. growing slowly), the patient may be able to avoid receiving adjuvant treatment. On the other hand, if it is identified as being potentially aggressive and life-threatening, the patient would be recommended to have additional therapy, such as adjuvant chemo(radio)therapy. Until recently, histological and clinical criteria mainly aided in addressing the above questions. However, these criteria are still universally used since a limited number of biomarkers have become available to supplement the traditional criteria for determining patient's prognosis nowadays. These include, for instance, the tissue-based biomarkers, urokinase-type plasminogen activator, and plasminogen activator inhibitor-1 as prognostic biomarkers for certain cancer types [77, 78], and Oncotype DX and MammaPrint gene expression panels for assessing breast cancer patient's prognosis [79, 80]. Other biomarkers widely used to aid the patient's prognosis include AFP, human chorionic gonadotropin (HCG), and testicular lactate dehydrogenase in patients with testicular cancer (non-seminomatous type) [81], and PSA in patients with prostate cancer [82]. Also, *BRAF* mutations are indicative of patients' poor outcome in metastatic colorectal cancer [83].

Predictive biomarkers: Therapy predictive biomarkers aim for prospective identification of patients who are likely to respond or be resistant to specific treatments. The necessity of predictive biomarkers is substantiated by the fact that patients with tumours of the same organ type respond very differently to a specific drug. Therefore, response rates for unselected patients with various advanced cancer types to currently available systemic therapies vary from < 10 % to > 90 % [79, 84]. Many of the newer biological or molecular therapies, in particular, have efficacy in only a minority of unselected patients (< 10 %). This finding, when combined with the high costs of some of these drugs, illustrates the importance of having accurate therapy predictive biomarkers. In recent years, several new

predictive biomarkers entered clinical use, and their measurement is mandatory prior to the administration of appropriate treatment (summarized in [65]). These include tissue biomarkers such as oestrogen/progesterone and HER2 receptors in breast cancer for predicting response to endocrine and anti-HER2, respectively; *KRAS/NRAS* genotyping for selecting colorectal cancer patients likely to be resistant to treatment with anti-EGFR antibodies; evaluating the microsatellite instability (MSI) status for predicting response to 5-fluorouracil (5-FU) in colorectal cancer; *BRAF* genotyping for predicting response to specific BRAF inhibitors in metastatic melanoma; *EGFR* genotyping and *ALK* rearrangements evaluation for predicting response to EGFR TKI and ALK-inhibitors, respectively, in patients with non-small cell lung cancer; and *KIT* and *PDGFRA* genotyping for prediction of response to c-KIT/PDGFRA inhibitors in gastrointestinal stromal tumours [44, 85]. Mutation testing of plasma circulating tumour DNA is likely to complement or possibly replace some of the existing biomarkers in the future [86]. However, there is still an urgent need to identify predictive biomarkers for specific cytotoxic drugs, anti-angiogenic therapies, and immunotherapies. Regarding immunotherapy, a recent publication highlighted that mismatch repair (MMR) deficiency (so-called MSI-high status) predicts response of solid tumours to immune checkpoint blockade with antibodies to programmed death receptor-1 [87]. As well as assessing treatment efficacy, predictive biomarkers may also potentially identify upfront an optimum drug dose and predict severe toxicities. Nevertheless, there are few validated biomarkers for these purposes at present.

Biomarkers for monitoring: These types of biomarkers may be used to detect recurrent/metastatic disease at a potentially curable stage with the assumption that early detection of disease recurrence/progression followed by the treatment initiation will result in a better patient's outcome than starting treatment when a recurrence is clinically evident. Biomarkers identifying early cancer recurrences that are used in clinical routine include HCG in trophoblastic malignancy, PSA in prostate cancer, carcinoembryonic antigen (CEA) in colorectal cancer, AFP and HCG in patients with germ cell tumours of the testis (non-seminomatous type), CA 15-3 in breast cancer, and CA 125 in ovarian cancer [88-98]. However, apart from CEA in colorectal cancer, there is little evidence that the early detection of recurrent disease and the initiation of new treatment enhances patient outcomes [88, 89]. The other purpose of the use of these types of biomarkers is the monitoring of patients with advanced cancer receiving systemic therapy. Although

imaging methods represent the gold standard for monitoring therapy response in oncology, the use of blood-based biomarkers is less expensive, more convenient for patients, and applicable in shorter intervals. Biomarkers widely used to monitor the therapy comprise the *BRC-ABL* translocation in chronic myeloid leukaemia, AFP and HCG in testicular germ cell cancers, CA 125 in ovarian cancer, PSA in prostate cancer, CEA in colorectal cancer, and CA 15-3 in breast cancer [88-98].

The concept of this Dissertation Thesis is based on investigating potential cancer biomarkers that are related to DNA damage and DNA repair. Therefore, the following chapters will be devoted to this subject to provide an overview of the importance of DNA damage and DNA repair in the context of cancer susceptibility, cancer development, and patient's therapy response and clinical outcome.

1.4 Genome-maintenance network in preventing malignant transformation

Genome instability is one of the leading forces driving the onset and progression of cancer. It is fuelled by DNA damage and errors made by the DNA damage response (DDR) system [99, 100]. Since the genome integrity is permanently challenged by DNA damaging agents (DDA), eukaryotic cells have evolved the mechanisms on how to deal with damaged DNA and limit genome instability. DNA lesions are detected, their presence is marked by a specific signal, and consequently, their repair is promoted by a variety of complex cellular pathways, which are collectively referred to as the DDR [101, 102]. The complex DDR system is encoded by almost 200 human genes [103, 104]. Cells defective in DDR generally display heightened vulnerability towards DDA, and subsequent accumulation of mutations in the genome thus eventually contribute to cancer development. On the other hand, unrepaired and excessively accumulated DNA damage can be toxic, promoting pathways of cell elimination such as apoptotic and necrotic death that are also thought to function as tumour suppressor pathways [105].

1.4.1 Sources and types of DNA damage

Nuclear DNA is continuously exposed to a myriad of DDA that have either endogenous or exogenous origin. Regarding *endogenous DDA,* the majority of the endogenously

induced DNA damage arises from the chemically active DNA engaging in oxidative and hydrolytic reactions with reactive oxygen species and water, respectively, that are naturally present within cells. [106-108]. In addition, non-enzymatic methylation also generates nucleobase lesions. DNA strand breaks (SBs) arising from physiological processes are caused by abortive topoisomerases activity, and DNA mismatches are occasionally introduced during DNA replication [102]. Other endogenous DDA are produced by gut microbiota metabolism [109], and some radicals (reactive oxygen and nitrogen compounds) are also produced by activated immune cells such as monocytes and macrophages as well [110].

Exogenous DDA comprise physical, chemical and specific biological agents from the environment. Examples include radiation (UV and IR), chemical mutagens (aromatic hydrocarbons, halogenated hydrocarbons, aromatic amines, and alkylating agents), high temperature, heavy metals (Ni, Cd and As), viruses and certain DDA produced by microorganisms, fungi, and plants (e.g., aflatoxin, mitomycin C) [111, 112]. The individual DDA causing the specific DNA lesions are summarized in Figure 9A.

DDA cause damage in DNA nitrogenous bases or its sugar-phosphate backbone. Furthermore, some DDA may cause the formation of covalent bonds between DNA molecule and proteins (DNA-protein cross-links), thus affecting DNA-histone and DNA-transcription factor interactions [112]. However, the vast majority of DDA affects the primary structure of the DNA double helix, which means that nitrogenous bases themselves are chemically modified. It is estimated that out of the 3×10^9 bases in the human genome, 25,000 of bases are altered in some way per cell per day [113]. Such modifications can, in turn, disrupt the regular helical structure of DNA by the introduction of non-native chemical bonds or bulky adducts that do not fit in the standard double helix. As a consequence, these lesions cause DNA single-strand breaks (SSBs). Moreover, when two SSBs arise in close proximity, or when the DNA-replication apparatus encounters a SSB or certain other lesions, DNA double-strand breaks (DSBs) are formed. While DSBs do not occur as frequently as the other lesions listed above, they are difficult to repair and extremely toxic [114]. If they remain unrepaired, mutations and chromosomal rearrangements which are causal events in oncogenic transformation and tumour progression occur [111]. The specific consequences of DNA damage are summarized in Figure 9B.
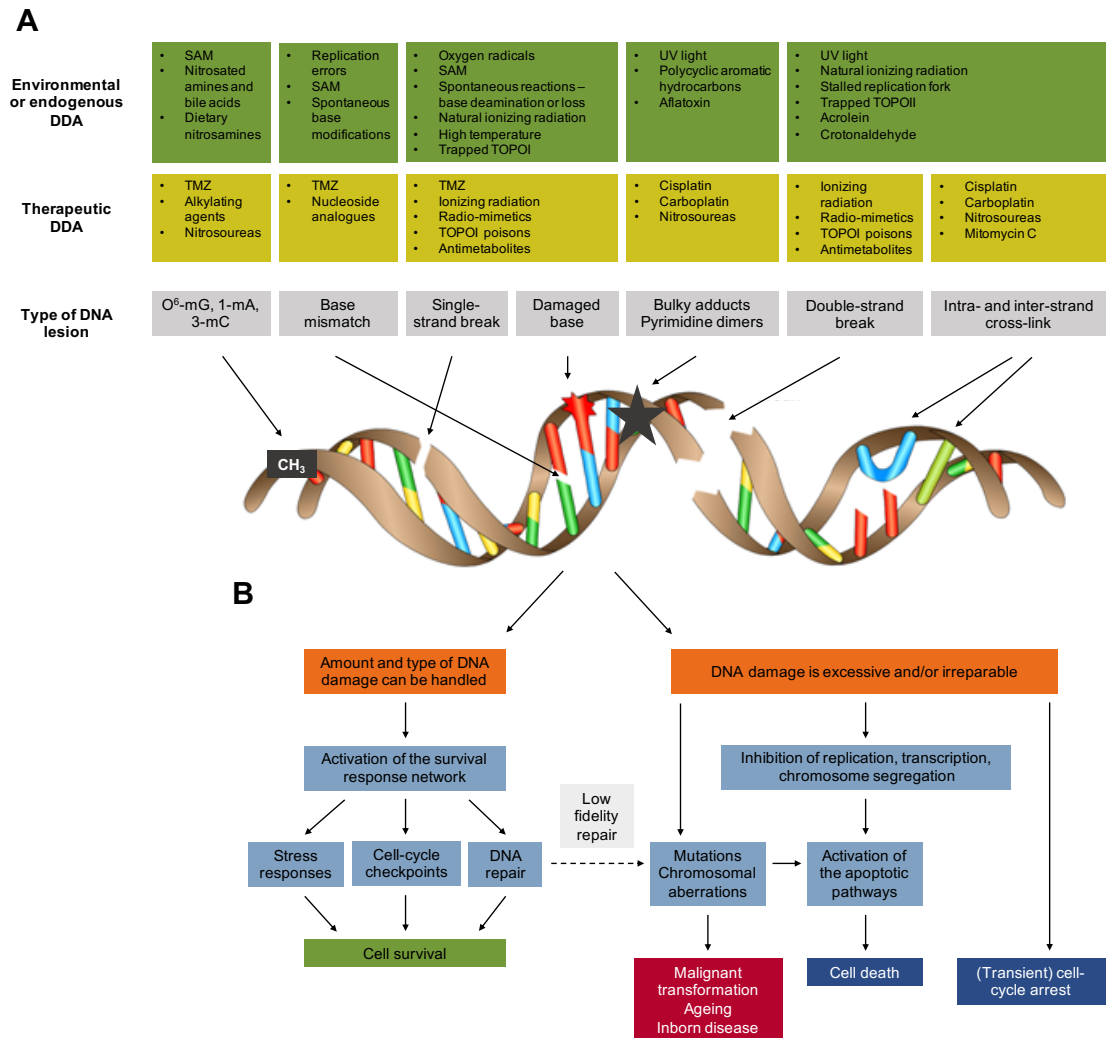
Figure 9. A summary of various DNA damaging agents and types of DNA damage induced (A) along with the consequences arising from DNA damage (B) (modified according to [115, 116]).

Figure 9A summarizes sources and types of DNA damage. Environmental and endogenous sources of DNA damage are shown in green boxes, examples of therapeutic DNA damaging agents are shown in yellow boxes, and the types of lesions induced by particular agents are shown in grey boxes. Figure 9B summarizes the consequences arising from DNA damage. Abbreviations: 1-mA – 1-methyladenine, 3-mC – 3-methylcytosine, O^6-mG – O^6-methylguanine, SAM – S-adenosyl methionine, TMZ – temozolomide, TOPO – topoisomerase, UV – ultraviolet.

1.4.2 DNA damage repair systems

DNA repair represents a complex biological system that comprises several distinct pathways repairing different types of DNA damage. Besides, the evidence for extensive interactions among proteins involved in distinct DNA repair pathways continues to emerge. No single pathway efficiently repairs all types of DNA lesions, and some lesions serve as substrates for more than one pathway [117]. Interestingly, if canonical DNA repair pathways are deficient, alternative repair mechanisms may be employed to compensate for that lack of function [118, 119]. Particular DNA repair pathways include: i) repair of base DNA damage by direct reversal DNA repair and base excision repair (BER); ii) repair of multiple and bulky base damage by nucleotide excision repair (NER), MMR, inter-strand cross-link repair (i.e. Fanconi anaemia (FA) pathway), and translesion synthesis; and iii) repair of DNA breaks by SSB repair pathway and DSB repair pathway (comprising homologous recombination – HR* and non-homologous end joining – NHEJ) [106]. SSB repair and BER are often assumed to be synonymous because they involve the same components and are similar after the initial recognition step [115]. Individual DNA repair pathways, their function and key enzymes involved are summarized in Table 1.

Table 1. The summary of DNA repair pathways responsible for repairing the individual DNA lesions along with a short description of their function and key genes involved (modified according to [118, 120]).

Repair pathway	Type of DNA damage	Function	Number of genes involved	Key genes
Direct reversal repair	Base modifications, including O6-methylguanine, 1-methyladenine, 3-methylcytosine, and N-methylated adenosine and cytosine	Direct repair of modified bases by enzymatic processes: demethylation.	3	*MGMT, ALKBH1*
Base excision repair (BER) (incl. Short patch repair, Long patch repair, and SSB repair)	Damaged and modified bases, SSBs	Monofunctional and bifunctional DNA glycosylases and endonucleases excise damaged base to generate a basic site. Abasic sugars following spontaneous deamination, oxidation or alkylation to form SSBs, followed by nicking, resynthesis, and SSB repair.	42	*OGG1, NEIL1, NEIL2, NEIL3, APEX1, PARP1, PARP2, XRCC1, POLB, LIG1, LIG3, FEN1, PNKP, MUTYH*
Nucleotide excision repair (NER) (incl. Transcription-coupled NER and Global NER)	Bulky DNA adducts, helix-distorting adducts (inter- and intra-strand cross-links)	Damage recognition and unwinding of local DNA, nuclease excision, resynthesis, and SSB repair.	66	*RAD23B, DDB1, RPA1, RPA2, ERCC1, ERCC2 (XPD), ERCC3, ERCC5, ERCC6, ERCC8, GTF2H1, GTF2H2, GTF2H4, GTF2H5, GTF2F2, CDK7, MMS19, MNAT1, XPA, XPC, CCNH, PCNA, RFC1*
Mismatch repair (MMR)	Base mismatches (single nucleotide mutations and small insertions/deletions) mainly caused by replication errors	Recognition and removal of mismatched base followed by resynthesis of correct base and SSB repair.	27	*MLH1, MLH3, MSH2, MSH6, PMS2*
Inter-strand cross-link (ICL) repair, i.e. Fanconi anaemia (FA) pathway	Inter-strand cross-links	Cross-links are excised and then repaired by HR (or other mechanisms).	22	*BRCA2, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCG, FANCF, FANCI, FANCL, BRIP1, FANCM, FAAP20, FAAP100*
Translesion synthesis (TLS) (DNA damage bypass rather than repair)	DNA adducts	Error-prone polymerases synthesize DNA past regions of damage, especially bulky DNA adducts (If damaged DNA bases or adducts are not repaired before replication has initiated, they may stall replication forks, contributing to genetic instability. Specialized TLS DNA polymerases are recruited to synthesize the DNA at these sites.)	19	*POLH*
Homologous recombination (HR*)	DSBs	Unwinding and resection at DSB to generate single-strand end, strand invasion, homologous recombination with sister chromatid, resynthesis, and resolution. Results in exact repair using sequences from sister chromatid.	52	*BRCA1, BRCA2, RAD51, RAD52 TP53BP1, RBBP8 (CTIP), EXO1, RPA1, RPA2, BLM, PALB2,* MRN complex: *MRE11, RAD50, NBN* (NBS1)
Non-homologous end joining (NHEJ)	DSBs	Processing and re-ligation of DSB ends. Error prone due to processing steps and because the homologous template is not used for repair.	27	*PRKDC* (DNA-PKcs), *XRCC5* (Ku80)*, XRCC6* (Ku70)*, LIG4, XRCC4, POLQ, NHEJ1, DCLRE1C* (Artemis)*, PARP1, PARP2, XRCC1*

Abbreviations: BER – base excision repair, DSBs – double-strand breaks, FA – Fanconi anaemia, HR* – homologous recombination, ICL – inter-strand cross-links, MMR – mismatch repair, NER – nucleotide excision repair, NHEJ – non-homologous end-joining, SSBs – single-strand breaks, TLS – translesion synthesis.

1.5 DNA damage and DNA repair in relation to cancer

Genome instability represents one of the most pervasive characteristics of tumour cells. It arises as a consequence of the combined effect of DNA damage, tumour-specific DNA repair defects, and a failure to stall or stop the cell cycle before the damaged DNA is passed on to daughter cells [10]. Not surprisingly, in many cancers, DNA repair, DNA damage tolerance and DDR pathways are disrupted or deregulated, which increases mutagenesis and genome instability, thereby promoting cancer progression [121-123]. On the other hand, DNA repair appears to play a substantial role in cancer therapy response. Therefore, a better understanding of the cellular response to DNA damage will not only refill our knowledge of cancer onset and development but also help to refine the cancer classification as well as treatment [124]. Based on these findings, a recent study by Chae *et al.* has compiled and analysed a comprehensive list of DNA repair genes utilising the large databases Catalogue Of Somatic Mutations In Cancer [125] and The Cancer Genome Atlas [126, 127]. The study provided a list of candidate DDR genes that may serve as potential biomarkers for genome instability, novel therapeutic targets, or predictors of therapy efficacy [103].

An integral part of this Dissertation Thesis is a chapter which reflects long-lasting (approximately 25 years) experience of our Department with the study of DNA damage and repair in relation to cancer as well as the most recent findings, accomplished during my PhD studies. In recent years, we have published four review articles on this topic (Publications IX–XII), which represent a part of this Thesis, and their content is summarized in this chapter.

1.5.1 DNA damage repair and cancer susceptibility and development

Each individual has a different risk of developing cancer that is mainly determined by genetic background and exposure to carcinogens. Genetic predisposition affects not only hereditary forms of cancer but also applies to sporadic tumours, which represent approximately 90-95 % of all cancers [128, 129]. Inter-individual differences in DNA repair systems may play a role in modulating the individual risk of developing cancer. Among the genes playing a role in cancer susceptibility, DNA repair genes are prominent candidates as cancer is associated with inherited deficiencies of DNA repair [124].

Alterations in genes involved in DNA repair pathways are associated with the development of several malignancies, summarized in [103]. Inactivating mutations and hypermethylation in MMR genes (i.e. *MSH2, MSH6, MLH1, PMS1,* and *PMS2*) lead to the development of hereditary non-polyposis colorectal cancer and MSI, conferring a 70% lifetime risk of colorectal cancer and an increased risk of developing other cancers, such as endometrial, ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain, and skin [130]. Germline mutations in *BRCA1* and *BRCA2*, involved in HR* and FA repair pathways, increase the risk of developing, among others, breast cancer by 40-80 % and ovarian cancer by 11-40 % [131]. Moreover, defects in *ATM*, another gene involved in HR* pathway, are associated with ataxia-telangiectasia and up to a 25% lifetime malignancy risk, particularly lymphomas and leukaemia as well as other cancers [132]. Regarding NER defects, these are responsible for xeroderma pigmentosum, which is linked to a 70% risk of skin cancer by eight years of age [133].

Nevertheless, it remains ambiguous whether all alterations in DNA repair genes are truly causal events in driving tumorigenesis, as mutations in "mountain" genes, or are a by-product of the malignancy and represent more infrequently mutated "hills" [134]. In support of the former is the "mutator phenotype" and the concept that early mutations in critical genes, such as those involved in DNA repair, resulting in genome instability and subsequent hypermutability, accounting for the high mutation rate seen in tumours [135]. This theory of causality was further supported by studies documenting that MMR mutations and MSI are commonly seen in early adenomas and early stages of colorectal cancer [136, 137].

1.5.2 DNA damage repair and patients' treatment and monitoring

Cancer treatment strategies, from which the most widely used are chemotherapy and radiation therapy, are designed to have cytotoxic effects on rapidly dividing cells (such as cancer cells) via induction of DNA damage. In response to induced DNA lesions, the DDR machinery allows cells to activate cell cycle checkpoints and proapoptotic signals to preserve genome integrity. Depending on the cell or tissue type, persistent genotoxic insults could trigger either cellular death by mitotic catastrophe or autophagy or induce a replicative stress-induced state of cellular senescence [102]. Although DDR dysregulation is causative and permissive of malignant transformation of normal cells and cancer progression, it can provide a weakness that can be exploited therapeutically in both

conventional cytotoxic therapy and targeted therapy using DDR inhibitors [115, 121, 124, 138]. Tumour cell sensitivity to chemo- and radiotherapy can highly depend on the cellular capacity to repair DNA damage within and between tumour types. Therefore, annotation of functional defects in DDR may allow for the development of novel prognostic biomarkers as well as could be used to predict therapeutic response, including predicting responses following inhibition of DNA repair.

Several studies found the association of upregulation of DNA repair genes with resistance to chemo- and radiotherapy in multiple tumour types [121] and with the tumour's ability to metastasize [139, 140]. Thus, while the loss of DNA repair function is significant in cancer initiation, the gain of function of similar genes and re-activation of lost DNA repair pathways is involved in cancer progression [141, 142]. Targetable DNA repair inhibition has been shown to enhance tumour responses to therapy. For instance, PARP1 inhibitors are used to treat patients with advanced breast and ovarian cancer harbouring *BRCA1/BRCA2* deleterious mutations, as loss of BRCA sensitizes these tumours to further inhibition of DNA repair resulting in a synthetic lethality [143, 144]. PAPR1 inhibitors also evince potential in many other cancer types with DNA repair deficiencies [145], and inhibition of other DNA repair genes is being evaluated to induce synthetic lethality, including PRKDC inhibition in MYC-overexpressing tumours [146]. Therapeutic targets encompassing DDR pathways are reviewed in [147]. Besides, the emerging field of personalized immunotherapies directed specifically against mutated cancer "neo-antigens" may ultimately prove to be strongly linked to impairment in DNA repair [148].

1.6 Candidate cancer biomarkers associated with DNA damage and DNA repair

This chapter briefly describes those biomarkers studied in this Dissertation Thesis, which can serve as potential biomarkers of susceptibility and/or patients' clinical outcome.

1.6.1 Single nucleotide polymorphisms (SNPs)

While mutations in DDR genes are associated with high degrees of individual cancer risk, these rare events explain only a small fraction of all cancers [149]. Given the importance

of DNA damage to cancer development, it is plausible that common variants of DNA repair genes would contribute to cancer risk. Such a risk could be measured in large, genome-wide association studies (GWAS), or the studies of candidate genes. GWAS represent a hypothesis-free (or technology-driven) approach that was applied in Publication V and VI. The latter, a hypothesis-based (or hypothesis-driven), approach was used in Publication IV and VII. No conclusion has been reached about which of these two approaches is more effective/convenient in studying SNPs [150].

SNP, a substitution of a single nucleotide occurring in > 1 % within a population, represents the most frequently studied type of DNA variation potentially being associated with the altered susceptibility to cancer. SNPs are reproducible and possible to measure at any point in time (may be used in both prospective and retrospective studies). Moreover, the identification of SNPs is becoming increasingly routine, including limited genotyping of tumour DNA and screening of somatic (non-tumour) DNA for mutations that predispose to cancer or alter treatment response [59].

GWAS are feeding into the clinical use of DNA variants and have identified hundreds of SNPs and susceptibility loci associated with risk for various cancers [151-161]. Nevertheless, only few GWAS have identified cancer susceptibility loci near DNA repair genes at stringent levels of significance that have also been shown to function through altered DNA repair [156, 159, 161, 162]. These data suggest that common variants in DNA repair genes may not make significant contributions to cancer susceptibility and that cancer susceptibility may be mostly conferred by high-risk, rare variants within this class of genes. However, it is possible that underpowered GWAS could miss common variants with weak effect sizes and also, one of the limitations of GWAS is that only common SNPs are captured, which by themselves may only contribute a small amount of risk to developing cancer. Recently, a pooled analysis of thousands of SNPs in DNA repair genes for most common cancers has been performed by analysing data from 32 GWAS in order to reveal increase the power to detect common variants associated with cancer susceptibility [163]. GWAS are also identifying predictors of sensitivity to radiation therapy [164] and the pharmacodynamics of anticancer drugs [165]. Nevertheless, SNP-based research to identify oncogenic DNA abnormalities remain a significant challenge, due to the difficulty of separating these cancer-causing abnormalities from genetic and epigenetic "noise" [166].

1.6.2 Chromosomal aberrations (CAs)

Accumulation of chromosomal abnormalities contributes to genome instability, specifically to chromosomal instability. Acquired chromosomal abnormalities can be structural or numerical. In the studies involved in this Thesis (Publication I, II, IV, V, and VI), we focused on structural chromosomal abnormalities, i.e. CAs in peripheral blood lymphocytes (PBLs). Structural CAs arise as consequences of direct mutagenic effect and/or due to DNA repair dysfunction [167]. Unrepaired or insufficiently repaired DSBs, as well as telomerase dysfunction, are substantial players in the formation of structural CAs [168, 169]. Morphologically distinct types of chromosome-type aberrations (CSAs) and chromatid-type aberrations (CTAs) emerge depending on the types of clastogens to which individuals are exposed and at which stage of the cell cycle SBs appear as primary lesions that consequently result in CAs. Usually, G0 or G1 phase-dependent clastogens such as IR or bleomycin create DSBs that are either incompletely repaired by NHEJ or remain unrepaired and eventually give rise to CSAs, including dicentric and ring chromosomes. CTAs include chromatid breaks and exchanges that affect only one chromatid of a chromosome. They are induced by chemical or environmental clastogens during the S or G2 phase and arise from SBs. These breaks are later converted into DSBs possibly through failed or incomplete HR*, giving rise to chromatid breaks [170, 171] (Figure 10).

CAs can be further divided into non-specific and specific. The non-specific CAs that we focused on in our studies represent non-recurrent, non-clonal karyotypic alterations. They are derived from a single abnormal cell that can remain in the cell for its lifespan and thus can be detected by the standard cytogenetic analysis [172, 173]. The specific CAs are often recurrent and are analysed by molecular cytogenetic methods, such as sequencing and fluorescent *in situ* hybridization. Many human cancers and neoplastic cells exhibit specific CAs, especially translocations and related gene fusions [174]. In the recent past, a majority of studies has been focussed on clonal specific CAs as they relate to a specific disease or condition and non-specific CAs have been largely ignored since they were considered as insignificant genetic "noise" [175]. The evidence linking non-specific CAs with cancer is not as overwhelming as it is for specific CAs. However, an examination of non-specific CAs in PBLs has been conventionally used in individuals exposed to mutagens and potential carcinogens for decades as a surveillance mechanism for

genotoxic effect [176-178]. Elevated non-specific CAs in PBLs have been detected during the course of cancer evolution, making them an early biomarker of cancer susceptibility based on the hypothesis that genetic damage in PBLs reflects similar damage in other body cells undergoing carcinogenesis [179, 180]. Therefore, it is important to investigate the basis of the origin and the accumulation of CAs both in healthy individuals and cancer patients.

1.6.3 Telomere length (TL)

Telomeres are unique nucleoprotein structures composed of TTAGGG tandem repeats that cap ends of linear eukaryotic chromosomes. In the normal human population, the length of telomeres is heterogeneous, ranging between 5 and 15 kb. Also, TL is influenced by the genetic background and environmental factors [181]. The telomere complex regulates a "cellular mitotic clock" and protects chromosomes against exonucleolytic degradation, DNA damage and chromosomal instability [182-184]. Telomeres progressively shorten through the cellular lifespan. At each cell division, human telomeres lose 50-200 bp [185]. The telomeric loss at between 9 and 147 bp per year, depending on the organ/tissue has been determined [186]. Telomere shortening can cause the proliferation arrest and apoptosis of the cell through the loss of protection at chromosome ends. Critically shortened telomeres may be poorly end-capped and recognized as DSB by repair machinery [187]. These processes may underlie end-to-end chromosome fusions, the initiation of breakage-fusion-breakage cycles, and lead to telomere crisis with consequent genome instability that can induce the development of numerical and structural chromosomal abnormalities [188-191]. On the other hand, the reactivation of telomerase stabilizes telomeres and immortalizes premalignant cells, thus enabling cancer progression [192]. Altogether, TL plays a critical role both in genome integrity maintenance [193, 194] and cancer initiation/progression [195, 196] (Figure 10). There is growing evidence of shorter telomeres caused by low telomerase activity in somatic cells being associated with the increased frequency of CAs in PBLs, in particular with CSAs [197, 198]. Therefore, we focused on measurement of TL in PBLs in Publication II and III to examine whether the telomere shortening contributes to the formation of CAs and whether TL may be used as biomarkers on cancer susceptibility and/or predictive/prognostic biomarkers.
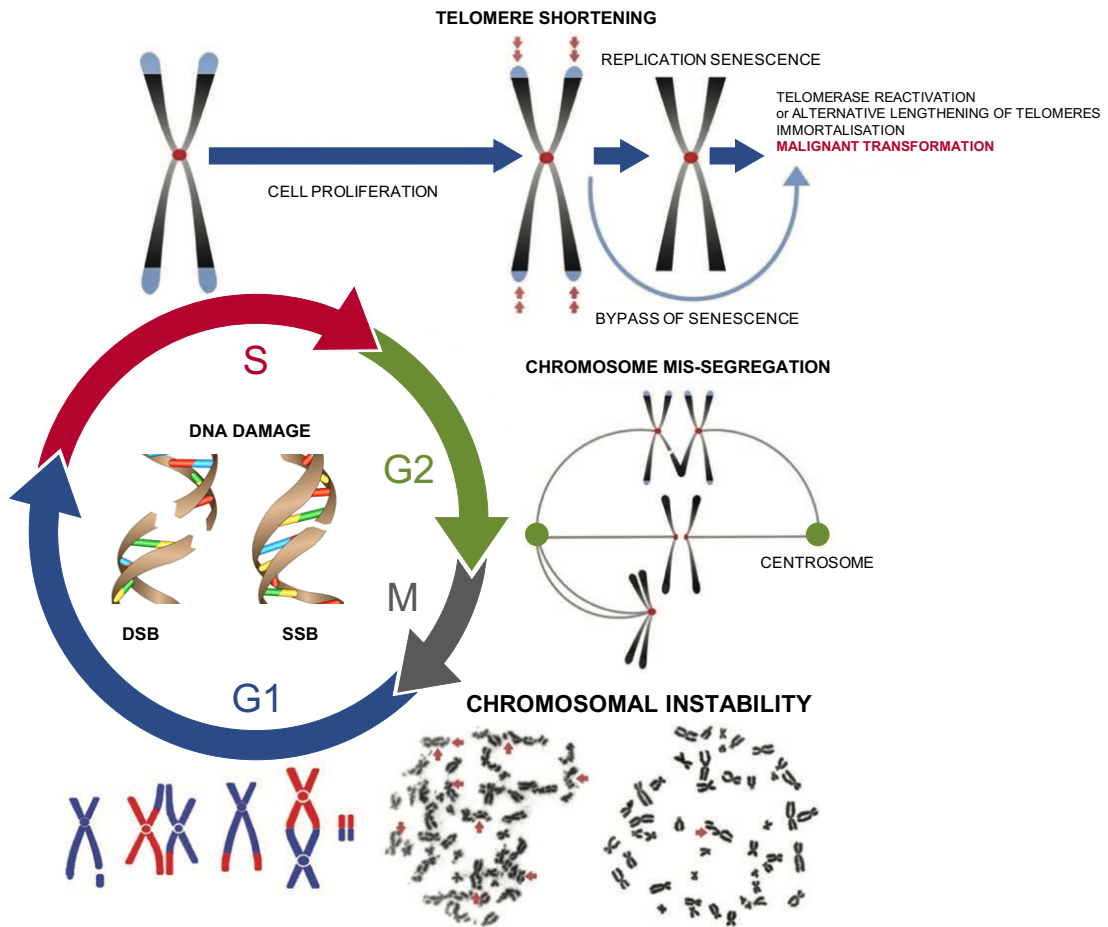
Figure 10. Sources of chromosomal instability (modified according to [199, 200]).

Single-stranded DNA damage, if unrepaired, gives rise to DSBs and CTAs. Shortened telomeres may be poorly end-capped and recognized as DSBs by repair machinery, resulting in the development of numerical and structural CAs.

Abbreviations: CAs – chromosomal aberrations; CTAs – chromatid-type aberrations; DSB – double-strand break; SSB – single-strand break.

1.6.4 Phenotyping DNA repair as DNA repair capacity (DRC)

As evident from current knowledge on DNA repair, it represents a multiprotein and multistep process which works in a synchronized and coordinated way, together with simultaneous participation of DNA damage signalling and cell cycle control. Therefore, a real multivariate approach needs to be undertaken to understand this complex system. Although the genetic variants of DNA repair genes may provide useful information on their association with cancer susceptibility and patient's clinical outcome, their functional consequences are usually predicted *in silico*. Thus, DNA analysis does not provide full information on the overall DRC. Similarly, individual gene and protein expression levels

did not prove to be sufficiently informative about the overall DRC; several studies have reported an inconsistency between transcript level and respective protein quantity [201], or actual protein/pathway activity [202, 203].

Moreover, genetic predisposition does not exclusively modulate DNA repair activity of individuals [204], it is also influenced by environmental and lifestyle factors via various mechanisms, such as modulation of the activity of DNA repair enzymes, the pool of DNA precursors, and regulation of expression of DNA repair genes [205]. Summarizing all mentioned above, a better characterization of DNA repair at the functional level as DRC (used in Publication III and VIII), the true phenotypic endpoint that comprises the variability of both hereditary and environmental components, gives the information of actual DNA repair activity of the cell/tissue/organism [117, 206].

To conclude the Introduction, the constant increase of cancer incidence and the enormous costs of (new) treatments make searching for novel cancer biomarkers a crucial goal in order to maintain sustainable public health systems across the world. Carcinogenesis is a multistep process, which allows time for active intervention that requires well-defined risk classification. Consequently, personalized strategies and specific treatments can be applied to cohorts with a documented increased cancer risk. Also, understanding of different patients' responses to particular anti-cancer treatment remains insufficient. Since each patient is genetically unique, there is a growing need for novel predictive and prognostic biomarkers in order to aid oncologists in the selection of optimal type, combination and dose of drugs for each patient to improve their outcome and minimizing treatment-related toxicity. Further development of these strategies in an efficient and timely manner requires investment in the discovery and validation of surrogate cancer biomarkers to detect and monitor the efficacy of interventions in clinical trials and beyond.

2.   Hypotheses & Aims

The overall aim of this Dissertation Thesis reflects the current interest in identifying the nature and biological regulation of DNA damage and DNA repair and the impact of their accumulation and deregulation, respectively, on the cancer development, patients' therapy response and clinical outcome. In our studies, we focused on searching for potential novel biomarkers related to DNA damage and DNA repair, and on confirmation of the validity of already existing biomarkers. We also explored the biological basis of different biomarkers and their associations.

The work was divided into three main parts in which the working hypotheses and experimental work were driven by these major assumptions:

1) Genome instability as one of the leading forces driving cancer onset and progression is caused by the accumulation of DNA damage and deregulation of DNA repair. Therefore, increased levels of chromosomal damage, telomere shortening and inter-individual variations in DSB repair capacity may play a significant role in the individual cancer susceptibility and patients' clinical outcome after diagnosis.

2) Due to extensive interactions between individual DNA repair pathways, inter-individual differences in DRC may be caused by genetic variants in different DNA repair genes and other related genes and their gene-gene interactions. These genetic variants may thus contribute to different levels of chromosomal damage in both in disease-free population and occupationally exposed population, as well as in cancer patients.

3) In many cancers, DNA repair pathways are disrupted or deregulated, thereby promoting cancer progression. We thus assumed that inter-individual differences in DRC might be associated not only with cancer susceptibility, but they may also affect patients' therapy response and clinical outcome after diagnosis.

Explicitly, we stated the following aims:

1) To examine whether CAs, TL and DSB repair capacity in PBLs are associated with cancer susceptibility and patients' clinical outcome, and also, whether the telomere shortening contributes to the formation of CAs and whether inter-individual variation in DSB capacity may influence the levels of DNA damage and TL.

2) To explore the genetic basis of inter-individual variations in CA frequency in PBLs and whether it depends on the level and type of exposure by finding novel SNPs predisposing to the formation of CAs and potentially to cancer; and to examine whether SNPs in DNA repair genes and other genes and their interactions are associated with the levels of CAs.

3) To search for differences in DNA repair in cancer patients which may aid in stratifying patients according to predicted therapy response and patients' survival. It will lead to an individual approach to patients and may be an attractive target for therapeutic intervention strategies.

3.   Material and Methods

3.1   Study populations

All studies included in this Thesis were performed on human biological samples, largely on peripheral blood cells and a few of them also on tissue samples. The design of all studies was approved by appropriate Ethic committees of participating institutions/hospitals. All studies had adhered to the ethical guidelines as set out in the Helsinki Declaration – all participants were sufficiently informed about all aspects of the study, agreed with the study purpose and procedures to be undertaken (including the storage of biological samples and personal data) and provided informed consent.

Publication I: The study population consisted of three groups of the incident (i.e. newly diagnosed) and histologically confirmed individuals with breast (N=158), colorectal (N=101), and lung (N=87) cancer, and a group of healthy control individuals without any personal cancer history (N=335) from the Czech Republic and Slovakia. All individuals were sampled for peripheral blood. Baseline characteristics such as demographics, family history of cancer, smoking habit, occupational history, body mass index, and the presence of other diseases and drugs received were collected prior to blood collection using a structured questionnaire. Moreover, patient disease characteristics, including tumour location, TNM stage, histopathological grade, histological classification, and the presence of hormonal receptors in breast tumours, were collected after surgical resection.

Publication II: The study was conducted on almost the same groups of patients and healthy control individuals, as in Publication I. The total numbers of cancer patients in individual groups only slightly differed: breast cancer patients (N=151), colorectal cancer patients (N=96), lung cancer patients (N=90). The number of individuals in the control group remained unchanged (N=335). In this study, we also collected the follow-up data.

Publication III: The study population comprised newly diagnosed and histologically confirmed individuals with breast (N=47) and colorectal (N=44) cancer patients and two healthy control groups (N=46 and N=44) from the Czech Republic. The individuals were sampled for peripheral blood and the same baseline and patient's disease characteristics, as in Publication I, were collected.

Publication IV: The studied group involved in this study consisted of healthy individuals (N=2196) from the Czech Republic and Slovakia. The population comprised individuals with defined occupational exposures as well as an unexposed reference group. The individuals were sampled for peripheral blood and completed a questionnaire regarding the job category, mode and duration of exposure, various exogenous factors (such as smoking and dietary habits, alcohol consumption, drug usage, and exposure to X-ray radiation) prior to blood collection.

Publication V: The study population consisted of healthy individuals (N=1473) from the Czech Republic and Slovakia, which were divided into two groups based on the measurable exposure to genotoxins due to their occupation and smoking habits. The exposed and unexposed reference group consisted of 607 individuals and 866 individuals, respectively. The individuals were sampled for peripheral blood and completed the same questionnaire as in Publication IV.

Publication VI: The sample set (discovery set) comprised healthy individuals (N=639) from the Czech Republic and Slovakia. The subjects consisted of individuals with defined occupational exposures as well as an unexposed reference group. The individuals were sampled for peripheral blood and completed the same questionnaire as in Publication IV. The replication was conducted on two different sample sets. The first replication set (replication 1) consisted of 482 individuals (newly diagnosed primary cancer patients – described in Publication I, and self-reported smokers). The second set (replication 2) was composed of 1288 individuals (occupationally exposed individuals and self-reported smokers). All individuals were sampled for peripheral blood.

Publication VII: This study was carried out on a discovery cohort from the Czech Republic comprising newly diagnosed and histologically confirmed individuals with sporadic colorectal cancer (N=1832) and healthy control individuals (N=1172), and replication cohort from Austria comprising newly diagnosed and histologically confirmed individuals with sporadic colorectal cancer (N=950) and healthy control individuals (N=820). Patients were sampled for non-malignant colon/rectal tissue or peripheral blood and healthy controls for peripheral blood. The same baseline and patient disease characteristics, as in Publication I, were collected from both cohort participants, along with the type of cancer therapy regimen received and the follow-up data.

Publication VIII: The set of patients in this study comprised newly diagnosed and histologically confirmed individuals with the sporadic form of colon cancer (N=123) from the Czech Republic. For each patient, we have collected paired samples of tumour tissue and non-malignant adjacent mucosa (5-10 cm distant from the tumour). The same baseline and patient's disease characteristics, as in Publication I, were collected along with the type of cancer therapy regimen received and the follow-up data.

3.2 SNPs analysis

3.2.1 Selection of candidate SNPs

The candidate SNPs were selected using different *in silico* bioinformatic tools to examine their functional consequences (Publication IV – SIFT and PolyPhen; Publication V and VI – Locus zoom, UCSC genome browser, Haploreg, Regulome DB; Publication VII – F-SNP, GERP, SiPhy, ELASPIC and DUET) and according to relevant published literature. The SNPs were filtered for their minor allele frequency (MAF > 5-10 % depending on the particular Publication) in Caucasian populations to reach an appropriate representation of all genotypes in sets of patients and healthy individuals (using UK10K—1000 Genomes Project). Besides, functional consequences of highly associated SNPs were predicted including their location with respect to the genes in the region, the presence of regulatory elements, linkage disequilibrium and expression effects from expression quantitative trait loci studies. Individual genes and SNPs, together with their selection, are described in detail in the corresponding Publications.

3.2.2 Genotyping

Genomic DNA from blood samples was isolated using standard procedures. If the blood sample was not available, non-malignant colon/rectal tissue was used to obtain DNA by the DNeasy Blood and Tissue Kit (Qiagen). SNPs were determined by Restriction Fragment Length Polymorphism technique, TaqMan Allelic Discrimination Assay (Applied Biosystems), KASP™ Genotyping Assay (LGC genomics), Axiom Genome-Wide CEU 1 Array (Affymetrix), and Illumina HumanOmniExpress Exome 8v1.3 chip array (Illumina). More details about genotyping procedures can be found in Publications IV–VII.

3.3 DNA damage assays

3.3.1 Evaluation of CAs

Cytogenetic analysis was performed on cultured PBLs as described in [177, 207-209]. Briefly, whole-blood samples were cultured in EKAMTB-100 complete medium with phytohemagglutinin (EuroClone S.p.A.), (Publication I, II, and IV), or in RPMI medium (Roswell Park Memorial Institute) along with L-glutamine and $NaHCO_3$ (Gibco) supplemented with 20% foetal calf serum (Gibco), antibiotics (penicillin and streptomycin, Gibco), and phytohemagglutinin (Murex), (Publication V and VI), for 50 hours at 37°C. After 48 hours of cultivation, cell division was stopped by colchicine (Sigma-Aldrich) in the first metaphase of mitosis. After a cytogenetic procedure, microscopic slides were stained by conventional Giemsa-Romanowski solution (Sigma-Aldrich). Microscopical analysis of 100 metaphases with 46 ± 1 chromosomes has been blindly performed by two independent scorers. The percentages of aberrant cells (ACs), total CAs (CAtot), CTAs (i.e. chromatid breaks and exchanges), and CSAs (i.e. chromosome breaks, terminal and interstitial deletions, dicentric and ring chromosomes with their difragments, abnormal chromosomes) were detected. Concerning CAs scoring, standardization procedure has been applied in former Czechoslovakia (and later in both separate countries) [210]. The arbitrary cut-off point between individuals with high and low CA frequency, chosen on the basis of long-term experience with this kind of biological monitoring in the Czech and Slovak Republics [211], was 2 % for CAtot and 1 % for CSAs and CTAs.

3.3.2 Measurement of TL

TL was measured in Publications II and III as relative telomere length (RTL). Briefly, genomic DNA from blood samples was isolated using standard procedures. RTL measurement was conducted using the monochrome multiplex PCR assay previously described by Cawthon [212] with slight modifications [213, 214]. All details about standard and calibration curves, DNA concentrations, negative and quality controls, master mix, conditions for telomere sequence, and albumin gene amplification are described in Publications III. All reactions were performed in triplicates in an optical 384-well reaction plate. Real-time PCR experiments were carried out on Viia 7 Real-time PCR

System (Applied Biosystems) with the use of two simultaneous programs to acquire the respective cycle threshold values for telomere sequences and the albumin gene. RTL was expressed as the ratio (T/S ratio) between telomere (T) and albumin (S; single-copy gene).

3.3.3 γ-H2AX concentration measurement

This assay documents differences of γ-H2AX levels in human PBLs and was used in Publication III. γ-H2AX is a form of histone 2AX that is produced after phosphorylation in response to DSBs and apoptosis [215]. Briefly, whole-blood samples were cultured in EKAMTB-100 complete medium with phytohemagglutinin (EuroClone S.p.A.) for 72 hours at 37°C. Five hours before harvesting (late S and G2 phase of the cell cycle), bleomycin (Sigma-Aldrich) was added in one of the two cultures; the other culture served as a reference. The immobilized γ-H2AX antibody in the wells of a 96-well plate captures γ-H2AX from sample lysate. Incubation with an H2AX detecting antibody (Trevigen), followed by addition of a Goat anti-mouse horseradish peroxidase conjugate and a chemiluminescent horseradish peroxidase substrate yields relative light units that directly correlates with the amount of γ-H2AX in the sample.

3.4 DNA repair assays

3.4.1 Mutagen sensitivity assay

Mutagen sensitivity assay (MSA) was performed according to the previously described protocol with minor modifications [216] and was used in Publication III. Briefly, two whole-blood samples from each subject were cultured for 72 hours in EKAMTB-100 complete medium with phytohemagglutinin (EuroClone S.p.A.). Five hours before harvesting (late S and G2 phase of the cell cycle), bleomycin (Sigma-Aldrich) was added in one of the two cultures; the other culture served as a reference. For three hours, cells were allowed to repair DSBs caused by bleomycin treatment. Two hours prior to harvest, Colcemid (Calbiochem) was added to arrest cells in metaphase. After a cytogenetic procedure, microscopic slides were stained by conventional Giemsa-Romanowski solution (Sigma-Aldrich). Microscopical analysis of CTAs of 100 metaphases with 46 ± 1 chromosomes has been blindly performed by two independent scorers. CTAs were cytogenetically assessed in samples of all groups after the bleomycin treatment, while not

affected samples served for detection of baseline CAs level. Mutagen sensitivity was expressed as the average number of CTAs per cell. Cells with ≥ 12 CTAs were assessed as those with a high level of chromosome damage, i.e. low DSB repair capacity.

3.4.2 Comet-based in vitro DNA repair assay

The comet-based *in vitro* DNA repair assay is a modified version of the comet assay (also known as single-cell gel electrophoresis assay) to assess DRC at the functional level. In Publication VIII, specifically BER capacity was measured. Briefly, protein extracts from samples were incubated with agarose-embedded substrate nucleoids ("naked" supercoiled DNA), containing specifically induced DNA lesions known to be recognised and repaired by the BER pathway [217]. During the incubation, BER enzymes contained in the samples' extract induced DNA SBs at the sites of specific DNA lesions in the substrate nucleoids. The accumulated DNA SBs (repair incisions) were measured after alkaline treatment by electrophoresis, similarly as in the case of the standard comet assay [218]. DNA loops containing DNA SBs were drawn towards the anode forming a comet-like image, subsequently visualised by a fluorescence microscope and analysed using semiautomated scoring software. The frequency of DNA SBs (represented by the proportion of total DNA in the comet tail) reflected the DRC of the extract. Background, treatment and specificity controls were used for all samples to calculate final BER capacity.

3.4.3 Assessment of MSI

MSI-high status correlates with the loss of expression of the main proteins involved in MMR (MLH1 and MSH2) and can, therefore, be used as a marker of a defect in the MMR pathway. This approach was used in Publication VIII. DNA for MSI status determination was extracted from tumour tissue and non-malignant adjacent mucosa using the DNeasy Tissue Kit (Qiagen). MSI status was ascertained using molecular testing of 5 mononucleotide-repeat markers (BAT 25, BAT 26, NR 21, NR 24, NR 27) run as a pentaplex using fluorescently labelled primers [219] and standard PCR chemistry. Fragment analysis was performed on ABI 3130 (Applied Biosystems). The final comparison between tumour and non-tumour DNA short tandem repeat profiles was performed with GeneMapper v4.1 software (Applied Biosystems). A tumour specimen was classified as MSI-high when two or more loci were unstable.

3.5 Statistical analysis

Individual studies used different statistical approaches according to the purpose of the study and were predominantly performed by an experienced biostatistician. Detailed pieces of information are reported in the enclosed Publications I–VIII. Statistical significance was set at $\alpha = 5\%$ threshold (P-value = 0.05). In GWAS, regions that were highly associated with CA frequency as determined by generally accepted suggestive significance threshold of $P = 1 \times 10^{-5}$ and the genome-wide significance threshold of $P = P = 5 \times 10^{-8}$ were further analysed with *in silico* online bioinformatics tools. Multiple testing corrections were performed using the Bonferroni test or the Benjamini-Hochberg false discovery rate. SNPs frequencies in healthy control individuals were tested for Hardy-Weinberg equilibrium. Descriptive statistical analyses were carried out for the measured parameters on the whole data set as well as on individual groups.

In case-control studies, the differences in investigated biomarkers between individual groups were tested by nonparametric tests (Mann-Whitney U-test, Kruskal-Wallis test, Median Two-Sample). The effect of each studied biomarkers on the cancer risk or the effect of SNPs on CA frequencies was determined by (multivariate) logistic and linear regressions and was calculated by estimating the odds ratios (ORs) with the 95% confidence intervals (CIs). Besides, adjusted ORs (aORs) for potential covariates (age, sex, smoking status, and occupational exposure) were used. Meta-analyses for GWAS and replication sets was performed, and meta P-values, ORs, effect sizes and heterogeneity index were recorded.

The relationships between individual biomarkers were tested using Chi-square test or Spearman's correlation, expressed by Spearman's rho (r_s) and graphically plotted by linear regression. Clinical outcomes were evaluated by calculating patients' 5-year overall survival (OS) and recurrence-free survival (RFS) or event-free survival (EFS). The standard Kaplan-Meier definition of events/censored data was used for OS and RFS/EFS analysis, depending on the particular study. The relative risk of death or recurrence was estimated as a hazard ratio (HR) with 95% CIs, with the use of the Cox regression. Moreover, multivariate analyses referred to as a classification & regression tree (CART) [220] using Cox regression model to identify the most prognostically significant interactions between investigated factors and patients' 5-year OS and RFS/EFS.

4. Results and Discussion

The individual parts of this section describe the results we have obtained based on the three main aims we have stated. The major findings from each publication (**Publications I–VIII**) included in this Thesis are discussed.

4.1 CAs, TL and DSB repair capacity and their association with cancer susceptibility and patients' clinical outcome

In this part, we aimed to examine whether CAs, TL and DSB repair capacity in PBLs are associated with cancer susceptibility and patients' clinical outcome. Also, we studied whether the telomere shortening contributes to the formation of CAs and whether inter-individual variation in DSB capacity may influence the levels of DNA damage and TL. The results are fully documented in attached **Publications I–III**.

4.1.1 CAs in PBLs as a biomarker of cancer susceptibility

First, we aimed to prove the hypothesis that CAs in PBLs may serve as a biomarker of cancer susceptibility. The results were published in the cross-sectional study entitled *"Structural chromosomal aberrations as potential risk markers in incident cancer patients."* by Vodenkova S *et al.* (2015) (**Publication I**, page 92). In this study, we evaluated the levels of CAs in PBLs in newly diagnosed colorectal, lung and breast cancer patients, and corresponding healthy control individuals. Besides, the attempt to relate CA frequencies to the clinicopathological characteristics was addressed for the first time. This study represented a free continuation of the study published by our Department in 2010 [209].

One of the major findings of the study identifies significant differences in distributions of all types of chromosomal damage in lung and breast cancer patients compared to corresponding control groups. In colorectal cancer patients, only CTAs were significantly elevated in comparison with controls. Frequencies of chromosomal damage in individual groups of cancer patients and healthy controls are summarised in Table 2.

Table 2. Frequencies of chromosomal damage in cancer patients and control healthy individuals.

Chromosomal damage (%)	General controls (N = 300)			CRC cases (N = 101)			P-value
	Mean ± SD	Median	Range	Mean ± SD	Median	Range	
ACs	1.82 ± 1.32	2	0–6	2.14 ± 1.43	2	0–6	0.057[a]
CAs	1.95 ± 1.47	2	0–7	2.27 ± 1.64	2	0–8	0.089
CTA	1.11 ± 0.99	1	0–4	**1.45 ± 1.28**	1	0–7	**0.031**
CSA	0.84 ± 1.13	0	0–6	0.82 ± 1.00	1	0–4	0.818

Chromosomal damage (%)	General controls (N = 300)			Lung cancer cases (N = 87)			P-value
	Mean ± SD	Median	Range	Mean ± SD	Median	Range	
ACs	1.82 ± 1.32	2	0–6	**2.86 ± 1.45**	3	0–6	**≤0.001**
CAs	1.95 ± 1.47	2	0–7	**2.90 ± 1.49**	3	0–7	**≤0.001**
CTA	1.11 ± 0.99	1	0–4	**1.86 ± 1.30**	2	0–6	**≤0.001**
CSA	0.84 ± 1.13	0	0–6	**1.05 ± 0.98**	1	0–4	**0.01**

Chromosomal damage (%)	Female controls (N = 158)			Breast cancer cases (N = 158)			P-value
	Mean ± SD	Median	Range	Mean ± SD	Median	Range	
ACs	1.82 ± 1.38	2	0–6	**2.62 ± 1.57**	3	0–7	**≤0.001**
CAs	1.93 ± 1.48	2	0–6	**2.73 ± 1.64**	3	0–7	**≤0.001**
CTA	1.11 ± 1.09	1	0–4	**1.65 ± 1.35**	1	0–6	**≤0.001**
CSA	0.80 ± 0.97	1	0–5	**1.08 ± 1.02**	1	0–4	**0.007**

The frequencies of chromosomal damage were tested with the non-parametric Mann-Whitney U-test. The significance level is 0.05. Significant values are in bold. [a]On the borderline of significance. Abbreviations: ACs – aberrant cells, CAs – total chromosomal aberrations, CTA – chromatid-type aberrations, CRC – colorectal cancer, CSA – chromosome-type aberrations, SD – standard deviation.

The results of our cytogenetic analysis were further supported by the use of binary logistic regression models, documenting the significant association of elevated CA frequencies with the susceptibility of particular cancer types. aORs with 95% CIs are summarized in Table 3. Elevated ACs, CAs, CTAs and CSAs significantly increased the risk of lung and breast cancer (except for CSAs in lung cancer). The only association of increased risk of colorectal cancer was found in case of elevated levels of CTAs in our set of patients. Our results confirmed the findings from large epidemiological prospective studies (summarized by Bonassi *et al.* [179, 221, 222] and Norppa *et al.* [223]). While the cohort study from Central Europe suggested that CSAs (rather than CTAs) are predictors of cancer risk [224], a pooled analysis of thousands of control individuals revealed an equally strong cancer predictivity of both CTAs and CSAs [225].

Table 3. Binary logistic regression models to discern the modulation of incident cancers by chromosomal damage end points and major confounders, such as age and smoking.

	CRC			Lung cancer			Breast cancer		
	aOR	CI	*P*-value	aOR	CI	*P*-value	aOR	CI	*P*-value
ACs (%)	1.11	0.93–1.31	0.256	**1.48**	**1.21–1.81**	**0.000**	**1.41**	**1.19–1.66**	**0.000**
Age (years)	**1.04**	**1.02–1.06**	**0.000**	**1.07**	**1.05–1.10**	**0.000**	0.99	0.97–1.01	0.189
Smoking (0 for non-smokers)	**1.82**	**1.12–2.94**	**0.015**	**9.09**	**4.54–20.0**	**0.000**	**2.56**	**1.56–4.17**	**0.000**
CAs (%)	1.07	0.92–1.25	0.369	**1.33**	**1.11–1.59**	**0.002**	**1.36**	**1.16–1.60**	**0.000**
Age (years)	**1.04**	**1.02–1.06**	**0.000**	**1.07**	**1.05–1.10**	**0.000**	0.99	0.97–1.01	0.153
Smoking (0 for non-smokers)	**1.85**	**1.14–3.03**	**0.013**	**10.0**	**4.76–20.0**	**0.000**	**2.56**	**1.54–4.17**	**0.000**
CTAs (%)	**1.25**	**1.01–1.55**	**0.037**	**1.70**	**1.33–2.18**	**0.000**	**1.33**	**1.09–1.62**	**0.005**
Age (years)	**1.04**	**1.02–1.06**	**0.000**	**1.08**	**1.05–1.10**	**0.000**	0.99	0.98–1.01	0.407
Smoking (0 for non-smokers)	**1.75**	**1.09–2.86**	**0.021**	**10.0**	**5.00–20.0**	**0.000**	**2.56**	**1.56–4.17**	**0.000**
CSAs (%)	0.91	0.73–1.13	0.402	0.97	0.76–1.24	0.815	**1.45**	**1.13–1.86**	**0.004**
Age (years)	**1.04**	**1.02–1.06**	**0.000**	**1.07**	**1.05–1.10**	**0.000**	0.99	0.97–1.00	0.141
Smoking (0 for non-smokers)	**2.00**	**1.23–3.23**	**0.005**	**11.1**	**5.56–20.0**	**0.000**	**3.03**	**1.85–5.00**	**0.000**

The significance level is 0.05. Significant values are in bold. Abbreviations: ACs – aberrant cells, aOR – odds ratio adjusted for main confounders, CAs – total chromosomal aberrations, CI – confidence interval, CTA – chromatid-type aberrations, CRC – colorectal cancer, CSA – chromosome-type aberrations, SD – standard deviation.

Furthermore, we have found significant differences in the distribution of terminal deletions between breast cancer patients and female controls (0.39 ± 0.64 vs 0.18 ± 0.41, $P \leq 0.05$), and the binary logistic regression revealed the association between frequency of terminal deletions and the risk of breast cancer (aOR = 1.73, 95% CI = 1.04-2.89, P = 0.03). Whether this observation is attributable to the importance of DSB repair in the etiopathogenesis of breast cancer or is rather representing random finding remains to be investigated [171].

Since some authors assume that chromosomal damage in PBLs in cancer patients may reflect progression (stage) of the tumour rather than being a biomarker of cancer susceptibility [226], we, therefore, compared the differences in CA frequencies for particular TNM stages and histopathological grades but did not record any association. CAs were neither associated with additional clinicopathological characteristics. In conclusion, this study supported the concept of using CAs in PBLs as a biomarker of early carcinogenic effect and clearly suggested the role of CAs as biomarkers of breast and lung cancer susceptibility, whereas their prognostic value warrants further investigation.

4.1.2 The impact of telomere shortening on the levels of CAs and their association with patients' clinical outcome

The study *"Chromosomal damage and telomere length in peripheral blood lymphocytes of cancer patients."* by Vodenkova S *et al.* (2020) (**Publication II**, page 100) represents

a free continuation of Publication I. Several years elapsed since its publishing, enabling us to focus on the patients' follow-up in the present study. Moreover, we supplemented the study by measurement of TL. The primary purpose of this study was to investigate the relationship between frequencies of particular types of CAs and TL in PBLs of incident cancer patients and corresponding control individuals. Obtained results were analysed together with collected baseline, clinicopathological, and follow-up data.

From descriptive statistics, by comparison of TL between cases and controls, breast cancer patients showed significantly longer TL compared to control women (1.54 ± 2.0 vs 1.88 ± 9.0, P < 0.0001). Moreover, a group of breast cancer patients had the longest TL out of all investigated groups (colorectal and lung cancer patients and all control individuals). This observation may be explained by the fact that females have longer telomeres than males [227]. Several hypotheses have been postulated to clarify this association, one of which suggested that this is caused by the presence of oestrogen [228, 229]. An oestrogen-responsive element is present in a catalytic subunit of the enzyme telomerase [230]. Oestrogen may, therefore, stimulate telomerase to add telomere repeats to the chromosome ends. It is also known that overexpression of oestrogen is one of the typical features of breast tumours and predispose the risk of breast cancer. Indeed, those individuals with longer telomeres were at increased risk of breast cancer by 65 % (aOR (adjusted for age and sex) = 6.49, 95% CI = 3.00-14.04, P < 0.0001). However, the previously published studies, both prospective and retrospective, showed that the association of TL in PBLs with breast cancer risk is still conflicting [231-238].

Regarding the relationship between CAs and TL, Li *et al.* [197] and Xu *et al.* [239] provided the evidence that CAs may rather arise as a consequence of telomere shortening than as a result of the direct DNA damage. Therefore, we have further correlated TL with the frequencies of all types of CAs, and both TL and CAs with age. We have found a negative correlation between ACs, CAtot, CSAs and TL in the whole group of controls (Figure 11) as well as the negative correlation between TL and age (r_s = -0.62, P < 0.0001). Our data were in accordance with previously published data by Hemminki *et al.* [198]. Nevertheless, except for the correlation between TL and age in control individuals, all the remaining statistically significant results should not be over-interpreted as the Spearman's rho were small. It means that even if the relationships may be statistically significant, they may not be biologically as important. Cancer patients did not exhibit any

relationship between either TL and CA frequencies or TL and age, except for lung cancer patients (data not shown). It may be probably due to the fact that in complex diseases such as cancer, more interactive pathways contribute to the formation of DNA damage and chromosomal instability.
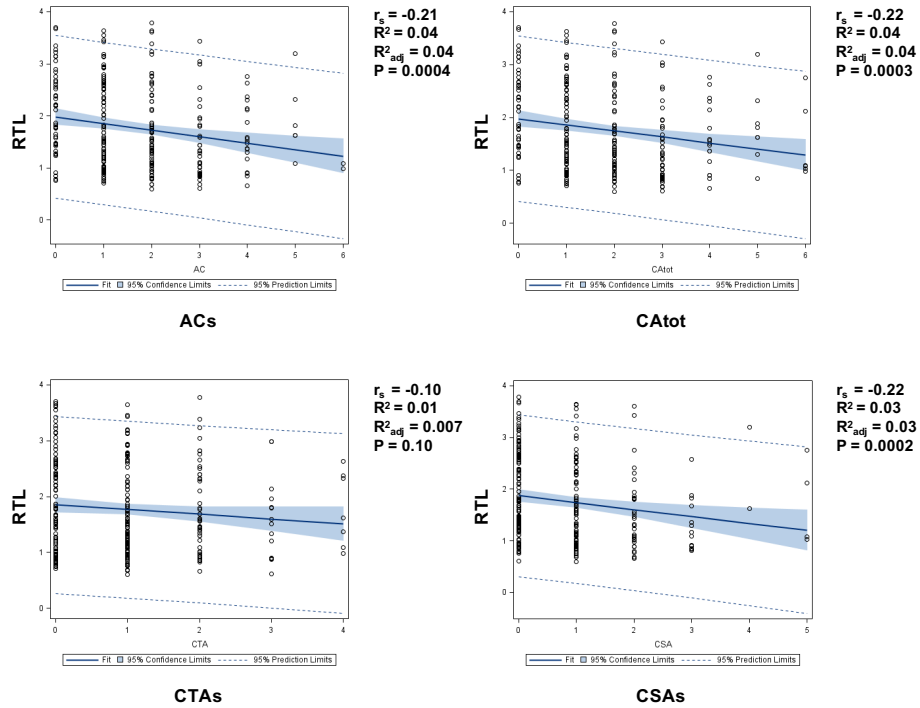
Figure 11. Spearman's correlation between ACs, CAtot, CTAs, CSAs and TL in control individuals.

The figure represents the relationships between ACs/CAtot/CTAs/CSAs and TL, which were investigated using Spearman's correlation, expressed by Spearman's rho (r_s) and graphically plotted by linear regression. CA frequencies are presented on the x-axis, and the value of TL expressed as RTL on the y-axis. Abbreviations: ACs – aberrant cells, CAtot – total chromosomal aberrations, CSAs – chromosome-type aberrations, CTAs – chromatid-type aberrations, RTL – relative telomere length, TL – telomere length.

Until now, the prognostic value of CAs and TL in PBLs in cancer patients remains unclear since conflicting results are reported [240-244]. The hypothesis that different CA frequencies and TL variations are determinants of prognosis is plausible to explain the heterogeneity in clinical outcomes of cancer patients. In our sets of patients, we did not observe any association between any type of CAs and OS or RFS using univariate survival analysis. However, outputs obtained from the OS CART analysis showed involvement of CTAs in the determination of patients' survival/mortality (Figure 12). Regarding TL, we did not find any association of telomere shortening with OS and RFS in our groups of cancer patients, either using a univariate or multivariate survival model.

In summary, we provided pilot data on CAs and TL in PBLs in the context of cancer susceptibility, patients' prognosis, and long-term survival. We observed that individuals with longer TL in PBLs were at increased risk of breast cancer. Accumulation of CTAs in PBLs was associated with decreased OS in breast and colorectal cancer patients after their stratification according to disease characteristics. In contrast to control individuals, cancer patients did not exhibit any relationship between either TL and CA frequencies or TL and age. We propose that there is a need to conduct more studies to elucidate the association between CAs and TL in PBLs of cancer patients.
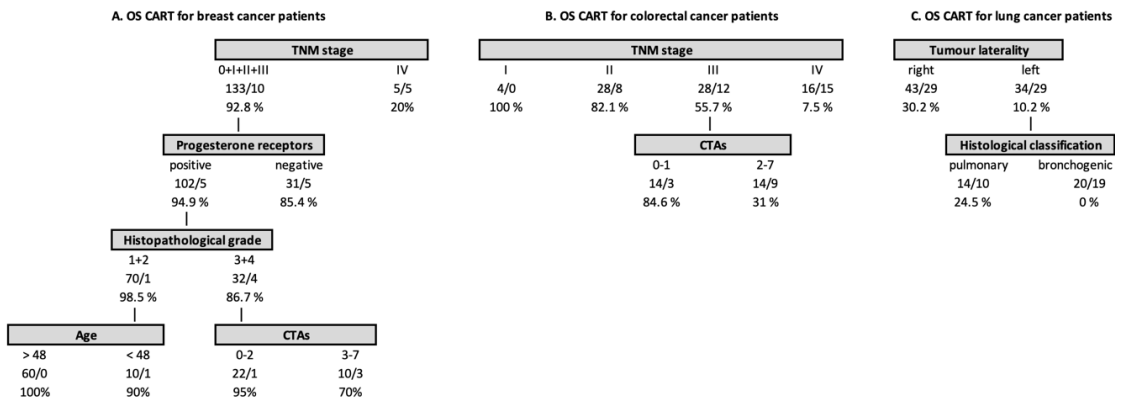
Figure 12. Overall survival l classification & regression trees for breast (A), colorectal (B), and lung cancer (C) patients.

Classification & regression trees represent the results of multivariate survival analysis (using the Cox regression hazard model). Numbers under each node show the total number of cases in a particular subcategory/number of events and percentages of patients with 5-year OS. Abbreviations: CART – classification & regression tree, CTAs – chromatid-type aberrations, OS – overall survival, TNM – tumour-node-metastasis.

4.1.3 Variations in DSB repair capacity and their impact on cancer susceptibility, chromosomal damage and TL

Finally, the study *"Bleomycin-induced chromosomal damage and shortening of telomeres in peripheral blood lymphocytes of incident cancer patients."* by Kroupa M *et al.* (2018) (**Publication III**, page 129) was aimed to employ the mutagen sensitivity for assessment of the potential interactions between induced DSBs in PBLs and individual's predisposition to breast and colorectal cancer. Inter-individual variations in DSB repair capacity were evaluated *in vitro* utilizing the G2 chromosomal MSA to quantify bleomycin-induced CTAs [216]. Besides, we also measured TL and compared mutagen sensitivity to γ-H2AX phosphorylation which represents a hallmark of DSB [215]. To our

knowledge, this was the first study describing DSB repair capacity in breast and colorectal cancer patients in correlation with a shortening of telomeres.

The mutagen sensitivity has been employed as one of the well-established markers for cancer susceptibility [245]. It is expressed as a mean number of CTAs per cell at metaphase PBLs and measured by following bleomycin exposure in the G2 phase of the cell cycle. Several studies have considered that this functional test may reflect the inter-individual differences in DRC [216, 245-252]. The reported increased amount of CTAs correlates with suboptimal ability to repair DSBs, suggesting that the outcome from MSA may reflect DSB repair capacity [253].

Results of this study suggested that altered DSB repair in PBLs occurs particularly in colorectal patients (158 ± 0.6 vs 130 ± 0.4, $P = 0.03$). However, the bleomycin sensitivity profile of breast patients was similar to that of the control population (data not shown). These results were in agreement with Hsu *et al.* [216] who confirmed that bleomycin response profile in PBLs differs in the colorectal, lung, and head/neck cancer patients (but not in breast cancer patients) compared to healthy controls. On the other hand, some studies have shown strong bleomycin sensitivity not only for familial breast cancer but also for sporadic patients [249, 254, 255]. A possible explanation for these discordant results could be different aetiology of familial breast cancer as compared to the sporadic form and the heterogeneity of breast tumours. Mutated *BRCA1* and *BRCA2* genes in familial breast cancer, which are involved in DSB repair machinery [256], could cause higher DNA damage in bleomycin exposed PBLs. Natarajan *et al.* postulated that mutagen sensitivity phenotype is a risk factor for breast cancer [254].

Mutagen sensitivity profiles were compared with the measurement of γ-H2AX as a hypothesized alternative approach for MSA that is time-consuming. We postulated that quantification of chromosomal damage after bleomycin treatment in PBLs might be comparable with the concentration of γ-H2AX. Unfortunately, our results did not show any correlation between these two approaches. In contrast, the concentration of γ-H2AH was higher in colorectal cancer patients who exhibited higher level of CTAs, suggesting a lower DSB repair capacity. We thus assumed that γ-H2AX is the first acute response of the cell to cope with DSB; however, CTAs are the final results of unrepaired DSBs with the absence of γ-H2AX. Therefore, the γ-H2AX measurement could not be used instead of MSA.

We further focused on the measurement of TL and the results were used for the study of associations between TL and mutagen sensitivity outcomes. We confirmed the observation from Publication II that women show longer TL than men (P = 0.0008), and therefore, results were adjusted both for age and sex. It has been documented that telomere shortening has also been linked to reduced DSB repair capacity [257]. Our results showed significant correlation between telomere shortening and mutagen sensitivity profile in a pooled group of cancer patients (r_s = -0.36, P = 0.02); however, the same trend was not detected in a control group.

In summary, results of this study suggested that altered DSB repair in PBLs is mainly associated with colorectal cancer susceptibility. Our results showed a significant correlation between telomere shortening and mutagen sensitivity profile in a pooled group of cancer patients; however, the same trend was not detected in a control group. Above observations added further information to the chain of evidence on the interplay between telomere complex and DSB.

4.2 Genetic basis of inter-individual variations in CA frequency

In this part, we aimed to explore the genetic basis of inter-individual variations in CA frequency in PBLs and whether it depends on the level and type of exposure by finding novel SNPs predisposing to the formation of CAs and potentially to cancer. We also examine whether SNPs in DNA repair genes and other genes and their interactions are associated with the levels of CAs. The results are fully documented in attached **Publications IV–VI**.

4.2.1 Interactions of SNPs in DNA repair genes and their association with CAs

The study *"Interactions of DNA repair gene variants modulate chromosomal aberrations in healthy subjects."* by Vodicka P *et al.* (2015) (**Publication IV**, page 139) was aimed at investigating functional variants in DNA repair genes in relation to CAtot, CTAs, and CSAs in healthy individuals. DNA repair represents a key player in the formation of structural CAs [169] and individual DRC in response to DNA damage, effectively preventing an accumulation of CAs, is often modulated by the gene variants in different DNA repair pathways [258-260]. Therefore, we examined the hypothesis that SNPs in

the BER (*XRCC1*, *hOGG1* and *APE1*), NER (*XPA*, *XPC*, *XPD* and *XPG*) and DSB repair (*XRCC2*, *XRCC3*, *NBN* and *RAD54L*) genes and their gene-gene interactions may modulate frequencies of structural CAs in a large set of healthy subjects.

In the whole set of 2196 individuals, the mean ± SD frequencies of CAtot, CTAs and CSAs were 1.54 ± 1.54 %, 0.74 ± 0.98 % and 0.80 ± 1.16 %, respectively, with median and range being 1 (0-11), 0 (0-6) and 0 (0-10). CAs as well as the constituent CTAs and CSAs were significantly increased in occupationally exposed subjects (OR = 2.36, 95% CI = 1.97-2.83, P < 0.01; OR = 1.73, 95% CI = 1.45-2.06, P < 0.01; and OR = 1.64, 95% CI = 1.38-1.96, P < 0.01, respectively). These observations were in accordance with previously published reports for different compounds [177, 207, 261-265].

By assessing functional SNPs in individual DNA repair genes, we observed a strong association between variant GG genotype in *XPD* rs13181 and decreased CTA frequency (OR = 0.64, 95% CI = 0.48-0.85, P = 0.004; n = 1777 subjects). Our study on such a large cohort confirmed our earlier observations on 225 healthy subjects [266] and later study on 140 subjects with higher age [267]. XPD represents an important helicase involved in NER, which communicates with other DNA repair gene products in dealing with exogenous DNA damage [268], but the functional role of *XPD* rs13181 remains unclear. Further, a novel observation was found, a significant association of CT genotype in *RAD54L* rs1048771 with increased CSAs was also observed (OR = 1.96, 95% CI = 1.01-4.02, P = 0.03; determined in 282 subjects with available genotype). RAD54L exhibits a DNA-dependent ATPase and supercoiling activities and plays a role in the HR* pathway [269, 270]. However, this association was less robust due to the number of subjects with available genotype. Individually, a small risk is irrelevant, but the combination of several low-risk alleles can add up to substantial risks, even in the absence of multiplicative statistical interactions [271].

By addressing pair-wise gene-gene interactions, we have discovered 14 interactions significantly modulating CAs, 9 CTAs and 12 CSAs frequencies. Highly significant interactions always included pairs from two different pathways. **Regarding CAs**, significant gene-gene interactions were mainly observed for genes involved in BER (*APE1, hOGG1*), NER (*XPC, XPD*) and DSB repair (*XRCC3*) together with other DNA repair gene variants (*NBS1, XRCC2* and *XPG*). Interestingly, *NBS1* rs1805794 appeared most often in these interactions; although interactions with BER gene variants resulted in

the higher CA frequency, the opposite effect was recorded for the interactions with NER gene variants. NBS1 plays an important role in the maintenance of genome integrity by being involved in the cellular DDR. The opposite effect on CA frequencies in the interplay of *NBS1* variants with either BER or NER SNPs is certainly interesting and may reflect the specificity of these two excision repair pathways towards different types of DNA damage.

For CTAs, the combinations of homozygous variant genotypes of *XPD* rs13181 with *XPG* rs17655 (OR = 0.54, 95% CI = 0.34-0.84, P = 0.006) or *XRCC1* rs25487 (OR = 0.68, 95% CI = 0.48-0.96, P = 0.03) genes showed decreased frequencies of CTAs. So did the combination of variant alleles in *hOGG1* and *XRCC3* (OR = 0.52, 95% CI = 0.27-0.98, P = 0.04). On the contrary, a combination of variant alleles in DSB repair genes (*XRCC3* rs861539 and *XRCC2* rs3218536) resulted in the significant increase of CTAs (OR = 2.56, 95% CI = 1.02-6.40, P = 0.05). These results point again to an effect of the G allele of *XPD* Lys751Gln on CTA frequency modulation as stated above.

For CSAs, again variant alleles in *XRCC1* rs25487 and *hOGG1* rs1052133 (both BER genes) in combination with homozygous variant genotype in *XPG* rs17655 resulted in significantly decreased frequencies of CSAs (OR = 0.22, 95% CI = 0.08-0.66, P = 0.007, and OR = 0.72, 95% CI = 0.54-0.97, P = 0.03, respectively). Interestingly, variant G allele in *hOGG1* rs1052133 in combination with variant alleles in genes involved in NER or DSB repair resulted in decreased frequencies of CAs, CTAs and CSAs, despite the fact that variant G allele is associated with the lower capacity to repair oxidative DNA damage [272]. This phenomenon may be connected with the fact that 8-hydroxy-deoxyguanine adducts may block replication fork, thus preventing the accumulation of CAs.

In summary, CAs arise as a consequence of the interaction between occupational exposure to various genotoxicants and individual genotype configuration. In this study, we tested the impact of functional SNPs in DNA repair genes on the frequency of CAs. Although individual variants in genes encoding DNA repair proteins modulated CAs only modestly, several gene-gene interactions evinced either enhanced or decreased CA frequencies. As suggested by Melis *et al.* [273] and now confirmed by us, the complex mechanism of CAs accumulation requires complex interplay between different DNA repair pathways. However, the mechanism may not be tracked without the knowledge of the experimentally proven functional impact of DNA repair gene variants.

4.2.2 Finding novel SNPs predisposing to the formation of CAs and potentially to cancer

Based on the results from Publication IV and other previous candidate gene studies which have mainly focused on genes important for the maintenance of genome integrity, such as DNA repair, mitotic checkpoint and metabolic pathways, as reviewed in Publication IX, in the following two publications (**Publication V** and **Publication VI**), we designed two GWAS. The main goal was to discover previously unknown, potentially functional loci predisposing to CAs. These studies were the first GWAS of this nature.

In the study entitled *"Distinct pathways associated with chromosomal aberration frequency in a cohort exposed to genotoxic compounds compared to general population."* by Niazi Y *et al.* (2019) (**Publication V**, page 148), we conducted two GWAS on healthy individuals in the presence (exposed group) and absence (reference group) of apparent genotoxic exposure with the primary aim to explore the genetic basis of the variation in CA frequency between individuals and whether it depends on the level and type of exposure.

In the exposed group, the proportion of individuals with high CAtot was 56 %, while it was only 29 % in the reference group, and the distribution of CA frequency differed significantly between the two groups ($P = 4.46 \times 10^{-19}$). GWAS were performed on both groups and several associations at the suggestive level of significance ($P \leq 1 \times 10^{-5}$) with *in silico* predicted functionality were found for all three CA phenotypes (CSA, CTA and CAtot) in both logistic and linear models. Since the samples sets and CA frequency differed, and because the CAs are measured as a number of aberrations per 100 cells, we used both of these models to evaluate the associations between the SNPs and CA frequencies.

In **the reference group**, 18 different loci showed an association at the suggestive level of significance. *In silico* analysis predicted functional consequences for five of the loci (see Table 2 in Publication V). In the CAtot analysis, logistic regression model implicated the locus p15.2 on chromosome 11 with rs10585869 as the top hit. In the linear model, two loci 7q11.21 and 8q22.3 showed significant associations and *in silico* predicted functional consequences with top SNPs rs9647884 and rs2293982, respectively. Additional two loci 5q35.2 and 12q22 were associated with CTAs and CSAs, respectively, in the linear model. GWAS on **the exposed group** revealed 11 associations at the suggestive level of

significance. After the *in silico* analysis, four loci were selected from the CTA and CSA linear and logistic models (see Table 3 in Publication V). Both the top SNPs in the CTA linear model analysis (rs56217929 at 2q33.3) and CTA logistic model analysis (rs10040952 at 5p15.31) almost reached the genome-wide significance. We further performed **a meta-analysis** in an attempt to identify loci predisposing to CAs independent of exposure. Three loci had P-values $\leq 1.0 \times 10^{-5}$, none to moderate heterogeneity between the two groups and *in silico* predictions suggesting functionality. These were rs11792561 at 9q22.2 in the CAtot linear model analysis, rs2933639 at 5q23.1 in the CTA logistic regression model analysis and rs8054859 at 16q12.2 in the CSA logistic regression analysis (see Table 4 in Publication V).

In this study, we identified novel loci that were located in or near genes related to DNA repair/DDR and chromatin modulation and chromosome segregation. Interestingly, apart from *COPRS* [274] and *FTO* genes [275], which were observed in the analysis on exposed group and meta-analysis, respectively, all other loci were identified from the reference group's analysis. These included *PSMA1*, *UBR5*, *PMS2P4*, *STAG3L4*, and *BOD1* genes [276-280]. Besides, several top hits from both groups were located in the genes related to tumour progression or suppression. All these SNPs are located within regulatory elements with the potential to affect the expression of the respective genes – for detail, see Publication V. It is also interesting to note that various SNPs in *ITGB3* have also been found to be associated with autism aetiology, a disease associated with chromosomal abnormalities [281]. Other two loci found associated with autism were identified in the CTA analysis of the exposed group. These were 5p15 (rs10040952) [282] and 2q33.3 (rs56217929) [283] near the *KLF7* gene. In the reference group, CAs may have arisen as a result of internal factors, reduced DNA repair or epigenetic deregulation. We can speculate that the higher burden of CAs induced by exposure to different genotoxic agents is not only influenced by individual variability in the genes dealing with different types of DNA damage but also in other genes related to tumorigenesis (including genes involved in metabolism and transport). Thus, distinct causes of CA increase between exposed and reference groups could explain the differences between the GWAS findings.

The following study entitled *"Genetic variation associated with chromosomal aberration frequency: A genome-wide association study."* by Niazi Y *et al.* (2019) (**Publication VI**, page 157)was also based on the GWAS approach in investigating SNPs related to CA

frequencies. To have a broad insight into the genetic susceptibility associated with CA frequency, the sample sets included in this study composed not only of non-exposed and differentially exposed individuals but also of newly diagnosed, untreated cancer patients, who may represent a population with increased susceptibility to CAs. We first conducted the GWAS on discovery sample set followed by replication on two independent sample sets (replication 1 and 2).

Occupational exposure significantly influenced CA frequency in the GWAS discovery sample set ($P = 1.21 \times 10^{-9}$). In replication 1, the most significant variable affecting CA frequency was cancer status ($P = 7.56 \times 10^{-6}$), while in replication 2, the effect of occupational exposure was moderate ($P = 0.009$). Both logistic and linear regression models for the same reasons as in Publication V were applied for the analysis of all three CA phenotypes (CSA, CTA and CAtot). No SNP associations at the level of $P \leq 1 \times 10^{-5}$ were found in CSAs. However, it is known that CSAs are affected to a lesser extent by chemical mutagens, to which our populations were mainly exposed, as compared to CTAs [284]. Altogether 11 loci, six from the CAtot and five from the CTA analysis, were chosen for replication and the most significant SNPs with $P \leq 1 \times 10^{-5}$ from these loci were selected on the basis of *in silico* analyses (see Table 3 in Publication VI).

Regarding CAtot, the logistic regression model showed more significant associations than the linear model; however, almost all the loci showed similar trends in the linear model as well. For all SNPs, except for rs16931167, replication 1 showed ORs on the same direction as in the GWAS and the strongest associations in the meta-analysis were for rs1383997 at 8q13.3 (OR = 0.6, 95% CI = 0.49-0.73, $P = 3.44 \times 10^{-7}$) and rs2824215 at 21q21.1 (OR = 1.57, 95% CI = 1.29-1.91, $P = 8.7 \times 10^{-6}$). Replication 2 did not give much support for the GWAS associations, and the strongest association in the meta-analysis of all populations with $P = 4.01 \times 10^{-5}$ was for rs12002628 at 9q21.13. Rs1383997 and rs12002628 are located in the gene related to transient receptor potential (TRP) cation channels *TRPA1* and *TRPM3*, respectively. TRP channels regulate the Ca^{2+} ions homeostasis in response to environmental and chemical factors. Any deregulation in Ca^{2+} distribution patterns can promote the signs of cancer development such as proliferation, enhanced survival and invasion [285]. The other SNP from the CAtot analysis, rs2824215 is located in a long intergenic noncoding RNA, and deletion in this locus has been linked to autistic features with complex chromosomal rearrangements [286]. Interestingly, two

other SNPs, which we selected for replication, rs17215792 (2q33.3) and rs2837619 (21q22.2) are located in the genes associated with autism and Down syndrome, *KLF7* [283, 287] and *DSCAM*, respectively [288, 289].

For the CTA analysis, on the other hand, higher associations were found in the linear model as compared to the logistic model. Five SNPs showed an association at the suggestive level of significance. Here also, the GWAS and replication 1 showed more similar associations than the GWAS and replication 2. In the meta-analysis of the GWAS and replication 1, one association, rs983889 at 5p15.1 remained statistically significant at the suggestive level (P = 1.06×10^{-5}), and no significant associations were observed in the meta-analysis of all three populations. Although the SNPs from the GWAS were selected based on the linear model, we also calculated the ORs and 95% CIs in the logistic model. For the most significant SNP, rs983889, the OR was 0.65 (95% CI = 0.52-0.80) in the meta-analysis of the GWAS and replication 1. Rs983889 is an intronic SNP in the *FBXL7* gene. FBXL7 belongs to F-box proteins, which are involved in phosphorylation-dependent ubiquitination of proteins and which display proapoptotic activity [290]. Incidentally, one of the targets of FBXL7 is the *AURKA* gene, a known oncogene, involved in the regulation of mitosis [291]. During the late G2 phase, AURKA is recruited to centrosomes [292] and later on promotes centrosome maturation and bipolar spindle formation [293]. Since CTAs also arise during S/G2 phase [171], an indirect involvement of AURKA can be anticipated to affect the frequency of CTAs.

In summary, our GWAS identified new SNPs associated with CA frequency, from which three were replicated at the suggestive level of significance in Publication VI. In Publication V, these variants were found in genes involved in DDR/repair, segregation of chromosomes and chromatin modification. Others were related to apoptosis, cell proliferation, angiogenesis and tumorigenesis. Three different variants are directly or indirectly related to autism/autistic traits, a condition linked to chromosomal abnormalities. In Publication VI, *in silico* predictions of functional consequences of the identified SNPs and their loci revealed that they were directly or indirectly related to different cancers. They included genes encoding TRP cation channel proteins, genes involved in autism and Down syndrome, and FBXL7, which interacts with AURKA, an important regulator of mitosis. Our results suggest a complex interaction of various genetic factors responsible for the inter-individual differences in CA frequency in the

presence and absence of evident exposure to genotoxins, many of which are still unexplored. Due to the sample size, the results of these GWAS are not definitive in terms of pointing out the exact rationale behind CAs development, they certainly point towards the probable loci that could be involved in the elevated frequency of CAs in the presence of environmental stress. Although further functional studies will be warranted to unravel the mechanism behind these interactions, the results of these studies help narrow down the essential genes and pathways behind them. Identification of new genetic variants for the frequency of CAs offers prediction tools for cancer risk in future.

4.3 DNA repair and its association with cancer susceptibility, patients' therapy response and clinical outcome

The primary aim of last two studies (**Publication VII** and **Publication VIII**) included in this Thesis was to find differences in DNA repair in colorectal cancer patients which may aid in stratifying patients according to predicted therapy response and patients' survival. It will lead to an individual approach to patients and may be an attractive target for therapeutic intervention strategies. While Publication VII was based on investigating genetic variants in DNA repair genes, Publication VIII investigated BER at the functional level as BER-DRC along with MSI.

4.3.1 SNPs in DNA repair genes and their association with cancer susceptibility and patients' clinical outcome

The next hypothesis-based study entitled *"Functional polymorphisms in DNA repair genes are associated with sporadic colorectal cancer susceptibility and clinical outcome."* by Jiraskova K *et al.* (2018) (**Publication VII**, page 170) was aimed at evaluating the relevance of 16 functional SNPs in 12 DNA repair genes (*EME1, FAAP24, FANCI, MUS81, NEIL3, POLE, POLN, POLQ, RAD51D, REV1, REV3L* and *RPA1*) on the risk of colorectal cancer development (in a case-control study) and modulation of patients' clinical outcome after cancer diagnosis (in a follow-up study). Selected SNPs were tested independently on two sample sets, the discovery and replication sets.

Regarding the case-control study, the carriers of the variant AA genotype in *REV3L* rs3204953 were observed as associated with an increased risk of colorectal cancer (P =

0.006) in the discovery set. SNPs in this gene in association with cancer susceptibility were also observed in published literature. The same SNP was recognized to be associated with a higher risk of breast cancer in Swedish cohort [294], and different SNPs in *REV3L* gene have been found to be associated with breast, stomach, and colorectal cancer [294-296]. Apart from the deleterious nature of the protein function, the amino acid change in REV3L was *in silico* predicted to decrease the protein stability [297]. In cancer cell lines, the importance of accurate regulation of *REV3L* expression was demonstrated; while its inhibition induced a growth arrest, the overexpression led to increased spontaneous mutation rates [298]. A decreased expression levels have also been reported in tumour tissue compared to the non-malignant adjacent mucosa in colon cancer [299, 300]. Unfortunately, these promising results obtained from the discovery sample set were not confirmed in the replication sample set. Nevertheless, since *REV3L* emerged several times as being a significant modulator of patients' OS and EFS in the replication set, we suppose that *REV3L* gene may have an impact on colorectal cancer susceptibility as well as on patients' survival and therapy response and further investigations are warranted.

In the follow-up study, several SNPs revealed to be associated with either patients' 5-year OS or EFS by investigating the interactive effects of genotypes and clinicopathological parameters using CART analysis. Only a few of these SNPs were shown as significant splits more than once in the final structure of the tree, suggesting their potentially greater relevance on patients' survival. SNPs in *POLQ* gene appeared as an optimal split factor in OS CART for the discovery set four times (rs1381057, rs3218649 twice, and rs3218651), and in the replication set four times as well (rs1381057 twice and rs3218651 twice (Figure 13). At least nine out of 23 known *POLQ* SNPs in the human are predicted to alter protein function [301], and several SNPs have also been associated with the risk of different cancers [294, 302-304]. In addition to the deleterious nature of the protein function, the amino acid change in POLQ was *in silico* predicted to decrease the final protein stability. In has been demonstrated that upregulation of *POLQ* was present in different tumour tissues, and this overexpression was in association with the patients' prognosis [305-308]. Based on the data from published studies, we suppose the significance of adequate *POLQ* functioning and regulation for tumour suppression.

Regarding the 5-year EFS CART analysis, *NEIL3* rs7689099 revealed twice as the optimal split factor in the discovery cohort (Figure 14). Different SNPs in *NEIL3* gene

were associated with the risk of several cancers [309-311]. Specifically, rs7689099 was associated with a decreased risk of differentiated thyroid carcinoma and prostate cancer [310, 311]. Similarly, as in previously mentioned *REV3L* and *POLQ*, upregulated expression levels of *NEIL3* were found in tumours of 20 cancer sites, including colorectal cancer [312, 313]. The overexpression was further observed in association with the progression to distant metastasis in melanoma [314]. The association of the SNP in the *NEIL3* gene with patients' survival was not detected in the replication sample set. However, considering the available data, we propose that the variation of the *NEIL3* gene also has the relevance for colorectal cancer susceptibility as well as patients' survival and therapy response.

In summary, this study evaluated the association of SNPs in DNA repair genes selected by likely functional relevance with colorectal cancer. The data suggested that even amino acid substitution causing subtle alterations in the specific proteins that function in DNA repair pathways may lead to inaccurate DNA repair, and thus play a role in colorectal cancer pathogenesis.

(A)
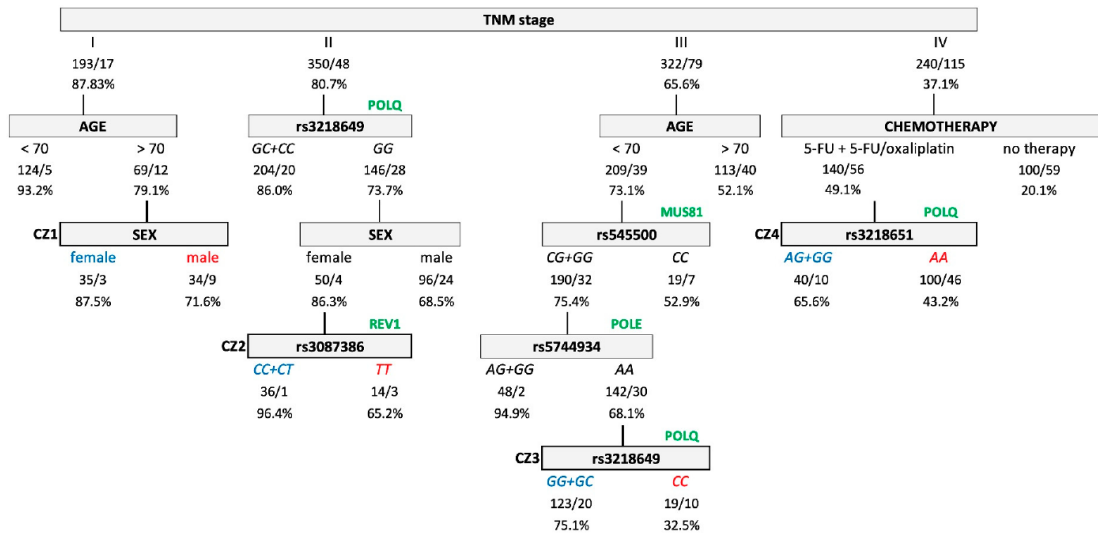
Figure 13. Overall survival classification & regression trees of colorectal cancer patients from the discovery sample set (A) and replication sample set (B).

Classification & regression tree represents the results of multivariate survival analysis (using Cox regression hazard model). Numbers under each node show the total number of cases in a particular subcategory/number of events and percentages of patients with 5-years OS. Corresponding Kaplan-Meier curves represent the differences in OS for each node. Abbreviations: 5-FU – 5-fluorouracil, TNM – tumour-node-metastasis.

(B)

TNM stage			
I	II	III	IV
186/32	210/33	331/84	114/90
82.8%	84.3%	74.6%	21.1%

AT7 — AGE
< 60 / > 60
54/3 — 132/29
94.4% — 78.0%

AGE
< 70 / > 70
146/13 — 64/20
91.1% — 68.8%

AT10 — AGE
< 70 / > 70
241/51 — 90/33
78.8% — 63.3%

RAD51D — rs4796033
CC / CT+TT
107/6 — 39/7
94.4% — 82.1%

AT9 — REV3L — rs3204953
GA+AA / GG
18/2 — 46/18
88.9% — 60.9%

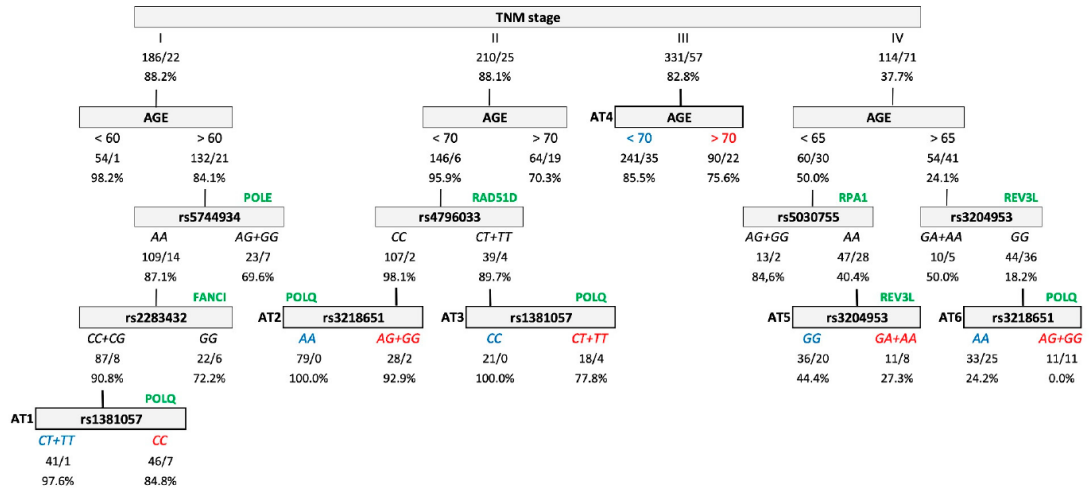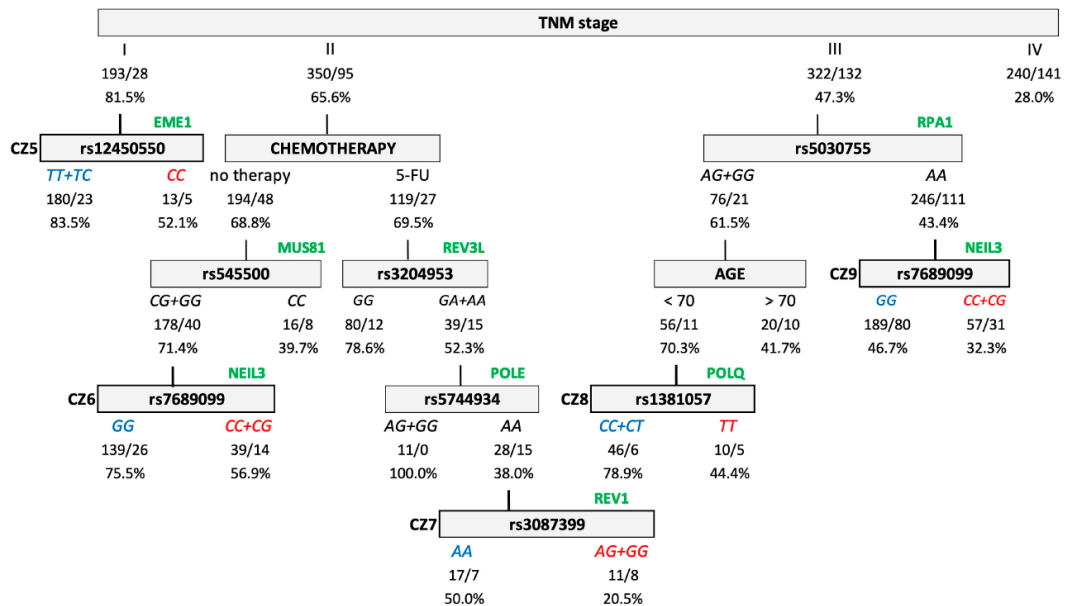AT8 — REV1 — rs3087386
CT+TT / CC
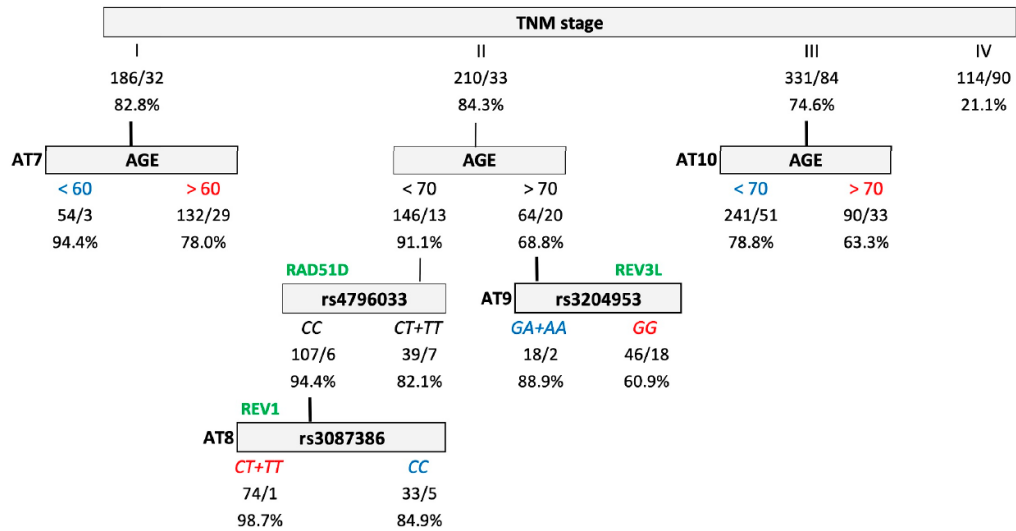74/1 — 33/5
98.7% — 84.9%

Figure 14. Event-free survival classification & regression trees of colorectal cancer patients from the discovery sample set (A) and replication sample set (B).

Classification & regression tree represents the results of multivariate survival analysis (using Cox regression hazard model). Numbers under each node show the total number of cases in a particular subcategory/number of events and percentages of patients with 5-years OS. Corresponding Kaplan-Meier curves represent the differences in OS for each node. Abbreviations: 5-FU – 5-fluorouracil, TNM – tumour-node-metastasis.

4.3.2 DNA repair capacity and its association with patients' therapy response and clinical outcome

The study entitled *"Base excision repair capacity as a determinant of prognosis and therapy response in colon cancer patients."* by Vodenkova S *et al.* (2018) (**Publication VIII**, page 193) was aimed at investigating BER-DRC and MSI in relation to 5-FU response of colon cancer patients as potential predictive and/or prognostic biomarker. Since BER recognizes and removes mis-incorporated uracil and 5-FU from DNA and MMR removes mismatched nucleotides and drives 5-FU-induced cytotoxicity [26,27], we thus hypothesized that the deregulation of and individual variation in BER and MMR might be significant factors in poor 5-FU response and decreased patients' survival. With this in mind, we designed this follow-up study of which the main aim was to investigate BER-DRC, further supplemented by MSI status determination in paired samples of tumour tissue and non-malignant adjacent mucosa of colon cancer patients.

This study failed to identify any significant differences overall in the level of BER-DRC between these two types of tissue (mean ± SD: 9.91 ± 10.32 vs 10.82 ± 12.01, P = 0.89).

However, we observed a significant correlation between BER-DRC in the paired samples ($r_s = 0.68$, $P < 0.0001$). The obtained results were in agreement with previously published studies [202, 315]. They may suggest that tumour cells do not become deficient in BER during the process of carcinogenesis but rather follow patterns characteristic for each individual and are comparable with non-malignant cells of the same origin. Besides, stromal cells play an important role in colon cancer development, progression and resistance to (mainly targeted) therapy [316].

Components of the BER pathway have increasingly been identified as predictive markers of cancer risk, prognosis, chemoresistance, and as potential therapeutic targets in a variety of cancers [317]. Published evidence also suggested an association between inaccurate BER and increased tumour invasiveness in colorectal cancer [318]. In this context, we observed the link between increasing BER-DRC tumour/mucosa ratio and advanced TNM stage of the disease (Figure 15). Analysis of a panel of BER pathway proteins showed their high expressions in gastric cancer patients in association with advanced stage and decreased patients' survival [319]. Recently, it has been pointed out that the prognostic significance of upregulated BER proteins supports the use of their measurement in refining the current TNM staging in colorectal cancer [320].
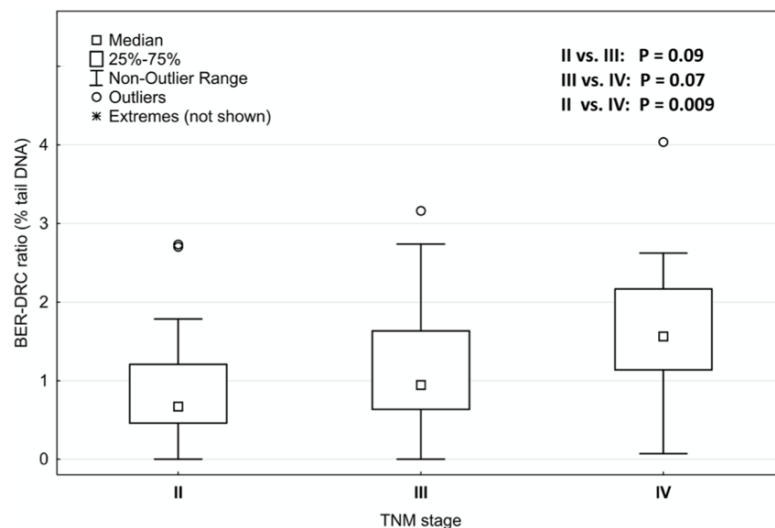
Figure 15. Differences in BER-DRC ratio between TNM stage II, III and IV of colon cancer.

BER ratio means a relative value of BER-DRC, calculated as the relative ratio of BER-DRC in tumour tissue over BER-DRC in non-malignant adjacent mucosa (i.e. BER-DRC in tumour tissue / BER-DRC in non-malignant adjacent mucosa). Differences in BER-DRC ratio between different TNM stages were calculated using Wilcoxon Two Sample Test. Abbreviations: BER – base excision repair, DRC – DNA repair capacity, TNM – tumour-node-metastasis.

Further, in univariate survival analysis, we observed that patients with a higher BER-DRC than cut-off in non-malignant adjacent mucosa exhibited significantly better 5-year OS and RFS (OS: HR = 0.36, 95%CI = 0.18-0.75, P = 0.007; RFS: HR = 0.52, 95%CI = 0.28-0.97, P=0.04). However, the level of BER-DRC in the tumour was not associated with clinical outcomes in our cohort. A possible explanation of this phenomenon might lie in a non-selective effect of 5-FU, resulting in adverse toxic side effects. Since colon epithelium is one of the most constantly regenerated tissues in the body and displays a large number of proliferating cells, it may have increased vulnerability to 5-FU-mediated DNA damage accumulation as well. As a consequence, higher BER-DRC in non-malignant mucosa may deal more successfully with uracil and 5-FU mis-incorporation to maintain genome stability and patients with this molecular characteristic may show better OS and RFS.

The prognostic utility of BER-DRC in non-malignant adjacent mucosa was further supported by CART survival analysis. The CART analysis explored the interactive effects of BER-DRC in paired tissues and MSI, together with clinicopathological data in association with 5-year OS and RFS. Patients in TNM stage II + III with good therapy response and higher BER-DRC than cut-off in non-malignant adjacent mucosa had 5-year OS increased by approximately 30 %. Moreover, the survival of these patients was even better in the presence of lower BER-DRC than cut-off in tumour tissue (Figure 16). These results supported our hypothesis that functional DDR is crucial for the maintenance of genome stability in non-malignant cells, whereas the suppression of DNA repair in malignant cells may increase the effectiveness of chemotherapy.

The presence of MSI-high status is a predictive marker for the detection of colorectal cancer patients in TNM stage II and III who might not benefit from adjuvant 5-FU chemotherapy and thus could reduce the risk of over-treatment [321]. MSI-high tumours accounted for 15 % of the whole set of samples, and they were mostly localized in the proximal colon (P < 0.0001), which was in accordance with world statistics [322]. Interestingly, we did not find any association of MSI-high tumours either with patients' and tumour characteristics or with therapy response and survival.

In summary, BER-DRC represents an integrated marker for evaluation of multistep DNA repair processes. As a functional measure of enzyme activity, it complements transcriptional and translational measurements of BER genes/proteins. The results of this

study suggested that the level of BER-DRC is associated with colon cancer patients' survival. Therefore, BER-DRC represents a potential prognostic biomarker, applicable for prediction of therapy response and useful for an individual approach to patients.
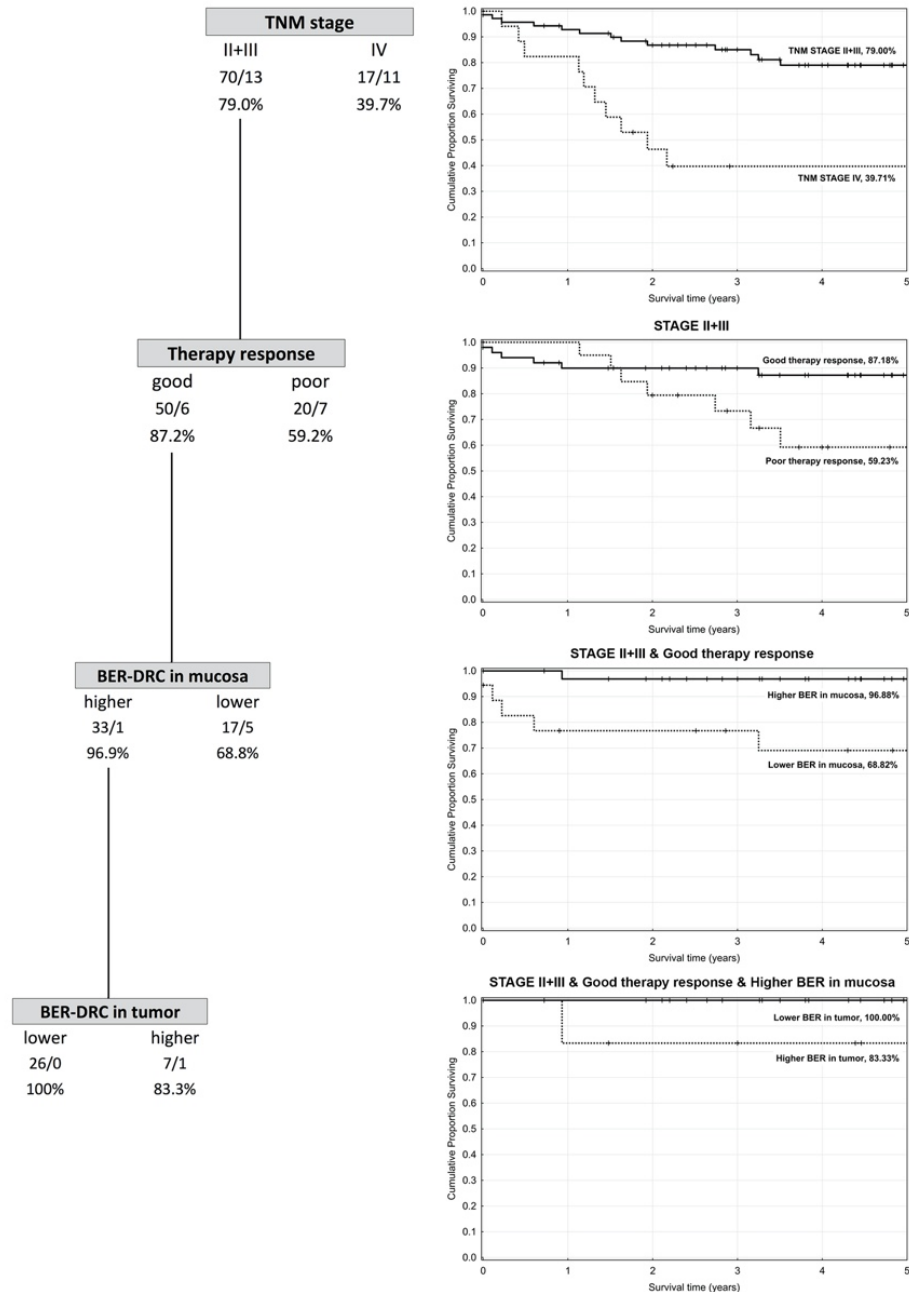
Figure 16. Overall survival classification & regression tree.

Classification & regression tree represents the results of multivariate survival analysis (using Cox regression hazard model). Numbers under each node show the total number of cases in a particular subcategory/number of events and percentages of patients with 5-years OS. Corresponding Kaplan-Meier curves represent the differences in OS for each node. Abbreviations: BER – base excision repair, DRC – DNA repair capacity, TNM – tumour-node-metastasis.

5. Conclusions

This Dissertation Thesis was performed in response to several unclear and unresolved issues of the role of DNA damage and repair in cancer pathogenesis. In this section, individual aims of the Thesis are provided by the primary outcomes of the own experimental work in light of existing literature and knowledge.

1) In **Publication I**, we supported the concept of using CAs in PBLs as a biomarker of early carcinogenic effect and clearly suggested the role of elevated CAs (including CAtot, CTAs, and CSAs) as a biomarker of cancer susceptibility, mainly breast and lung cancer. Colorectal cancer risk was only determined by the subset of CAtot, i.e. levels of CTAs. Further, in **Publication II**, we observed that individuals with longer TL in PBLs were at increased risk of breast cancer. Regarding patients' clinical outcomes, accumulation of CTAs in PBLs appeared to be associated with decreased OS in breast and colorectal cancer patients after their stratification according to disease characteristics. While we found the association of elevated CAs with telomere shortening in control healthy individuals, cancer patients exhibited no relationship between either TL and CA frequencies or TL and age. Results of **Publication III** suggested that altered DSB repair in PBLs is mainly associated with colorectal cancer susceptibility. Our results also showed a significant correlation between telomere shortening and mutagen sensitivity profile in a pooled group of cancer patients; however, the same trend was not detected in a control group. These observations added further information to the chain of evidence on the interplay between the telomere complex and DSB.

2) By investigating functional SNPs in DNA repair genes in relation to CAtot, CTAs, and CSAs in healthy individuals (**Publication IV**), we observed an association of variant GG genotype in *XPD* rs13181 with decreased CTA frequency and CT genotype in *RAD54L* rs1048771 with increased CSAs. By addressing pair-wise gene-gene interactions, we have discovered 14 interactions significantly modulating CAs, 9 CTAs and 12 CSAs frequencies and *NBS1* rs1805794 appeared most often in these interactions. However, these gene-gene combinations evinced either enhanced or decreased frequencies of CAs, CTAs and CSAs. The GWAS-based **Publications V and VI** indicated several new SNPs associated with CA frequency, from which three

were replicated at the suggestive level of significance. These variants were found in genes involved in DDR/repair, segregation of chromosomes and chromatin modification. Others were related to apoptosis, cell proliferation, angiogenesis and tumorigenesis. *In silico* predictions of functional consequences of the identified SNPs and their loci revealed that they were directly or indirectly related to different cancers, autism/autistic traits, Down syndrome, and a condition linked to chromosomal abnormalities. These results suggest a complex interaction of various genetic factors responsible for the inter-individual differences in CA frequency in the presence and absence of evident exposure to genotoxins, many of which are still unexplored.

3) Based on the results from **Publication VII**, we have identified the association of several functional SNPs in DNA repair genes with colorectal cancer susceptibility and patients' clinical outcome. *REV3L* rs3204953 was observed to be associated with increased susceptibility to colorectal cancer. Further, several other SNPs were shown to be associated with patients' OS and EFS using the multivariate survival analysis. Our data suggested that even amino acid substitution causing subtle alterations in the specific proteins that function in DNA repair pathways may lead to inaccurate DNA repair, and thus play a role in colorectal cancer pathogenesis. In **Publication VIII**, we pointed to the importance of studying DNA repair at a functional level, directly in tumour and non-malignant tissue to reveal its potential predictive and/or prognostic value. The results of this study suggested that the level of BER-DRC is associated with long-term survival of colon cancer patients. In accordance with published literature, we observed the link between increasing BER-DRC tumour/mucosa ratio and advanced TNM stage of the disease. Therefore, BER-DRC may represent a potential prognostic biomarker, applicable for prediction of therapy response and useful for an individual approach to patients.

This Dissertation Thesis suggested and/or verified several potential candidate biomarkers for predicting cancer susceptibility and patients' clinical outcome for further use in population monitoring and clinical practice. The majority of them represented already discovered biomarkers which were evaluated by well-defined, long-term used and validated methods. However, additional studies on larger independent cohorts are needed

to replicate our findings. Regarding the future perspectives, taking into consideration the fact that cancer represents a complex heterogeneous disease that is caused by the combination of several factors, multivariate approaches involving multiple biomarkers might contribute to the identification of reliable links between specific genetic/molecular features and increased cancer susceptibility, patients' therapy response and prognosis.

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Publication activity

Publications related to the Thesis (Publications I – XII)

Original Research Articles: Publications I – VIII

Publication I **Vodenkova S**, Polivkova Z, Musak L, Smerhovsky Z, Zoubkova H, Sytarova S, Kavcova E, Halasova E, Vodickova L, Jiraskova K, Svoboda M, Ambrus M, Hemminki K, Vodicka P. Structural chromosomal aberrations as potential risk markers in incident cancer patients. Mutagenesis. 2015 Jul;30(4):557-63. doi: 10.1093/mutage/gev018. Epub 2015 Mar 23. **IF (2015) = 2.297**.

Publication II **Vodenkova S**, Kroupa M, Polivkova Z, Musak L, Ambrus M, Schneiderova M, Kozevnikovova R, Vodickova L, Rachakonda S, Hemminki K, Kumar R and Vodicka P. Chromosomal damage and telomere length in peripheral blood lymphocytes of cancer patients. Oncology Reports. 2020. Under Review (Submitted 29-Nov-2019, Ref. No. 242392). **IF (2018/2019) = 3.041**.

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Publication IX   Vodicka P, Musak L, Vodickova L, **Vodenkova S**, Catalano C, Kroupa M, Naccarati A, Polivkova Z, Vymetalkova V, Försti A, Hemminki K. Genetic variations of acquired structural chromosomal aberrations.

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- **Vodenkova S***, **Azqueta A***, Collins A, Dusinska M, Gaivão I, Møller P, Opattova A, Vodicka P, Godschalk R, Langie S. The comet-based in vitro DNA repair assay: a standardized method to assess an individual's DNA repair activity. Nature Protocols 2020. Under review (Submitted 17-Mar-2020, Ref. No. NP-PI190829). **IF (2018/2019) = 11.361**.

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