

# Extended molecular testing of consecutive tumor specimen and subsequent treatment

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**UNIVERSITY OF SPLIT  
SCHOOL OF MEDICINE**

**Finja Marie Schwenkenbecher**

**EXTENDED MOLECULAR TESTING OF CONSECUTIVE TUMOR SPECIMEN  
AND SUBSEQUENT TREATMENT**

**Diploma Thesis**

**Academic year:**

**2021/2022**

**Mentor:**

**Assist. Prof. Christof Lamberti, MD, PhD**

**Coburg, September 2022**

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## **1. INTRODUCTION**

Cancer is the second leading cause of death worldwide and the overall incidence rate of cancer and mortality are still rising (1). In 2020 alone there were an estimated 19.3 million new cases of cancer and almost 10.0 million deaths from cancer (2). Researchers are therefore constantly striving to develop new, effective therapies that are better suited to patients. In cancer patients the development of neoplasms, an abnormal and excessive growth of tissue, is driven through a stepwise accumulation of alterations affecting the structure and function of the genome (3). During the last few years, more and more molecular alterations have been identified as drivers of cancer development and progression by deep molecular analysis. For the identification of specific genes or mutations, Next-Generation Sequencing can be used. It made genetic testing more affordable and faster over the last years (4). Moreover, generally available test panels like the OncoPrint Comprehensive Panel<sup>®</sup> or the OncoPrint Focus Assay<sup>®</sup> enable a fast detection of frequent genetic mutations in cancer cells. Depending on the molecular set up, cancer can be fragmented into molecular subtypes against which targeted therapies can be developed. The first successful molecular-based medicine was the use of endocrine therapy in luminal breast cancer patients (2). Today, more targeted therapies with increased efficacy and/or reduced toxicity are used in the routine clinical practice (5). In the year 2020 alone the FDA approved 28 targeted therapies defined by specific molecular biomarkers (6). The biomarker-driven approach is proving particularly beneficial for unlocking new personalized treatments for cancer patients with high unmet medical need (7).

This change from an organ-centric concept guiding treatment choice towards deep molecular analysis, driving a personalized approach, is a tremendous advancement in modern oncology enabling the development of many novel therapies (8). However, some of these novel therapies are not yet clinically approved and are only used in the setting of clinical trials or are known to be effective only in vitro or in animals. Applying those experimental but promising therapies in clinical practice is still difficult and often there is a significant gap between the treatment recommendations according to the detailed genetic test result and the treatment the patients actually receive.

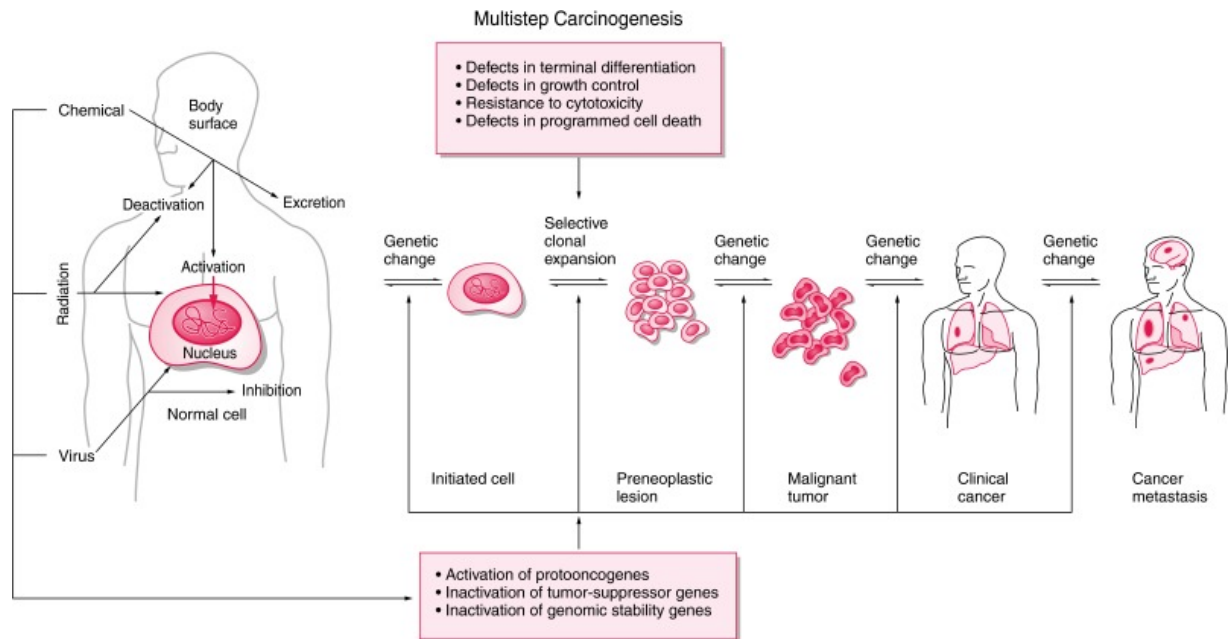
## **1.1 Carcinogenesis**

Carcinogenesis describes the development of a malignant tumor. Normal cells transform into invasive cancer cells. The cells gain malignant properties, including dedifferentiation,

fast proliferation and metastasis. Also evasion of apoptosis, immunosurveillance and a dysregulated metabolism and epigenetics have been generalized as hallmarks of cancer (9). Hereditary predisposition due to mutations in one or both of germinal cell alleles can lead to cancer (10). Also cancer can be sporadic and the genetic evolution that occurs during the tumorigenesis is influenced by environmental factors. Exposure to carcinogenic agents like tobacco smoke or agents such as asbestos or ultraviolet radiation can cause alterations in cancer associated genes. Additionally, infectious agents including certain viruses like the human papillomavirus or hepatitis viruses can initiate and drive the development of cancer (11).

Several steps are required for the change of properties within cells and the process of carcinogenesis can be divided into the following four stages: initiation, promotion, malignant conversion and progression (Figure 1). The initiation phase starts with an initiating damage to the DNA. Generally defense mechanisms can repair the DNA defect but through a dysfunction of (proto) oncogenes, repair genes, apoptosis regulating genes and tumor suppressor genes a neoplastic transformation is promoted eventually. Those mutations that lead to a selective advantage are maintained. The promotion of cancer compromises the selective clonal expansion of initiated cells and involves changes in the expression of the genome mediated through promoter-receptor interactions (12). In the stage of malignant conversion preneoplastic cells transform into cells that express the malignant phenotype (13). Characteristic for the final irreversible stage of progression is karyotypic instability. Additional mutations occur within cells of the tumor with the tendency of malignant cells to acquire more aggressive characteristics over time leading to malignant growth. The malignantly transformed cells proliferate and eventually can metastasize.





**Figure 1.** Multistage carcinogenesis

Source: Weston A, Harris CC. Multistage Carcinogenesis. In: Kufe DW, Pollock RE, Weichselbaum RR. Holland-Frei Cancer Medicine. 6th edition. Hamilton: BC Decker; 2003. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK13982/>

## 1.2 Genes Relevant to Carcinogenesis

As previously mentioned the development of cancer is a multistep process with a gradual progression of normal cells to malignancy. There are more than 3000 genes that have been considered as ‘cancer-related’, including the classical oncogenes and tumor suppressor genes (14). Mutations in cancer-relevant genes lead to changes in the gene sequence altering their expression levels and activities. This can lead to the activation of oncogenes and/or the inactivation of tumor suppressor genes being a critical step in tumor development. Different mutations in multiple oncogenes and tumor suppressor genes accumulate and affect complementary signaling pathways that regulate cell proliferation and survival.

### 1.2.1 Oncogenes

Oncogenes are mutated forms of proto-oncogenes and contribute to the development of cancer. Proto-oncogenes are important cell-regulatory genes controlling proliferation, differentiation and survival of cells (15). Many of the proteins encoded by proto-oncogenes regulate normal cell proliferation. During tumor development oncogenes are generated from proto-oncogenes by mutations or DNA rearrangements. Oncogenes are capable of inducing cell transformation and can contribute to the abnormal behavior of malignant cells. An

elevated expression or activity of the oncogene proteins drive an uncontrolled proliferation of cancer cells (15). There are several ways for the conversion from proto-oncogenes to oncogenes. One of them is a point mutation. Also abnormalities in chromosome structure, including translocations, duplications and deletions are displayed in many cancer cells. It is a large group of oncogenes that encode growth factor receptors, mostly protein-tyrosine kinases. Also many oncogenes encode transcriptional regulatory proteins that are normally induced in response to growth factor stimulation. Ultimately, the signaling pathways activated by growth factor stimulation regulate components of the cell cycle machinery and a progression through the restriction point in  $G_1$  is promoted (15). In addition, other oncogene products contribute to other aspects of cancer cell behaviors, such as defective differentiation or failure to undergo programmed cell death. Several oncogenes encode proteins that act to promote cell survival being dependent on growth factor stimulation in most animal cells.

### **1.2.2 Tumor Suppressor Genes**

Another genetic alteration involved in tumor development is the inactivation of tumor suppressor genes. Tumor suppressor genes represent the opposite side of cell growth control to oncogenes. They normally act to inhibit cell proliferation, tumor development and survival. These genes are often lost or inactivated in tumors. Thereby, negative regulators of cell proliferation are removed, resulting in an abnormal proliferation of tumor cells (15).

Tumor suppressor genes are involved in the development of both inherited and non-inherited forms of cancer. With inheritance of one defective copy of a tumor suppressor gene, a second somatic mutation is required. One example for an inherited condition is the childhood retinoblastoma. Compared to the general population, an inactivating mutation in the RB1 gene causes a 10.000-fold increased risk of developing retinoblastoma (16). In non-hereditary cases two normal genes are inherited and two somatic mutations are required to inactivate both copies. Many types of cancer including ovarian, lung, colorectal cancer and pancreatic cancer can be associated with a loss of function mutation in a tumor suppressor gene.

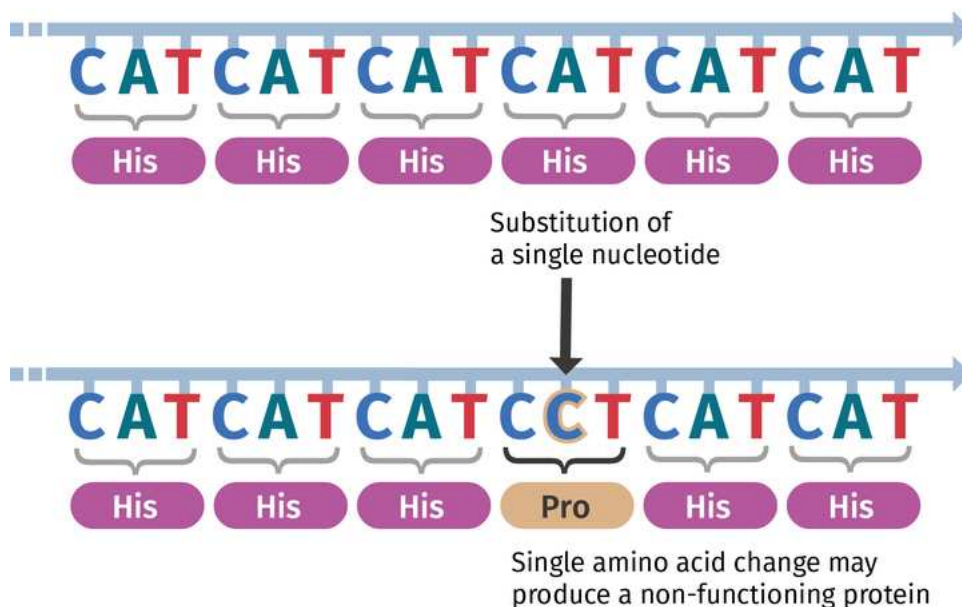
### **1.2.3 Stability Genes**

Stability genes like the BRCA 1 and 2 and the ATM gene are involved in check-point control of cell-cycle progression and repair of double-strand breaks in DNA. The inactivation of stability genes has no direct effect on cell proliferation or survival but leads to a high frequency of mutations in oncogenes or tumor suppressor genes (15).

### 1.3 Tumor-Promoting Mutations

#### 1.3.1 Single-nucleotide Variant (SNV)

Single-nucleotide variants (SNVs) occur at a specific genomic position. A single DNA building block called a nucleotide (adenine, thymine, cytosine or guanine) is replaced and alters the genome sequence. Single nucleotide variants are also known as single-nucleotide polymorphisms (SNPs) if they are present in at least 1% of the population (17). They are the most common type of genetic variants. SNPs can also occur normally throughout a person's DNA and most SNPs have no effect on health or development. However, some of these genetic differences have proven to be a risk of developing diseases. If a single nucleotide variant occurs in a protein-coding region and the nucleotide substitution does not result in a change in amino acid, it is a synonymous change. Multiple codons can code for the same amino acid making a synonymous change possible (18). However, a nucleotide substitution can also lead to a nonsynonymous change or a missense variant (mutation) altering the protein function and structure. If the nucleotide substitution results in a stop codon and consequently premature truncation of the protein it is called a stop gain change or a nonsense variant (mutation) (18).



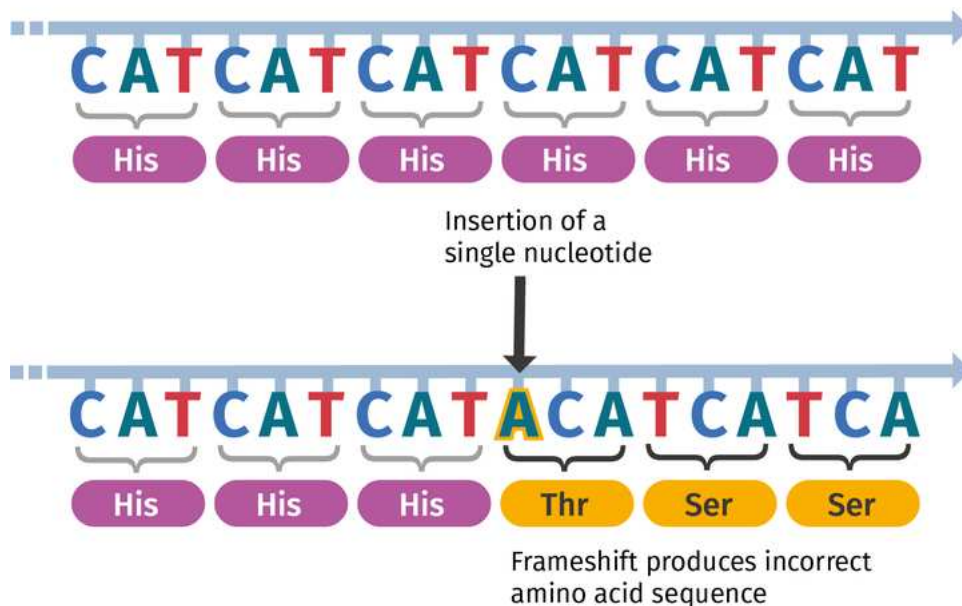
**Figure 2.** Single nucleotide variant (SNV)

Source: Types of variants [Internet]. Australia: Garvan Institute of Medical Research; 2021. Single nucleotide variant ; [24.07.2022]. Available from: <https://www.garvan.org.au/research/kinghorn-centre-for-clinical-genomics/learn-about-genomics/for-gp/genetics-refresher-1/types-of-variants>

### 1.3.2 Indel (Insertion-Deletion Mutation)

Insertion-Deletion Mutations (Indels) occur less frequently than SNVs but they are also widely spread across the genome and are commonly identified in cancers (19). Of 15 million known genetic alterations they comprise a total of 3 million (20).

Nucleotides of the genomic DNA are inserted and/or deleted leading to loss or gain of DNA on a smaller scale including less than 1000 bp (21). If only one or two nucleotides are lost and/or gained indel, variants can shift the reading frame. Subsequent codons to the variant will be “out of frame,” resulting in an entirely new set of amino acids and the variant will be termed a “frameshift” variant. A new amino acid will be encoded by the new frame and the amino acid sequence ends once a stop codon is encountered within the new frame. In contrast, indel variants with for example insertion of three nucleotides, or deletion of six nucleotides will maintain the codon frame and produce a “non-frameshift” variant. Indels are a common mechanism of kinase activation in cancer which is a feature exploited clinically by targeted therapy with kinase inhibitors (22).

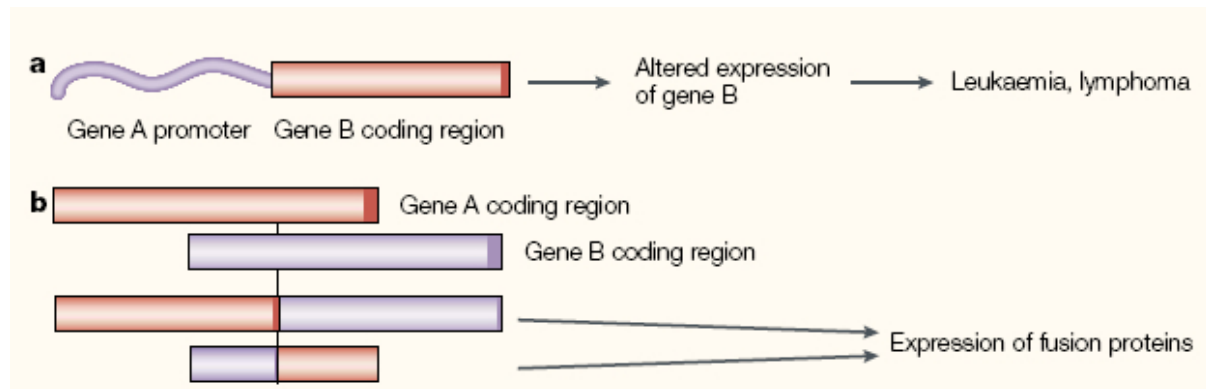


**Figure 3.** Insertion-Deletion Mutation

Source: Types of variants [Internet]. Australia: Garvan Institute of Medical Research; 2021. Indel; [24.07.2022]. Available from: <https://www.garvan.org.au/research/kinghorn-centre-for-clinical-genomics/learn-about-genomics/for-gp/genetics-refresher-1/types-of-variants>

### 1.3.3 Gene Fusions - Chromosome Translocations

In many cases molecular rearrangements and the generation of novel chromosomes via translocations are considered to be the primary cause of various cancers. In a translocation a Chromosome's segment is transferred to a new site on the same chromosome or to a nonhomologous chromosome (23). Genes are therefore placed in new linkage relationships and chromosomes are generated without normal pairing partners. This can result in the disruption or misregulation of normal gene function, depending on the chromosome breakpoints. Virtually all of the translocations observed in tumors are not inherited and have arisen through somatic mutations (23). There are 2 kinds of molecular rearrangements that are frequently linked to a malignant transformation. A translocation can place a coding sequence of one gene in proximity to a regulatory sequence of a different gene. In leukemia or in some lymphomas this translocation leading to juxtaposition of promoter/enhancer elements can be found. Also, translocations can fuse the coding sequences of two genes together to generate potent oncogenes (23). This can be seen in CML and in acute leukemia. A fusion protein that might have a new function is generated as it is the case for the BCR-ABL fusion protein encoded by the Philadelphia chromosome.



**Figure 4.** Chromosome translocations

Source: O'Conner C. Human Chromosome Translocations and Cancer. Nature Education. 2008;1:56

### 1.3.4 Copy Number Variations and Alterations CNVs

Copy number alterations (CNAs) play a major contribution in the development and progression of cancer. They comprise deletions, duplications or amplifications of fragments of genomic material resulting in gain or loss in copies of sections of DNA (24). DNA copy number variations (CNVs) affect a greater fraction of the genome than single nucleotide

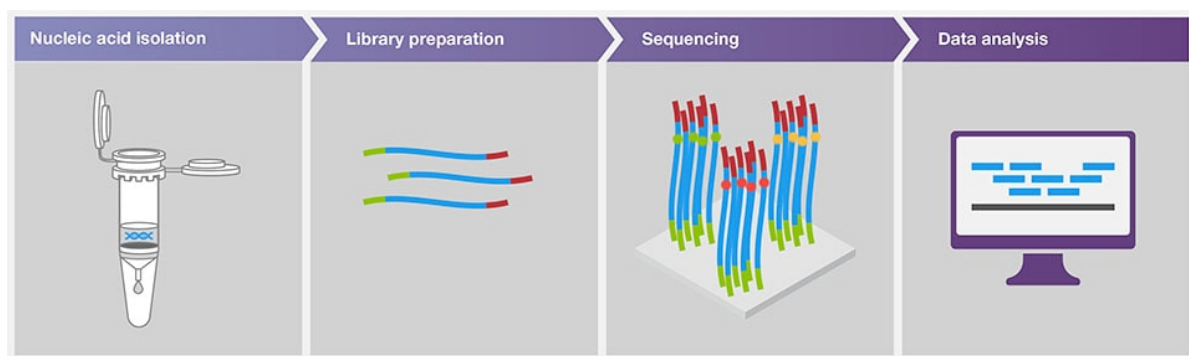
polymorphisms (SNPs) (25). The size of the fragments affected can be as low as a few kilobases or up to entire chromosomes. Copy number alterations are often longer than copy number variations and copy number variations are commonly observed in the germline as the DNA copy number is naturally variable. Copy number alterations being somatically acquired play a major contribution in cancer development and are particularly common. (24) Genes that have been identified to be affected by somatic CNVs include ERBB2, EGFR, MYC, PIK3CA, IGF1R, FGFR1/2, KRAS, CDK4, CCND1, MDM2, MET, CDK6 for amplification, and RB1, PTEN, CDKN2A/B, ARID1A, MAP2K4, NF1, SMAD4, BRCA1/2, MSH2/6, DCC, CDH1 for deletion (26). The identification and accurate detection of CNVs are important to improve cancer diagnosis and treatment decision. An example is Trastuzumab in breast cancer, which is effective only in ERBB2 amplified cancer (26).

#### **1.4 Next-Generation Sequencing**

To detect sequence alterations like mutations, insertions or deletions next-generation sequencing can be used. Next generation sequencing enables the parallel sequencing of thousands of nucleic acid fragments and is considerably faster compared to the previously used Sanger – sequencing. Sanger – sequencing is based on enzymatic techniques and only allows the analysis of DNA-fragments.

Different methods are available for massively parallel DNA sequencing, but the basic principle of Next-Generation Sequencing is based on four steps: Nucleic acid isolation, library preparation including fragmentation and adaption, amplification and data analysis. Nucleic acid isolation is crucial to enable proper lysis of the cells and tissue (27). In the second step, the library preparation for NGS, fragments of DNA and RNA are generated using enzymes or centrifugation. Also, oligonucleotides are added as adapters for each sample to be analyzed and are ligated to the ends of the genomic DNA fragments (27). In the third step, the process of amplification, the DNA fragments are bound to reaction media, for example a chip. The fragments of DNA are duplicated and clusters of identical DNA are generated. Afterwards, the clusters of DNA are sequenced and reassembled to form a genomic sequence. Different methods for sequencing are available. The process will be repeated for several rounds. The whole process of Next-Generation Sequencing creates millions of reads being a sequence of several hundred bases. In the last step, obtained data is stored in form of a DNA chip and complex bioinformatical evaluation algorithms analyze the

DNA sequences. The analyzed DNA of a patient is compared to a reference genome with regard to gene changes. Using mutation databases, in silico tools and the current literature these genomic alterations can then be evaluated for clinical relevance. The results can be summarized in one finding and detected alterations in the genome guide decisions on treatment choices.



**Figure 5.**

Source: Thermo Fisher Scientific. Next-Generation Sequencing Illumina Workflow-4 Key Steps [Internet]. Waltham: Thermo Fisher Scientific; 2022 [cited 2022 Jun 20]. Available from: <https://www.thermofisher.com/de/de/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/next-generation-sequencing/illumina-workflow.html>

## 1.5 Targeted Therapy

Targeted therapy is the foundation of precision medicine and targets proteins controlling cancer cell growth, division and spread (28). More and more of those targeted treatments are designed and come into use as researchers learn more about DNA changes and proteins that drive cancer. In contrast to chemotherapy which kills all cells that grow and divide quickly, targeted therapy interferes with specific proteins that help tumors grow and spread throughout the body (28). Small-molecule drugs and Monoclonal antibodies are the most common types of targeted therapy. Small-molecule drugs are so small that they can easily enter cells and can be therefore used for targets inside cells. Monoclonal or also called therapeutic antibodies are proteins designed in the laboratory to attach to specific targets found on cancer cells. Some of them are able to mark cells so they can be recognized and destroyed by the immune system. Others directly stop cancer cells from growing, cause self-

destruction or carry toxins to cancer cells. Biomarker testing helps to identify targets and a decision on treatment choices can be made accordingly (28).



## **2. OBJECTIVES**

## **2.1 Objectives**

The objective of this study is to confirm, whether detailed genetic testing of patients with advanced tumor stage provides the basis for new molecular targeted therapies. We also aimed to determine if detailed genetic testing extends treatment options and improves the outcome. In addition, the frequency of molecular alterations and their level of evidence were determined in an unselected group of patients. Furthermore, we investigated the frequency of targetable genomic aberrations and the proportion of patients actually treated with targeted therapy.

## **2.2 Hypotheses**

1. A substantial number of unselected incurable cancer patients exhibit targetable genetic alterations.
2. Most of the detected genetic aberrations exhibit a preclinical evidence level.
3. Comprehensive genetic testing of tumor tissues of incurable cancer patients provide the basis for new molecular targeted therapies, extend the treatment options and might improve outcome.

### **3. MATERIALS AND METHODS**

### **3.1 Subjects and Ethical Considerations**

This retrospective study was performed at the oncologic ambulatory health care center in Coburg, Germany between September 2021 and July 2022, and was approved by the Institutional Review Board in Coburg. We enrolled 54 patients with end-stage cancer in this study. In total, 58 detailed genetic tests were performed using the OncoPrint Comprehensive v3 panels or the OncoPrint Focus panels, in the year 2020.

#### **3.1.1 Inclusion Criteria**

Patients (total: 54 patients) receiving detailed genetic testing in the year 2020 to detect possible pathogenic mutations were included in this study. All of them were diagnosed with cancer and treated at the oncologic ambulatory health care center in Coburg.

#### **3.1.2 Exclusion Criteria**

Excluded were patients already being critically ill at the time of cancer diagnosis and therefore, no detailed genetic test was conducted. For those patients with more than one detailed genetic test being conducted in the year 2020 only one test result was taken into consideration. In the case that both OncoPrint Comprehensive and OncoPrint Focus Tests were performed, the less detailed ONCOPRINT-Focus test results were excluded. Test results of the metastatic specimen were excluded, if genetic testing was already performed in the primary tumor specimen. Also excluded were questionable or borderline mutations and amplifications.

### **3.2 Clinical Information and Diagnostic Tests**

Electronic medical records were reviewed for the patient characteristics, previous lines of therapy and outcomes of the patients. Only anonymous data was used for data interpretation.

For analysis of somatic genomic alterations with potential clinical relevance and examination of tumor-relevant and tumor-initiating mutations, tumor tissue was obtained from histological slice specimen using macrodissection. DNA and RNA were isolated using the RecoverAll-Kit according to the manufacturer's instructions (Thermo Fischer). The quantity and quality of recovered genomic DNA/RNA for next-generation sequencing (NGS)

analysis was determined by DNA/RNA concentration measurements, using the Qubit system 3.0 by Thermo Fischer, and DNA extraction control PCR. Consecutively, targeted mutation analysis was performed by Next-Generation-Sequencing technology (Ion Torrent-S5, Thermo Fischer) using the OncoPrint Comprehensive v.3 panels (Thermo Fischer) or the OncoPrint Focus panels (Thermo Fischer). OncoPrint is a Multi-Biomarker-Assay to detect Hotspots, SNVs, Indels, CNVs and Gene fusions from DNA and RNA. With OncoPrint Comprehensive v.3 panel sequencing approach a total of 135 genes in relevant gene regions of the complete gene are analyzed. Including 43 genes for focal alterations in gene copy number (CNV) as well as 51 genes for the occurrence of fusions. Using the OncoPrint Focus Panel sequencing approach, a total of 52 genes in the relevant gene regions are analyzed for relevant mutations, focal alterations in gene copy number (CNV), and the occurrence of driver fusions. The analysis of the data was performed using the Ion-Reporter software. Of the detected alterations, those that had an allele frequency above 5% were listed.

OncoPrint test results are summarized in an OncoPrint knowledge report. Oncologists and pathologists in Coburg discussed the results and relevancies of detected mutations, fusions, and CNVs. Their decision on treatment choices is based on the advanced bio-analysis and clinical- / study-analysis.

### **3.3 Evidence Classification System**

Published Evidence Classification systems help interpreting somatic variants and evaluate their diagnostic, prognostic and therapeutic implications (29). Several different classification systems exist, with the “Joint Consensus Recommendation”, the “ESMO Scale for Clinical Actionability of Molecular Targets” (ESCAT) and in Germany the NCT classification being the best known. For the evaluation of molecular alterations, in the context of this thesis, the NCT classification was used with slight modifications and additions.

The German NCT-Classification was established for the evaluation and development of therapeutic approaches for advanced and difficult-to-treat tumor diseases (29). As can be seen in Figure 6, stratification is made based on tumor type. The NCT-levels describe an association between a molecular biomarker and a drug for the same entity (m1) and for other entities (m2). Also, the NCT classification differentiates between preclinical and clinical evidence and the strength of clinical evidence is taken into account. As follows there is a

further stratification in m1 and m2 according to the study design with A being prospective studies, B retrospective studies and C including case reports, case series and smaller cohort studies. Molecular biomarkers whose drug association is derived from preclinical models are classified as NCT level m3. Biological rationales (prediction based on *in silico* data or analysis of signaling cascades) are classified as NCT level m4.

LoE		Explanation
m1A	Same entity	Predictive value of the biomarker or clinical effectiveness of the corresponding drug in a molecularly stratified cohort was demonstrated in a prospective study or a meta-analysis in the same tumor type.
m1B		Predictive value of the biomarker or clinical effectiveness of the drug in a molecularly stratified cohort was demonstrated in a retrospective cohort or case-control study in the same tumor type.
m1C		Case study or single unusual responder indicates the biomarker is associated with response to the drug in the same tumor type.
m2A	Different entity	Predictive value of the biomarker or clinical effectiveness of the corresponding drug in a molecularly stratified cohort was demonstrated in a prospective study or a meta-analysis in a different tumor type.
m2B		Predictive value of the biomarker or clinical effectiveness of the drug in a molecularly stratified cohort was demonstrated in a retrospective cohort or case-control study in a different tumor type.
m2C		Case study or single unusual responder indicates the biomarker is associated with response to the drug in a different tumor type.
m3	Preclinical	Preclinical data ( <i>in vitro</i> , <i>in vivo</i> models or functional genomics studies) demonstrate that the biomarker predicts response to a specific drug treatment, supported by scientific rationale.
m4	Biological rationale	Biological rationale that associates the biomarker with altered signaling pathway activity or drug sensitivity without direct clinical or preclinical evidence for response to the drug.
Additional modifiers	is—several <i>in situ</i> data and studies on patient material (e.g., IHC, FISH) support the biomarker and the level of evidence. iv— <i>in vitro</i> data Z—Drug is approved for use with the specific biomarker [Z = EMA approval, Z(FDA) = FDA approval] R—Biomarker predicts resistance to the drug.	

**Figure 6.** Classification scheme of the National Center for Tumor Diseases (NCT)

Source: Leichsenring J, Horak P, Kreutzfeld S, Heining C, Christopoulos P, et al. Variant classification in precision oncology. *IJC* 2019;145:2996-3010

In our study setting for the classification of genetic variations additional categories were introduced as depicted in Figure 7. Category I (Kat I) includes drugs already approved by the European Medicines Agency (EMA) or the Food and Drug Administration (FDA). The NCT classification is integrated in category 2 (Kat II). Pathogenic alterations but with no clear agent being available are described by category III (Kat III). Variants of unclear significance and likely benign or benign alterations are Category IV (Kat IV) and V respectively.

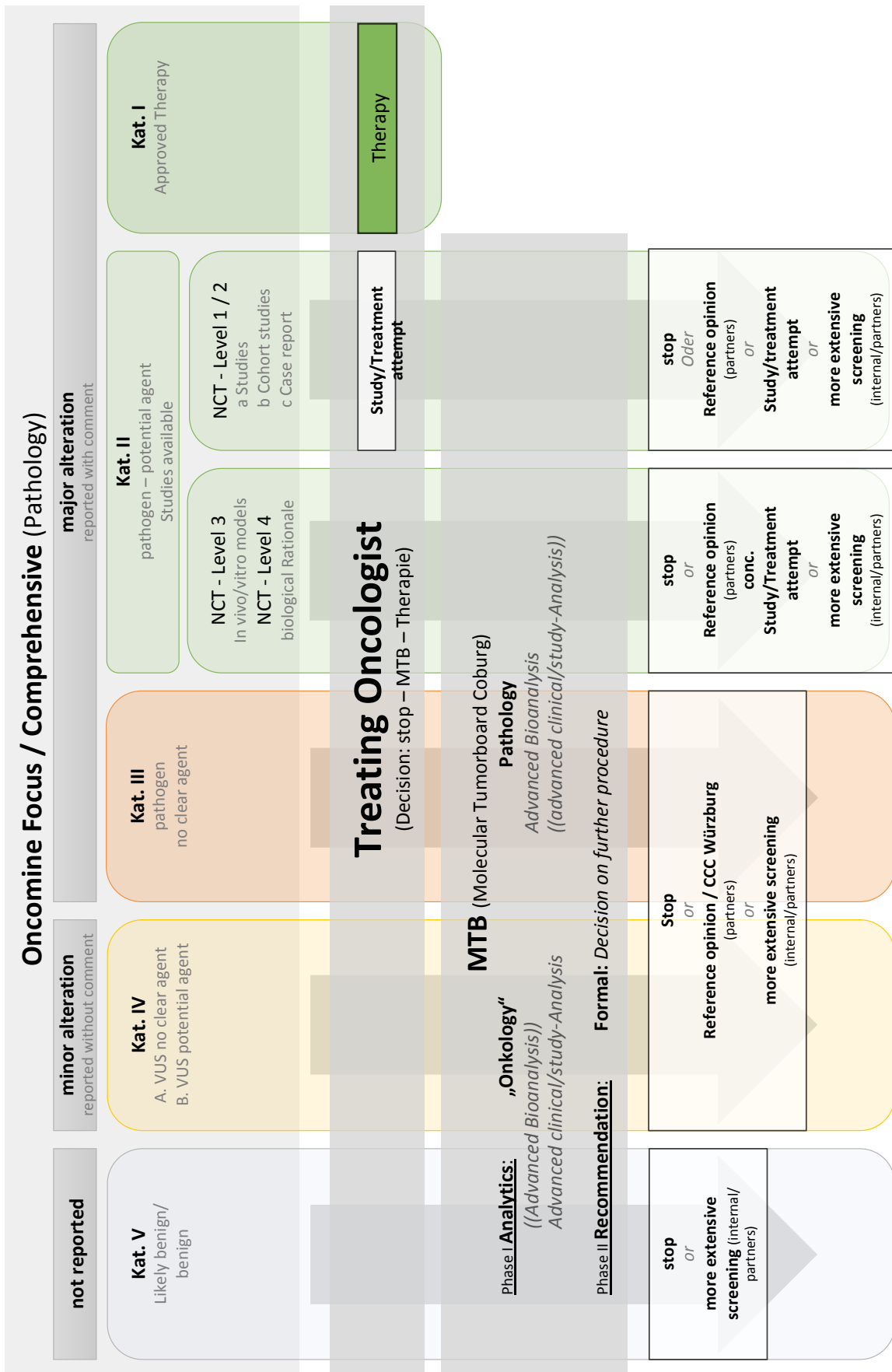


Figure 7. Classification concept

### **3.4 Statistical Analysis**

Data analysis was performed using the MedCalc<sup>®</sup> Statistical Software version 20.112 (MedCalc Software, Ostend, Belgium). Statistical significance was set to  $P < 0.05$  and statistical analysis of association of categorical variables was calculated by the chi-square test ( $\chi^2$ ), which gave us overall  $\chi^2$  and P.



## **4. RESULTS**

In this study 54 oncologic, adult patients (19 females and 35 males), who underwent detailed genetic testing, from January 2020 to December 2020, were included. Their ages ranged from 47 to 84 years with a median age of 68 years (Table 1). The females had a median age of 65 years (Min-Max: 47-83 years) and the males had a median age of 69 years (Min-Max: 51-84 years).

**Table 1.** Gender distribution and median age

	<b>AGE</b>		
	<b>Total</b>	<b>Female</b>	<b>Male</b>
<b>Number</b>	54	19	35
<b>Median Age</b>	68.00 ± 9	65.00 ± 13	69.00 ± 6
<b>Minimum Age</b>	47.00	47.00	51.00
<b>Maximum Age</b>	84.00	83.00	84.00

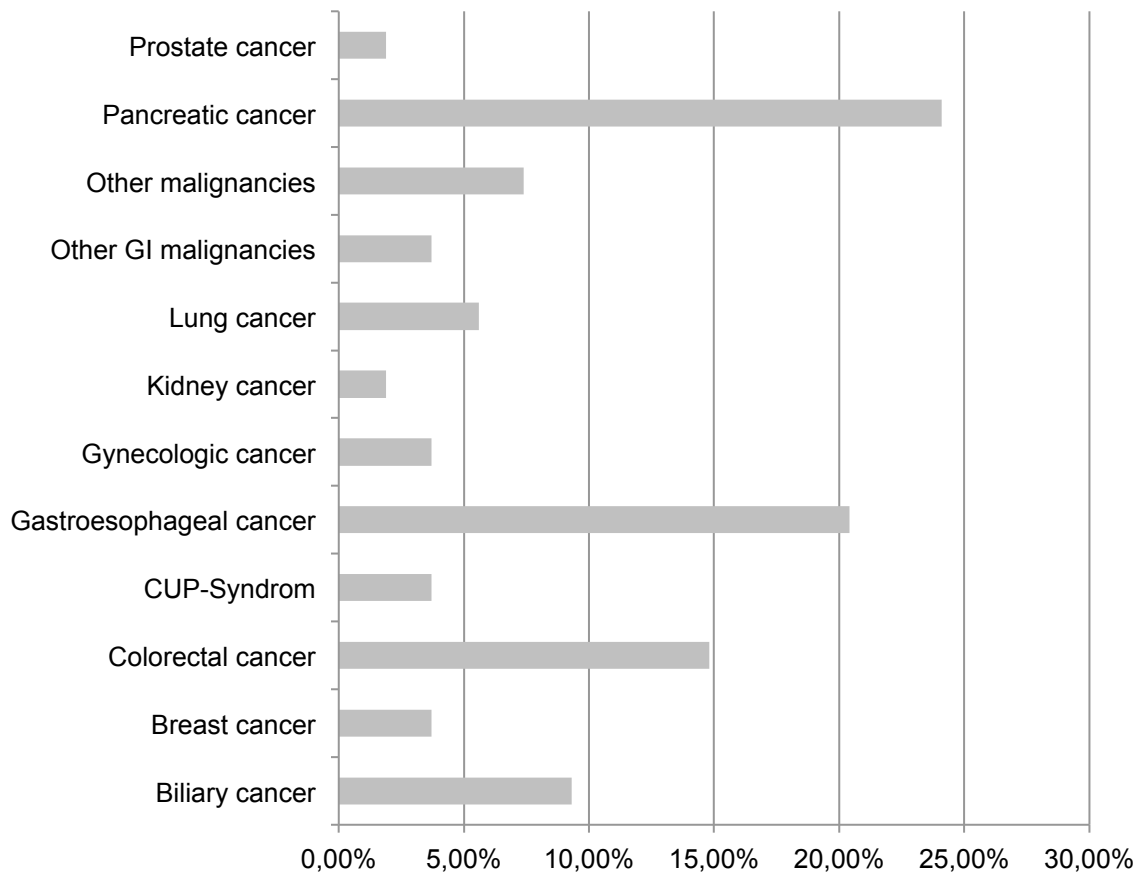
Data are presented as absolute numbers ± interquartile range

Most of the patients presented with stage IV cancer as presented in Table 2. Out of the 54 patients 53 (98.1%) presented with stage IV. Only one patient (1.9%) had stage III cancer at the time the detailed genetic test was conducted.

**Table 2.** Tumor stage

<b>TUMOR STAGE</b>	<b>Frequency (N)</b>	<b>Percentage (%)</b>
<b>III</b>	1	1,9%
<b>IV</b>	53	98,1%
<b>Total</b>	54	100.0%

As can be seen in Figure 7 the most common diagnosis was pancreatic cancer (24.1%, N=13) followed by Gastroesophageal cancer (20.4%, N=11), Colorectal cancer (14.8%, N=8) and biliary cancer (9.3%, N=5). Gynecologic cancer includes vaginal cancer (N=1) and cervical cancer (N=1). Other GI malignancies include duodenal cancer (N=1) and small intestinal GIST (N=1). Other malignancies include pleura mesothelioma (N=2), mediastinal cancer (N=1) and choroidal melanoma (N=1).



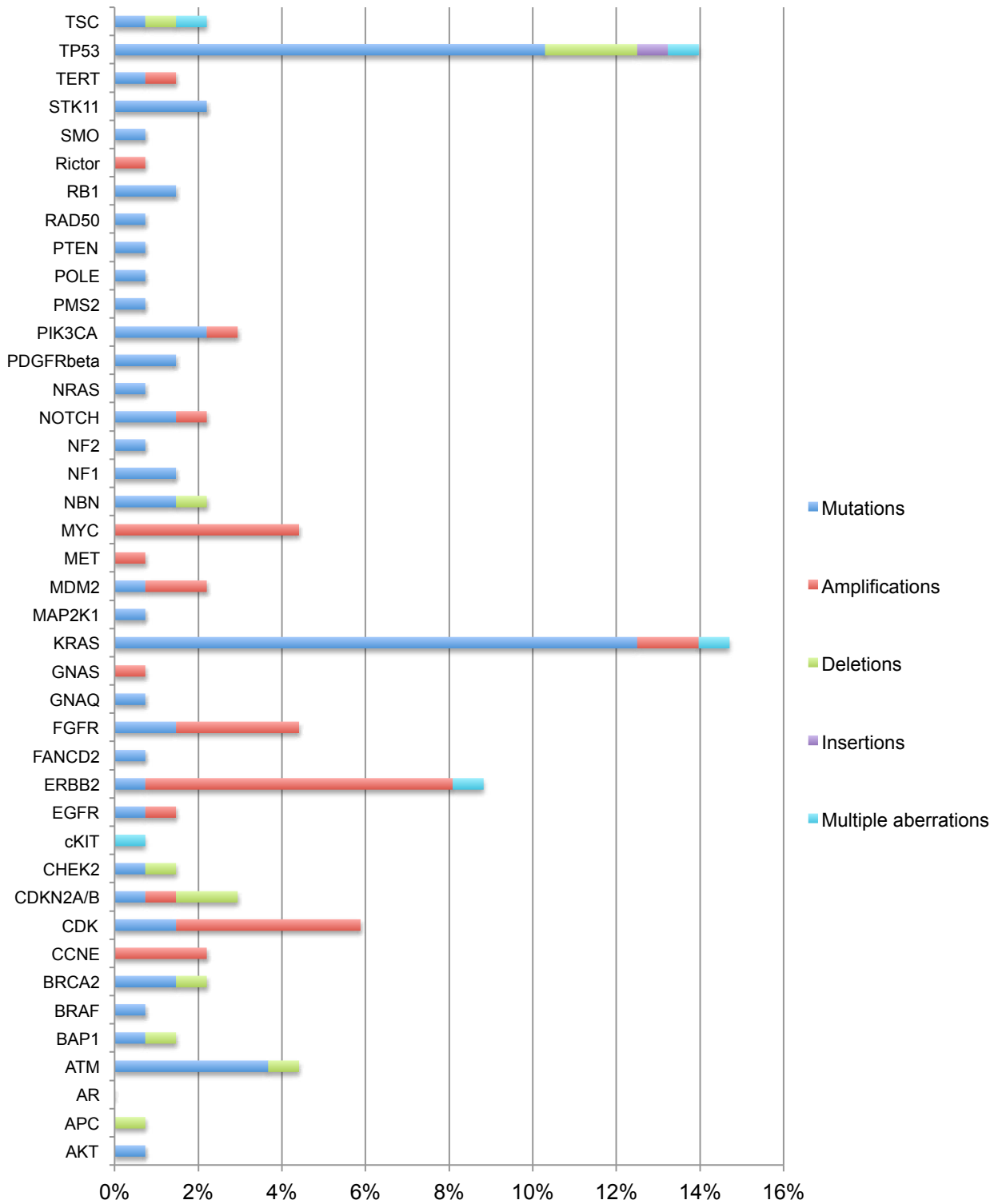
**Figure 7.** Frequency of different tumor diagnosis

Tissue Next-generation-Sequencing was performed to detect genomic alterations. In total 58 detailed genetic tests were performed in the year 2020 in patients with end stage cancer. Among the 58 presented patients, 4 patients presented more than once. Two of the patients received the Oncomine-Comprehensive testing twice. Once performed in the material of the primary tumor and a second time in the metastatic specimen. Another two patients received the Oncomine-Focus testing first and for further evaluation the Oncomine – Comprehensive testing afterwards. For those receiving the Oncomine-Comprehensive testing twice only the first test being conducted in the primary tumor specimen was taken into consideration. For those receiving the Oncomine- Comprehensive testing after an Oncomine-Focus test, only the more detailed Oncomine-Comprehensive test result was considered. In 50 patients (92.6%) a pathogenic alteration (Mutation, Fusion, CNV) or variant of unknown significance (VUS) was detected, as can be seen in Table 4. In 4 Patients (7.4%) no genomic alteration was identified via the next-generation panel sequencing.

**Table 4.** Frequency of patients with genomic alterations

<b>Presence of Genomic alteration</b>	<b>Frequency (N)</b>	<b>Percentage (%)</b>
<b>NO</b>	4	7,4%
<b>YES</b>	50	92,6%
<b>Total</b>	54	100.0%

Within the 50 patients a total of 138 pathogenic genomic alterations were noticed. Figure 7 shows the distribution of the genetic aberrations, including mutations, amplifications, deletions, insertions and multiple aberrations. KRAS was the most commonly altered (N=20, 15%), followed by TP53 (N=19, 14%), ERBB2 (N=12, 9%), CDK (N=8, 5%), MYC and FGFR (N=6, 4%). Additionally in 3 patients fusions (TMPRSS2-ERG, FGFR3-TACC3 and EGFR1-MET) were detected and were taken into consideration in this study.



**Figure 8.** Genomic alterations

The majority of patients had more than two prior lines of therapy (N=15, 27.8%) as depicted in Table 5. 14 Patients (25.9%) had only one prior line of therapy. 2 pretreatments were received by 13 oncologic patients (24.1%) and in 12 patients (22.2%) the detailed genetic test was conducted before any line of therapy was given.

**Table 5.** Prior lines of therapies

	<b>Frequency (N)</b>	<b>Percentage (%)</b>
<b>≥3 Pretreatments</b>	15	27,8%
<b>1 Pretreatment</b>	14	25,9%
<b>2 Pretreatments</b>	13	24,1%
<b>No previous therapy</b>	12	22,2%
<b>Total</b>	54	100.0%

To help with the interpretation of somatic variants, mutations, CNVs and fusions were categorized into Evidence Levels. Kat III was the most commonly present Evidence Level (N=57, 41.6%) as can be seen in Table 6. Followed by Evidence Level Kat II (N=50, 36.2%) with the majority being Kat II Level 3 (N=22, 15.9%) and Kat IV (N=23, 16.7%). Gene mutations for which there is already an EMA or FDA approved therapy were detected in only 5.8% (N=8).

**Table 6.** Frequency of categorized Evidence Levels

<b>Evidence Level</b>	<b>Frequency (N)</b>	<b>Percentage (%)</b>
<b>Kat I</b>	8	5,8%
<b>Kat II</b>	50	36,2%
Kat II Level 1a	12	8,7%
Kat II Level 1c	3	2,2%
Kat II Level 2a	13	9,4%
Kat II Level 3	22	15,9%
<b>Kat III</b>	57	41,3%
<b>Kat IV</b>	23	16,7%
<b>Total</b>	138	100.0%

Patients were evaluable for targeted therapy if the genomic variation was categorized as Evidence Level Kat. II or I. Targeted genetic therapy was eligible in 35 (70%) out of the 50 patients with identified genetic abnormalities (Table 7).

**Table 7.** Approach for targeted therapy

	<b>Frequency (N)</b>	<b>Percentage (%)</b>
<b>Targeted therapy can be considered (Study (Kat II) or approval (Kat I) for therapy available</b>	35	70%
<b>Targeted Therapy is not an option</b>	15	30%
<b>Total</b>	50	100.0%

Of the 54 patients in total and of those 35 patients eligible for targeted therapy, 14.8% (N=8 Patients) received a treatment based on the detailed genetic test findings (Table 8). 6 (11.1%) of the patients received an Evidence Level Kat I targeted therapy and only 2 patients (3.8%) were treated accordingly with an Evidence Level Kat II. 46 (85.2%) of the 54 patients received no targeted therapy but another therapy chosen by the treating physician.

**Table 8.** Treatment regime

<b>Therapy</b>		<b>Frequency (N)</b>	<b>Percentage (%)</b>
<b>Physician choice therapy</b>		46	85,2%
<b>Targeted Therapy</b>	Kat II Level 1a	1	1,9%
	Kat II Level 1c	1	1,9%
	Kat. I	6	11,1%
<b>Total</b>		54	100.0%

In the period up to June 2022 following the genetic testing in the year 2020 (December 2020 to June 2022), 38 (70.4%) out of 54 female and male oncologic patients deceased (Table 9). Of 46 patients receiving a physician's choice therapy and no targeted therapy 34 (73.9%) patients deceased compared to 4 (50%) of 8 patients in the group receiving a

targeted therapy. However, the difference between the two groups was not statistically significant ( $P=0.176$ ). Of those receiving a targeted therapy 6 oncologic patients received a targeted therapy Kat I and 2 of them Evidence Level Kat II therapy. 50% of those receiving Evidence Level Kat I (3/6 patients) and Kat II (1/2 patients) therapy deceased respectively.

**Table 9.** Distribution of deceased patients in the period up to June 2022

TREATMENT REGIME	DECEASED		<i>P</i> *	
	No	Yes		
Physician choice therapy	12 (26,1% RT)	34 (73,9% RT)	46 (85,2%)	0.176
Targeted Therapy	4 (50% RT)	4 (50% RT)	8 (14,8%)	
Kat II	1	1	2 (3,8%)	
Kat. I	3	3	6 (11,1%)	
	16 (29,6%)	38 (70,4%)	54	

Data are presented as absolute numbers. In parenthesis data are presented as percentages (%) as well as percentages of Row Total (% RT).

\* Chi-squared ( $\chi^2$ ) test

As shown in Table 10 within 3 months after the results of the detailed genetic test were obtained of those patients receiving a physician's choice therapy (N= 46), 10 patients (21.7%) died (Table 10) and in the group of patients with a targeted therapy treatment regime, 2 patients (25%) deceased within 3 months and 6 patients (75%) survived longer than 3 months after receiving genetic test results ( $P=0.839$ ). Subdivision of the targeted therapy group according to evidence levels showed that none of the two patients receiving evidence level Kat II therapy died and of those receiving a targeted therapy Kat I 2 of 6 patients deceased within the 3 months.



**Table 10.** Distribution of deceased patients within 3 months after the results of the detailed genetic test were obtained

<b>TREATMENT REGIME</b>	<b>DECEASED WITHIN 3 MONTH</b>			<b>P*</b>
	No	Yes		
<b>Physician choice therapy</b>	36 (78,3% RT)	10 (21,7%)	46 (85,2%)	0.839
<b>Targeted Therapy</b>	6 (75,0% RT)	2 (25,0% RT)	8 (14,8%)	
<b>Kat II</b>	2	0	2 (3,8%)	
<b>Kat. I</b>	4	2	6 (11,1%)	
	42 (29,6%)	12 (70,4%)	54	

Data are presented as absolute numbers. In parenthesis data are presented as percentages (%) as well as percentages of Row Total (% RT).

\* Chi-squared ( $\chi^2$ ) test

## **5. DISCUSSION**

Cancer is an extremely heterogeneous disease, which still leads to death in a considerable amount of patients, despite the field of oncology having developed and expanded dramatically in the last years (30). Therefore, it is clearly of interest to find more effective therapy strategies better suited to the individual patient. This study investigated, whether detailed genetic testing of unselected patients with advanced tumor stage provides the basis for new molecular targeted therapies and improves the outcome.

A total of 54 male and female patients with advanced tumor stage received detailed genetic testing in the year 2020 and were included in this study. Our patient collective had a median age of 68 years. Others also investigating the advantage of targeted therapy achieved promising results in their study with a similar patient median age of 61 years (31).

Our results did show that a substantial number of unselected incurable cancer patients exhibit targetable genetic alterations. Of the 54 patients included in this study in 92.6% (N=50) a genetic aberration was detected through the Next-Generation Sequencing panels and 70% (N=38) of 50 patients were evaluable for targeted therapy. In total, 138 pathogenic genomic alterations and 3 pathogenic fusions were detected. As observed in our study results, KRAS was the most commonly altered (N=20, 15%), followed by TP53 (N=19, 14%) and ERBB2 (N=12, 9%). Similarly, in other studies (31,33) TP53 and KRAS were the two most commonly genetic alterations. Both TP53 and KRAS are frequently mutated in diverse types of cancer, whereas others are rare and often restricted to only one cancer type (39).

Within our patient group the results of our study identified pancreatic cancer, followed by gastro-esophageal and colorectal cancer as the most common cancer types. In the similar study conducted by Kato S. *et. al.* the most common diagnosis was breast cancer (31). Colorectal and hematologic malignancies were the second and third most commonly detected tumor types. Contributing to these findings is that a detailed genetic test was preferably ordered if there is already EMA or FDA approved therapy available if the patients exhibit a certain mutation. Pancreatic cancer for example can be treated with a PARP inhibitor if the cells have changes in one of the BRCA genes (BRCA1 or BRCA2) or NTRK inhibitors can be used if pancreatic cancer patients exhibit changes in one of the NTRK genes (33).

The results regarding the assignment of Evidence Level did show that most of the pathogenic alterations were alterations with no clear agent being available (Kat III) (Table 6).

Of those mutations categorized under Kat II, a slight majority of actionable mutations were categorized into clinical Evidence Levels (Kat II Level 1 and 2). The MASTER molecular stratification program used the NCT classification system for categorization of actionable mutations and also identified that in nearly two-thirds of cases their decision is supported by clinical evidence and is in large parts based on clinical observations in other tumor entities (32). It should be noted however, that in individual cases classification of detected actionable molecular alterations is difficult and overall the classifications system in the setting of this study served primarily to distinguish whether there was already clear evidence and thus therapy indication or not. With regard to the excess of Kat III assigned genetic alterations one must take into account that the most commonly altered genes were KRAS and TP53. The Kirsten Rat Sarcoma Viral Oncogene Homologue (KRAS) has the highest mutation rate among all cancers and due to the intrinsic characteristics of KRAS proteins, targeting KRAS is quite challenging. Despite 40 years of proprietary drug efforts, there are still hardly any effective strategies targeting KRAS (34). But the recently discovered specific KRAS (G12C) mutation is raising the hope of drugging KRAS. Also it is a general finding that a dysfunction of the TP53 gene is a highly attractive target for the development of new anticancer drugs, but challenging so far (35). Although multiple strategies have been investigated to target TP53, only 2 of these, including MDM2/MDM4 inhibitors and mutant p53 reactivating compounds, have yielded compounds for testing in clinical trials (35) but it is still unclear if these agents have clinical efficacy. Regardless of the promising progress, further investigation is clearly warranted, not only regarding mutations in KRAS and TP53 but also in many other genetic aberrations.

Of the 38 patients eligible for targeted therapy, only 14.8% (N=8) received a targeted therapy. 6 of them received a therapy already approved by the EMA or FDA compared to two patients receiving a not yet approved and more experimental therapy approach. Despite the advances in technology a gap exists between the plethora of preclinical data and the lack of effective therapies (36). This is attributed to suboptimal drug development targeting driver mutations or other aberrations of human cancer, high costs of clinical trials and available drugs as well as limited access of patients to clinical trials (36).

Furthermore, our results showed that a large proportion of male and female patients of our older patient collective received more than two prior lines of therapy (Table 5).

Consequently genetic testing is often requested only after previous therapy attempts have been unsuccessful and targeted therapy is often only considered as a last measure.

As seen in our results, the majority of patients, regardless of whether they received targeted therapy or not, deceased between the time the test was performed in 2020 and the time the data was collected in June 2022. Some of them already passed away within the first 3 months after performing the test. The results of whether more patients who did not receive targeted therapy deceased within the first three months were not significant. Due to the short time frame chosen and the small study group without control group it was not possible to determine whether gene targeted therapy improves the overall survival (OS). However, the study of Haslem DS *et.al.* compared precision oncology to standard therapy or best supportive care and the results show a significant ( $P=0.008$ ) survival benefit for patients receiving targeted therapy compared to the cohort of patients receiving standard therapy (37).

When interpreting and applying the conclusions, several limitations to our study must be considered. First we had a very small patient collective. Of the 54 patients who underwent detailed genetic testing in the year 2020 only 8 patients received a targeted therapy. This made a valid comparison in regard of mortality between patients receiving a targeted therapy and those who received a physician's choice therapy difficult and left us with non-significant results. Also the data was retrospectively collected up to one and a half years following the conduction of the detailed genetic test. A substantial number of patients had a progression of disease and deceased within this time period; however, 30% of the patients were still alive at the time of data collection. A meaningful analysis in terms of overall survival was therefore not possible. Additionally, it was not a randomized controlled trial but rather reflected an unselected sample of oncologic patients. Another limitation refers to the assignment of evidence levels. The classification is partly subjective and is always time-dependent, since new data is constantly coming in.

To reach the ultimate goal of precision treatment of cancer with an effective drug delivery to each individual patient based on their molecular profiles, a considerable amount of basic research to understand the fundamentals of cancer heterogeneity is still required (38). Also when taking the often higher age of patients and the multiple pretreatments into account patients might benefit from earlier testing for potentially treatable genetic alterations. Overall,

precision oncology is a very promising area of oncology and it may be possible to treat and cure many more patients in the future.

## **6. CONCLUSIONS**

- Mostly detailed genetic tests are conducted in elderly patients with advanced tumor diseases and quite late in the course of therapy. Patients would benefit from earlier testing for potentially targetable genetic alterations.
- Often a detailed genetic test is ordered in a tumor type with a known and already EMA or FDA approved targeted therapy to exist for certain genetic aberrations.
- A substantial number of unselected cancer patients exhibit targetable genetic alterations and many of them are eligible for targeted therapy. However, predominantly there were no already approved targeted therapies available and only a small fraction of patients with existing preclinical or clinical evidence actually received a targeted therapy. This is attributed to suboptimal drug development targeting genomic aberrations, high costs of clinical trials and available drugs as well as limited access of patients to clinical trials.
- A high mortality of the vulnerable patients was observed in our study results. Furthermore our results did not show significantly fewer deaths in those patients receiving targeted therapy compared to those who received a physicians choice therapy within three months of test implementation or until the time of data collection. However, other studies presented promising results with a significant survival benefit for patients receiving targeted therapy.



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## **8. SUMMARY**

**Objectives:** The purpose of this study is to confirm, whether detailed genetic testing of patients with advanced tumor stage provides the basis for new molecular targeted therapies and to determine, if detailed genetic testing extends treatment options and improves the outcome. In addition, the frequency of molecular alterations and their level of evidence were determined in an unselected group of patients. Furthermore, we investigated the frequency of targetable genomic aberrations and the proportion of patients actually treated with targeted therapy.

**Materials and Methods:** 54 patients who received detailed genetic testing in the year 2020 to detect possible pathogenic mutations were included in this study. All of them were diagnosed with cancer and treated at the oncologic ambulatory health care center in Coburg. In total, 58 detailed genetic tests were performed using the OncoPrint Comprehensive v3 panels or the OncoPrint Focus panels. Electronic medical records were reviewed for the patient characteristics and Evidence Level classification was used to help interpreting somatic variants and evaluate their diagnostic, prognostic and therapeutic implications.

**Results:** Most (70%) of the patients with different cancer types exhibit a targetable genetic alteration but only 14.8% of the patients eligible for targeted therapy received a targeted therapy. The majority (70.4%) of patients, regardless of whether they received targeted therapy or not, deceased between the time the test was performed in 2020 and the time the data was collected. Also, there was no significant difference between the patients receiving targeted therapy and those receiving a physician's choice therapy regarding their death 3 months after performing the detailed genetic test.

**Conclusions:** Predominantly elderly patients with advanced disease and multiple prior therapies received detailed genetic testing. Even though a substantial number of unselected incurable cancer patients exhibit targetable genetic alterations and are eligible for targeted therapy only a few of the patients actually receive it. This is attributed to difficult drug development targeting genomic aberrations, high costs of clinical trials and available drugs as well as limited access of patients to clinical trials. Also a high mortality of the patients was observed regardless of whether they received targeted therapy or not.

## **9. CROATIAN SUMMARY**

**Naslov:** Prošireno molekularno testiranje uzastopnog uzorka tumora i zaključci za buduće terapije.

**Ciljevi:** Svrha ove studije je potvrditi da li je detaljno genetsko testiranje bolesnika s uznapredovalim stadijem tumora temelj za nove molekularno ciljane terapije. Uz to se ispituje, proširuje li detaljno genetsko testiranje mogućnosti liječenja, te poboljšava li ishod terapije. Štoviše, utvrđena je učestalost molekularnih promjena i njihova razina dokaza u neselektiranoj skupini pacijenata. Nadalje, istražili smo učestalost ciljanih genomskih aberacija i udio pacijenata liječenih ciljanom terapijom.

**Materijali i metode:** Uključeno je 54 pacijenata koji su 2020. godine primili detaljno genetsko testiranje za otkrivanje mogućih patogenih mutacija. Svima je dijagnosticiran rak i liječeni su u onkološkom ambulantnom zdravstvenom centru u Coburgu. Ukupno je provedeno 58 detaljnih genetskih testova korišćenjem panela Oncomine Comprehensive v3 ili panela Oncomine Focus. Pregledani su digitalni medicinski zapisi pacijenata iz kojih su izvađeni karakteristične podatke. Procjena razine dokaza je rađena kako bi se protumačile somatske varijante i procjenile njihove dijagnostičke, prognostičke i terapijske implikacije.

**Rezultati:** Većina (70%) pacijenata s različitim tipovima raka pokazuje genetsku promjenu pogodno za ciljanu terapiju, ali samo 14,8% pacijenata koji su kvalificirani za ciljanu terapiju primilo je ciljane terapije. Većina (70,4%) pacijenata, neovisno o tome jesu li primali ciljanu terapiju ili ne, preminula je između vremena provedenog testa 2020. godine i trenutka prikupljanja podataka. Također, nije bilo značajne razlike između pacijenata koji su primali ciljanu terapiju i pacijenata koji su primali terapiju po izboru liječnika, u pogledu na smrtnost 3 mjeseca nakon genetskog testa.

**Zaključci:** Pretežno stariji pacijenti u naprednom stadiju bolesti i više prethodnim terapijama primili su detaljno genetsko testiranje. Iako značajan broj neizlječivih pacijenata pokazuje genetske promjene koje se mogu liječiti ciljanom terapijom, samo manjina pacijenata zapravo i prima ciljanu terapiju. Ova činjenica se može pripisivati teškom razvoju lijekova usmjerena na genomske aberacije, visokim troškovima kliničkih ispitivanja i dostupnih lijekova. Uz to, otežavajući dolazi i ograničeni pristup pacijentima kliničkim ispitivanjima. Također je primijećena visoka smrtnost pacijenata bez obzira na primanje ciljane ili konvencionalne terapije.



## **10. CURRICULUM VITAE**

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Clinical Rotations in Emergency Medicine and Orthopedics February 2022 in Coburg and Lichtenfels Regiomed Kliniken

Clinical Rotations in Internal Medicine and Geriatrics January 2022 in Rheinfelden, Germany

Clinical Rotations in Gynecology August 2021 in Nürnberg, Germany

Nurse Internship 3 Months December 2015-March 2016 in Lörrach, Kreiskrankenhaus

Refugee help, Working group for health, Support during consultation hours and vaccination campaigns November 2015-July 2016

Internship at F. Hoffmann – La Roche AG in Basel – pharmaceutical Industry (Parenteralia) July 2015-August 2015