DISSERTATION

Next-generation sequencing in the context of useful and possible clinical applications in peripheral hospitals

submitted by

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for the academic degree of a

Doctor of Medical Science

(abbr. Dr. scient. med.)

at the

Medical University of Graz

Diagnostic and Research Institute of Human Genetics

under the supervision of

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2024

Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice “.

Erich Schaflinger

Graz, Juli 2024

Disclosures

Part of this thesis have been published in

- Schaflinger E, Dietmar E. The impact of next-generation sequencing on medical genetic diagnostics and counseling. Dtsch Med Wochenschr 2022; 147: 1336-1341.

- Schaflinger E, Blatterer J, Khan AS, Kaufmann L, Auinger L, Tatrai B, Abbasi SW, Ali MZ, Abbasi AA, Kaissi AA, Petek E, Wagner K, Khan MA, Windpassinger C. An exceptional biallelic N-terminal frame shift mutation in ZMPSTE24 leads to non-lethal progeria due to possible utilization of a downstream alternative start codon. Gene 2022; 833: 146582.

- Enko D, Schaflinger E, Müller DJ. Clinical application examples of a next-generation sequencing based multi-genepanel analysis. Dtsch Med Wochenschr 2023; 148: 695-702.

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- Ali MZ, Blatterer J, Khan MA, Schaflinger E, Petek E, Ahmed S, Khan E, Windpassinger C. Identification of a novel protein truncating mutation p.ASP98* in XPC associated with xeroderma pigmentosum in a consanguineous Pakistani family. Mol Genet Genomic Med 2020; 8: e1060.

- Ahmed J, Windpassinger C, Salim M, Wiener M, Petek E, Schaflinger E, Taj S, Hussain S, Abbas S, Abbas M, Younis I, Muhammad N, Khan S, Khan MA. Genetic study of Khyber-Pukhtunkhwa resident Pakistani families presenting primary microcephaly with intellectual disability. J Pak Med Assoc 2019; 69: 1812-1816.

I confirm that all co-authors have explicitly agreed to the use of their data in the thesis.

Acknowledgments

The doctoral candidate was trained within the frame of the Doctoral School of "Lifestyle-related Diseases" of the Medical University of Graz.

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Abbreviations

AML	Acute myeloid leukemia
BMP	Bone morphogenetic protein
bp	base pair
CNV	Copy number variant
DMT1	Divalent metal-ion transporter
DPYD	Dihydropyrimidine dehydrogenase
dNTPs	Deoxynucleotides
ddNTPs	Dideoxynucleotides
ELN	European LeukemiaNet
FAP	Familial adenomatous polyposis
FFPE	Formalin-fixed paraffin-embedded
GPI	Glykosyl-phosphatidyl-inositol
GTG	Gentechnikgesetz
HBOC	Hereditary breast and ovarian cancer
HNPCC	Hereditary non-polyposis colorectal cancer
IRIDA	Iron-refractory iron deficiency anemia
ITDs	Internal tandem duplications
MAP	*MUTYH*-associated polyposis
MDS	Myelodysplastic syndromes
MMR	Mismatch repair
MPN	Myeloproliferative neoplasm
MRD	Minimal residual disease
MSI	Microsatellite instability
NGS	Next generation sequencing
NIPT	Non-invasive prenatal test
NSCLC	Non-small cell lung cancer
NUDT15	Nudix hydrolase 15

PPAP	Polymerase proofreading-associated polyposis
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
STR	Short tandem repeat
TMB	Tumor mutation burden
TfR1	Transferrin receptor-1
TfR2	Transferrin receptor-2
TPMT	Thiopurine methyltransferase
VUS	Variant of uncertain clinical significance
WES	Whole exome sequencing
WGS	Whole genome sequencing
WHO	World Health Organization

Abstract in German

Next-Generation Sequencing (NGS) ist eine moderne Hochdurchsatz-Sequenziermethode, die sich in den nächsten Jahren auch in peripheren Krankenhäusern durchsetzen wird. Sie ist ein nützliches diagnostisches Instrument, welches eine präzise genetische Diagnose sowohl somatischer Mutationen in Tumoren als auch von Keimbahnmutationen bei erblichen Krebserkrankungen ermöglicht.

Ziel dieser Arbeit war es, sinnvolle Möglichkeiten und aussagekräftige Gen-Panels für die Etablierung einer NGS-Diagnostik kombiniert mit genetischer Beratung in peripheren Krankenhäusern außerhalb eines universitären Zentrums für Humangenetik zu untersuchen. Darüber hinaus wird aufgezeigt, welche Fragestellungen eine Zusammenarbeit mit einem Humangenetischen Zentrum erfordern.

Periphere Krankenhäuser könnten von der Anwendung der NGS-Technik in den Bereichen Onkologie (z. B. Dickdarmkrebs, Brustkrebs), Hämatologie (z. B. akute myeloische Leukämie, myelodysplastisches Syndrom, myeloproliferative Syndrome), sowie in der Diagnostik erblicher Tumorsyndrome (z. B. Lynch-Syndrom, familiäre adenomatöse Polyposis, erblicher Brust- und Eierstockkrebs) profitieren. Die Erhebung einer Familienanamnese in Kombination mit einer professionellen genetischen Beratung würde den Zugang zu einer individualisierter Präzisionsmedizin für Patientinnen und Patienten im jeweiligen Einzugsgebiet deutlich erleichtern. Weitere mögliche Beispiele für eine sinnvolle Anwendung der NGS-Technologie in peripheren Häusern sind die Abklärung spezieller Krankheitsbilder, wie z. B. genetisch bedingte Störungen des Eisenstoffwechsels, und nicht zuletzt das große Feld der Pharmakogenomik.

Spezielle Themen, wie die Erforschung konsanguiner Erkrankungen, kausale Zusammenhänge seltener Erkrankungen, oder wissenschaftliche Projekte, erfordern die Zusammenarbeit peripherer Krankenhäuser mit universitären Zentren für Genetik.

Die Ausweitung der NGS-Technologie auf periphere Krankenhäuser wird die diagnostischen und therapeutischen Möglichkeiten an den jeweiligen Standorten deutlich verbessern.

Abstract in English

Next-generation sequencing is a modern high-throughput sequencing method, which will be also established in peripheral hospitals within the next few years. It is a useful diagnostic tool, which enables a precise genetic diagnosis for somatic mutations in tumors as well as germline mutations in hereditary cancers.

This thesis aimed at investigating the possibilities of establishing meaningful areas and gen-panels for NGS diagnostics combined with genetic counseling in peripheral hospitals outside of University Centers of Human genetics. Furthermore, it is demonstrated, which issues require cooperation with a Human Genetic Center.

Peripheral hospitals would have a benefit from NGS-technique application in the fields of oncology (per example: colon cancer, breast cancer), hematology (per example: acute myeloid leukemia, myelodysplastic syndrome, myeloproliferative syndromes), and also in the diagnosis of hereditary tumor syndromes (per example: Lynch syndrome, familial adenomatous polyposis, hereditary breast and ovarian cancer). Family anamnesis combined with professional genetic counseling would significantly facilitate access to individualised precision medicine for patients in the respective catchment area. Further possible examples for a useful application of the NGS-technology in peripheral hospitals are the clarification of special clinical pictures, such as genetic disorders of iron metabolism, and last but not least, the big field of pharmacogenomics.

Special topics, such as the investigation of consanguineous diseases, causal relationships of rare diseases, or scientific projects, require the cooperation of peripheral hospitals with University Centers of Genetics.

The extension of NGS-technology to peripheral hospitals will significantly improve diagnostic and therapeutic options at the respective locations.

1. Introduction

The use of high-throughput sequencing methods in daily medical diagnostics routine and the resulting increase in knowledge about the human genome open up new dimensions in the prevention, prognosis assessment and the treatment of heterogeneous genetic diseases. The wide range of clinical applications includes tumor diseases (e.g. hereditary breast and ovarian carcinomas), hematological diseases, cardiac arrhythmias, cardiomyopathies, epilepsies, neurodegenerative and neuromuscular diseases, pharmacokinetics and non-invasive prenatal diagnostics.

The clinical-genetic interpretation of complex genetic data with high genetic variability is a challenge for both the diagnostician and the clinician. Genetic counseling in the era of this new high-throughput sequencing technology is gaining in importance and takes significantly more time compared to traditional genetic counseling focused on a corresponding causative target gene due to the large number of genes examined, the occurrence of unexpected results and the interpretation of so-called "variants of unclear clinical significance".

Next-generation sequencing (NGS) is a modern high-throughput method, which enables complex multi-gene analyses. It can be used for a better genetic diagnosis for somatic mutations in tumors and also for germline mutations in hereditary cancers respectively tumor disposition syndromes.

In the last decade, this unique technology has been established for a broad range of clinical disorders and will be further expanded in the clinics over the next few years. This will lead, that this technology will not only be use in University Hospitals or Centers but also in laboratories of General Hospitals.

In most peripheral hospitals, the current situation is that many patients with genetic medical issues have to come to the university hospital. This is a hurdle for many of those affected. Human genetics is currently not established in peripheral hospitals and there is a lack of family histories, which play a decisive role in the further diagnostic and therapeutic procedure for many genetic issues, per example for the hereditary colon carcinoma.

If both human genetic counseling and the corresponding genetic analysis can be established in peripheral hospitals, this will be of enormous benefit to patients in the respective peripheral

catchment area. The relevant expertise in breast cancer, for example, can be expanded and utilized there. Collaboration with the university centers is reserved for special issues, such as rare genetic diseases or scientific work and projects.

The first subchapter of this thesis provides a brief overview of Sanger sequencing and NGS.

1.1 Sequencing technologies: Sanger and NGS

Polymerase chain reaction (PCR)-based sequencing technologies enable the elucidation of unknown DNA segments up to the sequencing of the entire genome of an affected individual.

In 1977, Fred Sanger developed a new sequencing method in which new DNA is generated enzymatically by the so-called chain termination synthesis and then the DNA is analyzed (Sanger et al. 1977). In this type of sequencing, so-called dideoxynucleotides (ddNTPs), which do not carry a 3'-OH group, are added to the four normal deoxynucleotides (dNTPs). Once a ddNTP has been incorporated, the synthesis is terminated, as further incorporation of nucleotides can no longer take place due to the missing 3'-OH group.

The individual PCR products can be separated by gel electrophoresis based on the differences in length. The respective color of the incorporated, labeled ddNTPs is determined using a laser. The base sequence can be derived from this. This sequencing method can be used to search specifically for disease-causing changes in the DNA sequence if a monogenic disease is clinically suspected.

This sequencing technique is still in use today but is increasingly being replaced by NGS technology. NGS as high-throughput sequencing has led to a revolution in medical genetic diagnostics over the last 15 years. New methodological approaches are constantly appearing on the medical market (Slatko et al. 2018). The basic principle of this technology is based on four steps:

- Creation of a library: DNA fragments are generated, which are provided with adaptors at the 3' and 5' ends.
- Cluster amplification: The DNA fragments are bound to a flow cell and amplified. This generates clusters.

- Sequencing: Fluorescent nucleotides are incorporated accordingly. Due to the division into clusters, many sequencing runs can be performed simultaneously in a very short time.
- Data analysis: The data obtained is analyzed bioinformatically. The individual reads are combined and compared with a reference genome (Neveling et al. 2014).

Although the use of NGS technology is aimed at the rapid determination of large amounts of data, different amounts of data can be determined depending on the research interest and specific strategy (Deutscher Ethikrat 2013).

This method is suitable for the so-called "panel diagnostics", the search for a group of different genes at various gene loci in the human genome. Furthermore, all exons can be sequenced as part of "exome sequencing" ("whole exome sequencing" (WES)) or the entire genome as "whole genome sequencing" (WGS) (Holinski-Feder 2017).

Due to the rapidly increasing growth in the field of clinical NGS-based genetic testing, it is essential that clinical staff have a certain basic understanding of this new technology (Muzzey et al. 2015).

1.2 NGS is a revolutionary high-throughput method

1.2.1 High-throuput method

NGS is a very revolutionary and dynamic field of technology whose potential is far from exhausted (Kuß 2014).

The advantages of NGS-based diagnostics over conventional sequencing according to Sanger are the possibility of parallel sequencing of a large number of DNA fragments with a higher diagnostic sensitivity and a significantly more cost-effective processing compared to the "gene-by-gene" method.

The high sequencing capacity with precise genetic diagnosis also generates large amounts of digital data. Bioinformatics is playing an increasingly important role here. It makes it possible to translate the volumes of data obtained into analysable information.

While the time required for sequencing per se has been reduced many times over by NGS-based methods, the requirements for the clinical interpretation of the analyses have increased many times over due to the large amounts of data and are very complex.

1.2.2 Data analysis and quality control management

The digital primary data generated by the respective NGS platform is translated into a nucleotide sequence (read). The individual reads are subjected to quality control. The probability with which each nucleotide is actually present or has been incorrectly recognized is specified for each nucleotide (Weißmann & Gilissen 2014).

This is followed by the so-called "alignment". Computer programs are used to assign the individual reads to a position in the human reference genome. As part of the secondary data analysis, the so-called "variant calling", it is determined where and how the examined DNA differs from the reference genome in order to compare the variants of an individual with single nucleotide polymorphism (SNP) databases in the subsequent tertiary data analysis and to filter out frequent pathogenetically irrelevant variants.

A distinction must be made between actual changes that are present in the sequenced DNA and technical artifacts that occur, for example, due to sequencing errors or incorrectly assigned reads. The sequencing depth, i.e. the number of reads that can be assigned to a common position in the human genome, is the decisive measure for reliable variant calling. The greater the sequencing depth, the better it is possible to distinguish between actual variants and technical artifacts on the sequenced DNA segment (Weißmann & Gilissen 2014).

The high genetic variability of the human genome remains an enormous challenge for the clinical-genetic interpretation of data in everyday clinical practice. Here, the genetically trained diagnostic expert must decide whether and which of the variants found are actually disease-relevant (pathogenic). The implementation and clinical application of an NGS infrastructure, including the securing of large amounts of data, requires highly motivated scientific personnel who are also available on a long-term basis.

1.3 The impact of genetic diagnostics in daily medical practice

The human genome consists of a total of three billion base pairs and contains between 25,000 and 30,000 genes (Poeggel & Mewitinger 2017).

The targeted interaction of genes and their products controls all biological processes in the human body. As a result of biomedical research, medical genetics has undergone rapid development in recent years with an enormous increase in knowledge and now extends very deeply into everyday clinical practice.

When considering disease processes, the possibility of a genetic cause is increasingly being considered and at the same time traced back to its molecular roots. Increasing knowledge of the human genome enables a better basic understanding of the multifactorial interaction between genetic material and the environment. This opens up new possibilities in the prevention, diagnosis and treatment of genetic diseases (Schweizerische Akademie der Medizinischen Wissenschaften 2011).

Today, state-of-the-art high-throughput sequencing, e.g. using NGS, makes it possible to make medical diagnoses that were not possible until recently. Genetic diagnostics in modern medicine is not only available for targeted prevention and family counseling, but also for the causal treatment of diseases.

In order to be able to guarantee adequate clinical diagnosis and therapy for patients in daily clinical practice in the future, measures must be taken as quickly as possible to meet this demanding challenge. This includes, on the one hand, a necessary basic understanding of the various stakeholders in the healthcare system for the introduction of this innovative technology into everyday medical practice and, on the other hand, adequate measures in the context of education, training and further education of young medical professionals, which promote know-how in the areas of clinical genomics and genetic counseling (Huber et al. 2018).

The decoding of the human genome, a milestone in biomedical research, involved more than a thousand scientists for more than a decade. The cost of this international human genome project, which was successfully completed in 2003, was around 3 billion US dollars (Haller et al. 2018). In comparison, the current processing time for a complete genome sequencing including bioinformatics analysis is a few weeks at a cost of a few hundred euros (Haller et al. 2018).

Only the broad application of NGS in recent years has revealed the true extent of inter-individual genetic variability and its potential significance for everyday medical practice. These new findings open up the innovative path to genome-based personalized medicine for all clinical disciplines. It quickly became clear that humans are genetically much more variable than was assumed at the time the human genome project was completed in 2003.

There are actually millions of variants in the human genome, which occur with a frequency of >1% in the population. The most common form of variability is the so-called "single nucleotide polymorphism" (SNP). With the help of systematic genetic mapping in families and populations, numerous genetic variants that are partly responsible for the development of diseases have been identified in the period following the Human Genome Project (Collins et al. 2021). The disease-causing genes have been identified in more than five thousand rare single-gene diseases (Mendelian diseases), thus enabling genetic diagnostics and, in some cases, gene therapy for many patients (Collins et al. 2021).

The discovery of more than 100,000 associations between genomic regions and common diseases in humans has enabled the development of polygenic risk scores, which can be used to identify individuals with an increased risk of cardiovascular disease, breast cancer, etc. (Collins et al. 2021). Knowledge of disease-associated SNPs can be used to make reliable risk predictions and support diagnosis.

Gene-based medicine plays an important role in today's medical practice in the prevention and treatment of hemato-oncological diseases (Ramkissoon & Montgomery 2021, Castillo-Guardiola et al. 2022). The diagnostic application of NGS enables the identification of individuals at high risk due to hereditary germline mutations and also provides information about somatic driver genes in tumor development.

This enables early detection, particularly in breast, ovarian and colorectal cancer, combined with an important information about the response to certain therapeutic agents in everyday clinical practice. The aim of genetic diagnostics is to correctly assess disease progression as early as possible and to provide the best possible therapy for the affected individual.

Close cooperation between laboratory medicine centers, where genetic analyses are increasingly being carried out using NGS, and the various clinical disciplines is essential in order to be able to translate the latest findings in genetic diagnostics into targeted diagnosis and

better treatment in everyday clinical practice. In particular, the exchange of knowledge of new scientific findings, e.g. in the context of the introduction of new prognostic or diagnostic genetic markers, is crucial in this area.

Clinicians must be familiar with the diagnostic possibilities of sequencing technologies such as NGS. At the same time, those conducting the work in the genetic laboratory should also have the best possible knowledge of the clinical circumstances and the environment in which the examination and sample collection on the patient take place. Only in this way can the optimum treatment goal be achieved for the patient concerned.

The importance of NGS in daily clinical genetic testing is discussed in the next subchapter.

1.4 The importance of NGS for genetic diagnostics

With the increasing use of NGS technology and the establishment of new diagnostic panels, the goal of personalized medicine in everyday medical practice has come much closer.

NGS is particularly suitable for the investigation of heterogeneous genetic diseases that are caused by changes in numerous genes. These include tumor diseases (e.g. hereditary breast and ovarian carcinomas, colorectal carcinomas), cardiac arrhythmias, cardiomyopathies, epilepsies, neurodegenerative and neuromuscular diseases (Biskup 2010, Qin 2019, Lohmann 2014).

Using the specific example of patients with hereditary breast and ovarian cancer, current studies have shown that the implementation of NGS multigene panels in clinical diagnostics significantly improves the detection of high-risk patients (Molina-Zayas et al. 2022). Gene panel diagnostics using NGS provides a reliable, simple, fast, and cost-effective way of identifying hereditary tumor diseases in the oncological setting (Kamps et al. 2017).

The goal of diagnostic clarification of hereditary cancers and somatic alterations in tumors has come much closer in everyday medical practice.

One clinically relevant application example for NGS diagnostics is tumor predisposition syndromes. Here, multi-gene analyses (gene panels) with all known causative genes of the respective clinical picture are used. The detection of causative germline mutations enables reliable predictive genetic testing and at the same time the identification of high-risk family

members ("at-risk individuals") (Perne et al. 2020). In most cases, the reliable diagnostic detection of germline mutations in tumor disposition syndromes is based on leukocyte DNA from an EDTA blood sample (Perne et al. 2020).

A clinically relevant example of the use of multi-gene analysis in the context of clarifying a tumor disposition syndrome is hereditary breast carcinoma, in which the target genes *BRCA1, BRCA2, CDH1, PTEN, TP53, STK11, PALB2, ATM, CHEK2, NBN, RAD51C* and *RAD51D*, among others, are of significance (Slavin et al. 2015).

The genetic multi-panel clarification of familial ovarian cancer is also clinically relevant. The main target genes include *BRCA1, BRCA2, MLH1, MSH2, RAD51C, RAD51D* and *BRIP1* (Slavin et al. 2015). Using the specific example of patients with hereditary breast and ovarian cancer, current studies have shown that the implementation of NGS multigene panels significantly improves the detection of high-risk patients in clinical diagnostics (Molina-Zayas et al. 2022, Castillo-Guardiola et al. 2022).

Another example of an application for multi-gene analysis using NGS methods in the field of hereditary tumor syndromes is the risk assessment of familial colon rectal carcinomas. The target genes *APC, BMPR1A, EPCAM, MLH1, MSH2, MSH6, MUTYH, PMS2, SMAD4, STK11, PTEN, TP53, MUTYH, POLD1, POLE*, among others, are in the foreground (Slavin et al. 2015).

In addition to the detection of germ cell mutations, which are usually inherited from one parent and are present in all parts of the body, the NGS method is also suitable for the detection of somatic mutations that arise during tumorigenesis in neoplastic tissue and are restricted to the tumor cells (Perne et al. 2020). A clinically relevant example of this is hematological diseases, which place very high demands on NGS technology in terms of prognosis, diagnosis and detection of minimal residual disease.

High sensitivity and specificity combined with low turn-around times and costs are expected from modern NGS platforms and algorithms. Numerous diagnostic genetic markers for prognosis assessment and therapeutic decisions, especially for patients with acute myeloid leukemia, myeloproliferative neoplasia, and myelodysplastic syndrome, have been included in national and international guidelines. The implementation of NGS technology in recent years has made it possible to identify and describe the heterogeneous and unique molecular genetic map of these clonal hematologic diseases.

The implementation of NGS technology in recent years has made it possible to identify and describe the heterogeneous and unique molecular genetic map of these clonal hematologic diseases (Patel et al. 2021, Breinholt et al. 2022). Many of these mutations play a decisive role in the pathogenesis of the respective disease and are therefore crucial for targeted therapy and disease monitoring (Malinowska & Glodkowska-Mrowka 2016).

Another example of successful clinical application of NGS-based genetic diagnostics is being realized in the field of pharmacogenetics. In modern psychiatry, the focus is increasingly on testing genes that influence the metabolization of antidepressants and antipsychotics in the body. The established diagnostic gene panels include the pharmacokinetic genes of the cytochrome P450 superfamily. NGS technology is an adequate tool for the discovery of new variants for sometimes complex genotype-phenotype correlations in this field (Demkow 2016).

In recent years, NGS has also been increasingly used in prenatal diagnostics. The high sensitivity of the method enables the molecular diagnostic characterization of cell-free fetal DNA from maternal venous blood. The clinical diagnostic application of this non-invasive prenatal test (NIPT) enables very comprehensive statements to be made about the genetic characteristics of the unborn child at a very early stage of pregnancy. It is to be expected that NGS-based NIPT will find broad clinical application. With regard to the clinical integration of this procedure, ethical and social factors, including adequate genetic counseling, must also be considered (Cogulu 2016).

1.5 Genetic counseling in the NGS era

With the rapid increase in genetic data in daily medical diagnostics, the technical and bioinformatic prerequisites for these genetic diagnostics are increasingly being created and solved. The correct interpretation and transmission of findings in the context of genetic counseling are becoming increasingly important in the NGS era.

The indication for carrying out genetic diagnostics makes sense if a prophylactic or therapeutic consequence can be derived for the person seeking advice and/or their family members. In every genetic counseling session, it should be pointed out, before testing, that the right not to know exists at all times (Biskup 2010).

Regardless of the indication for genetic testing, making a correct suspected diagnosis has far-reaching consequences for all subsequent considerations regarding the risk of recurrence and prognosis assessment for other family members. Therefore, in the first step of genetic counseling, the collection of a personal and family history is mandatory for the development of a working diagnosis (Schweizerische Akademie der Medizinischen Wissenschaften 2011).

Once the appropriate genetic test has been ordered and performed, the final written genetic statement is an integral part of genetic counseling. The patient receives a letter containing the contents of the counseling, including the results and diagnoses of the corresponding genetic analyses, in generally understandable language. If possible, medical terms should be adequately paraphrased or explained (Deutsche Gesellschaft für Humangenetik 2018).

Traditional genetic counseling focuses on an appropriate target gene, which is considered with regard to the risk and cause of a disease (Vrijenhoek et al. 2015).

NGS technology can simultaneously produce information on different genetic variants with different functional and clinical consequences. The challenges of genetic counseling in NGS testing are the large number of genes studied, the occurrence of unexpected results, the interpretation of phenotype-genotype correlations of all genes studied, the lack of specific guidelines aimed at NGS, the lack of specific data for corresponding guidelines and standardization of NGS, and the interpretation of the so-called "variants of uncertain clinical significance" (VUS) (Fahrioğlu 2018, Marcus et al. 2015, Durmaz A & Durmaz B 2016).

Case studies from practice with the introduction of WES or WGS show that the NGS-based consultation process takes considerably longer and is thematically more comprehensive and complex (Machini et al. 2014). The challenges lie in presenting the benefits and limitations of testing, the possible final results and their clinical consequences for the patient or their family members in a way that is comprehensible to the patient (Machini et al. 2014).

These circumstances significantly increase the complexity of genetic counseling. Previous principles of genetic counseling must be rethought and worldwide scientific networks, which show the respective counselor different possibilities and ways of genetic counseling in the NGS era, must be used accordingly (Yang & Kim 2018).

Laboratory-based genetic counseling by the respective clinician includes, in addition to the consultation and the corresponding documentation of the case, in particular the selection of the correct genetic test or gene panel and the clinical interpretation of the test (Swanson et al. 2014).

The genetic counselor should also be familiar with the technical limitations of NGS technology. This requires close cooperation with the respective laboratory, which can answer questions and concerns regarding the testing process or genetic analysis (Facio et al. 2014). For many genetic counselors, the necessary comprehensive literature search for each gene of a diagnostic NGS panel is very time-consuming and challenging. Therefore, collaboration and discussion with other counselors and counseling centers seems extremely useful. In this way, consistent patient care can be ensured (Wolfe Schneider et al. 2014).

In recent years, medical genetic diagnostics has brought about decisive improvements in the basic understanding of the molecular mechanisms of numerous diseases. NGS technology can be used increasingly broadly in everyday medical practice in a targeted manner as part of personalized medicine.

Genetic counseling must be aligned and adapted accordingly in the light of the new complexity of genetic data information. In the coming years, a further rapid increase in knowledge can be expected in the field of NGS technology, which can be integrated into everyday clinical practice for the benefit of patients in all medical disciplines.

Genetic analyses are becoming increasingly important in medical research and practice. These are laboratory analyses that determine specific properties with regard to the number, structure or sequence of chromosomes, genes, DNA segments or DNA products and their specific chemical modifications and thus provide information about a carrier status, a disease risk, an existing disease or the course of a disease or therapy in a person.

In Austria, the handling of genetic analyses is regulated in the Austrian Genetic Engineering Act (Gentechnikgesetz - GTG). According to GTG §65, a total of four types are distinguished in genetic analyses for medical purposes:

- Type 1: Is used to determine an existing disease, to prepare a therapy or to monitor the course of a therapy and is based on statements about specific somatic changes in the number, structure, sequence or their specific chemical modifications of chromosomes, genes or DNA segments.

- Type 2: Is used to determine an existing disease that is based on a germline mutation.

- Type 3: Is used to determine a predisposition to a disease, in particular a predisposition to a genetic disease that may break out in the future or to determine a carrier status for which prophylaxis or therapy is possible according to the state of the art in science and technology.

- Type 4: Is used to determine a predisposition to a disease, in particular a predisposition to a genetic disease that may break out in the future or to determine a carrier status for which no prophylaxis or therapy is possible according to the state of the art in science and technology.

According to GTG §68, genetic analyses may only be carried out in authorized facilities:

- Genetic analyses (type 3 and type 4 analyses) may only be carried out in facilities licensed for this purpose and only at the instigation of a medical specialist trained in human genetics/medical genetics or a medical specialist responsible for treatment or diagnosis in the indication area.

- Approval shall be applied for from the Federal Minister of Health and Women's Affairs by the head of the institution in which such genetic analyses are intended to be carried out.

- Approval shall be granted by the Federal Minister of Health and Women's Affairs after consultation with the competent scientific committee, if necessary subject to appropriate requirements and conditions, if the personnel and equipment available ensure that the genetic analyses are carried out in accordance with the state of the art in science and technology.

- The Federal Minister for Health and Women's Affairs shall revoke the authorization if the requirements for its granting are no longer met or, in the event of serious deficiencies, shall impose other suitable conditions combined with the order not to carry

out any more genetic analyses pursuant to GTG §65 (type 3 and type 4) until these conditions have been met.

Genetic analyses can have a profound impact on the person affected and their family, and possibly also on their life planning. The GTG takes these particularities into account: According to §69 the person concerned must be informed in detail in advance about the nature, scope and significance of a genetic analysis.

This takes place in genetic counseling, where the person concerned receives information from specially trained doctors about the cause and manifestation of a genetic disease, the course of the disease and therapy and the probability of developing or transmitting the disease. Genetic counseling makes it possible to understand and discuss the personal significance of the information in the individual life context and to discuss it. Its possible significance for the relatives is also addressed (Bundesministerium für Gesundheit und Frauen 2005/2006).

In genetic counseling, the diagnostic measures necessary for the clarification of a genetic disease can be arranged. It should be seen as part of the most holistic, interdisciplinary long-term care of the affected person and their family (Bundesministerium für Gesundheit und Frauen 2005/2006).

The Guideline for Genetic Counseling from the "Bundesministerium für Gesundheit und Frauen" from 2002 comprises the following important points for clinicians who perform genetic counseling (Bundesministerium für Gesundheit und Frauen 2002):

- Counseling on the occasion of a genetic analysis should help those seeking advice to make autonomous decisions on the basis of the necessary information that are decisions that are acceptable in the long term. This counseling must not be conducted in a directive manner.

- Counseling prior to carrying out a genetic analysis includes clarifying the personal clarification of the personal question and the objective of the consultation. Furthermore, the collection of the personal and family health history (family tree survey, anamnesis), the evaluation of available medical medical findings or reports, detailed information about the disease in question and the prophylactic measures existing or missing prophylactic/therapeutic options.

- Counseling after a genetic analysis has been performed must include a comprehensive discussion of all test results and medical facts, social and psychological consequences and must not be directive. At the personal request of the person seeking advice or at the suggestion of the counselor, a psychotherapist should be directly involved in the counseling process.

- The person seeking advice has the fundamental right to knowledge with regard to all data relating to his/her results from the genetic analysis. However, the person seeking advice must be informed that he/she also has the right not to know all or some of the results. The contents of the counseling session(s) and its/their results are to be written form in a letter that is understandable for the person seeking advice.

- Counseling for genetic analyses on humans is the exclusive responsibility of a medical specialist trained in human genetics or a medical specialist responsible for the indication in question (§65 GTG).

- Psychotherapeutic counseling is provided by psychotherapists licensed under the Psychotherapy Act with a corresponding additional qualification in the field of human genetics.

2. Aims of the thesis

The purpose of this thesis is to demonstrate the possibilities of establishing meaningful areas of application for NGS diagnostics in everyday clinical practice in peripheral hospitals. The first part of this doctoral thesis relates in particular to the clinical fields of hematology, oncology and congenital tumor syndromes.

Nowadays the methodological application of NGS in everyday clinical practice is becoming increasingly simple. The aim is to show which areas of NGS application are also considered useful outside of a University Center of Human Genetics, with particular emphasis on useful application-related multigene panels.

The second part of this work deals with various rare consanguineous diseases, which in any case require cooperation with a Human Genetics Center, for example the Diagnostic and Research Institute of Human genetics at the Medical University of Graz, Austria.

The last part of the work covers the large field of possible NGS-application in the broad spectrum of pharmacogenomics.

3. Results and Discussion

3.1 Useful targeted NGS-based gene analyses in clinical practice

Targeted NGS-based multi-gene panel analysis with the selection of clinically relevant genes can be useful in oncology, hereditary tumor syndromes and underlying hematological diseases. Commercially available NGS panels tailored to the user's needs enable the precise identification of somatic mutations in solid tumors (e.g. lung carcinoma, gastrointestinal tumors) and thus contribute to a better prognostic and therapeutic assessment of the underlying disease.

The differential diagnostic clarification of germline mutations in increasingly genetically complex congenital tumor syndromes (e.g. hereditary breast and ovarian carcinoma syndrome, Lynch syndrome and polyposis) using NGS-based multi-panel diagnostics has proven its worth in routine diagnostics. The use of NGS multi-gene panel analysis in the field of hematology (e.g. acute and myeloid diseases) enables a diagnostic, prognostic and detectable change in the molecular genetic profile of the respective underlying disease.

The NGS technology is a powerful diagnostic tool that has changed and enabled the molecular genetic testing strategy from a “gene-by-gene approach” to “multi-gene panel testing”. Sufficient evidence-based knowledge of the causative genes of a disease is a prerequisite for genetic clarification before clinical NGS tests are performed.

NGS-based multi-gene panels developed by molecular genetics laboratories enable the simultaneous evaluation of several potential genetic causes of an underlying disease of the affected person, thus reducing time and costs.

In particular, diseases with genetic and clinical heterogeneity in the oncological and hematological field benefit from this testing strategy. In addition, hereditary tumor syndromes can be clarified more easily by molecular genetics and thus differentiated from one another in terms of differential diagnosis. The implementation of NGS-based multi-gene panel testing in daily routine not only influences and supports diagnostics, but also prognostics and targeted therapy concepts for underlying oncological and hematological diseases.

Possible disadvantages of NGS testing compared to single-gene testing are lower sensitivity, more complex analyses with higher costs and possible incidental findings that are not directly related to the primary tumor (e.g. clonal hematopoiesis).

In today's clinical setting, NGS testing is useful for certain tumors, such as the non-small cell lung cancer (NSCLC), colon cancer, myeloid diseases such as acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), but also for advanced tumor diseases in the context of molecular tumor boards.

The following subchapters of this thesis describe clinically useful application examples of an NGS-based muti-gene panel testing strategy in the fields of hereditary tumor syndromes, hematology, and oncology.

3.1.1 Hereditary tumor syndromes

A human genetic clarification of hereditary tumor syndromes appears to make sense for family members at high risk ("high-risk individuals") (Schaflinger & Enko 2022, Perne et al. 2020).

Approximately 5-10% of all carcinomas are caused by specific germline mutations in oncogenes and tumor suppressor genes. Many of these germline mutations are associated with tumor syndromes (Ramirez-Calvo et al. 2019, Paduano et al. 2022). This familial disposition leads to a high lifelong risk of actually developing the disease. Affected individuals and their families with specific hereditary tumor syndromes receive appropriate genetic counseling and molecular genetic testing (Ramirez-Calvo et al. 2019). Hereditary breast and ovarian cancer and Lynch syndrome are the best-studied tumor predisposition syndromes (Kamps et al. 2017).

Targeted multi-gene panel testing using NGS (targeted sequencing) is particularly suitable for the diagnosis of such hereditary cancers (Ramirez-Calvo et al. 2019). Circulating tumor DNA in peripheral blood results from necrotizing or apoptotic tumor cells and provides valuable information about somatic mutations and germ cell mutations (Gaspersic & Videtic Paska 2020). The detection of causative germline mutations from peripheral blood enables the rapid detection and predictive genetic testing of high-risk family members (Perne et al. 2020).

Validated NGS multi-gene panels aligned with the recommendations of the American College of Medical Genetics and Genomics (e.g. *APC, BRCA1, BRCA2, MEN1, MLH1, MLH2, MLH6,*

MUTYH, NTRK1, PMS2, PTEN, RET, RB1, SDHB, SDHC, SDHD, SDHAF2, STK11, TP53, TSC1, TSC2, VHL, WT1) for the clarification of hereditary tumor disposition syndromes are already integrated into daily routine diagnostics and have proven their worth (Shinriki et al 2020, Green et al. 2013).

The significance of important target genes for NGS panels of hereditary tumor diseases is shown in Table 1 (Shinriki et al. 2020, Green et al. 2013).

Table 1: Important target genes of hereditary tumor syndromes.

Phenotype	**Gene**	**Inheritance**
Hereditary breast and ovarian cancer	*BRCA1*	autosomal dominant
	BRCA2	
Li-Fraumeni syndrome	*TP53*	autosomal dominant
Peutz-Jeghers syndrome	*STK11*	autosomal dominant
Lynch syndrome	*MLH1*	autosomal dominant
	MSH2	
	MSH6	
	PMS2	
Familial adenomatous polyposis	*APC*	autosomal dominant
MUTYH-associated polyposis	*MUTYH*	autosomal recessive
Von-Hippel-Lindau syndrome	*VHL*	autosomal dominant
Tuberous sclerosis	*TSC1*	autosomal dominant
	TSC2	
Hereditary paraganglioma-pheochromocytoma syndrome	*SDHD*	autosomal dominant
	SDHAF2	
	SDHC	
	SDHB	

Consensus guidelines recommend the following panel diagnostics for the diagnosis of hereditary breast, ovarian and colon cancer (Taylor et al 2018):

- Breast cancer: *ATM, BRCA1, BRCA2, CHEK2, PALB2, PTEN, STK11, TP53*
- Ovarian cancer: *BRCA1, BRCA2, BRIP1, MLH1, MSH2, MSH6, RAD51C, RAD51D*
- Colon carcinoma/polyposis: *APC, BMPR1A, EPCAM, GREM1, MLH1, MSH2, MSH6, MUTYH, NTHL1, PMS2, POLE, POLD1, PTEN, SMAD4, STK11.*

Germline mutations of hereditary breast carcinoma include *BRCA1* and *BRCA2, PALB2* and, among others, Li-Fraumeni syndrome (*TP53* pathogenic variant), Cowden syndrome, (*PTEN* pathogenic variant), hereditary diffuse gastric carcinoma syndrome (*CDH1* pathogenic variant), and Peutz-Jeghers syndrome (*STK11* pathogenic variant) (Manahan et al 2019).

The use of NGS multi-gene panel analysis in the context of germline mutation testing for breast cancer enables an estimation of the cancer risk and risk-minimizing therapeutic measures to be derived from this. *BRCA1, BRCA2, PALB2, PTEN* and *TP53* are associated with high, *ATM, CHEK2* and *NBN* with moderate and *MLH1, MSH2, MSH6, RAD51C* with low risk of breast cancer (Tsaousis et al. 2022).

The most common tumor syndrome associated with breast cancer is called hereditary breast and ovarian cancer (HBOC) syndrome. Affected women also have a high risk of developing ovarian cancer. *BRCA1* and *BRCA2* are the two main genes for germline mutations in this tumor syndrome. Thanks to the development of NGS, other germline mutations associated with this syndrome (e.g. *CHEK2, BRIP1, ATM* and *PALB2* and others) can be detected simultaneously in affected families using NGS multi-gene panel analysis (Paduano et al. 2022, Castillo-Guardiola et al. 2022).

NGS studies show that HBOC can be associated with many germline mutations. Corresponding genes with high penetrance include *BRCA1* and *BRCA2, TP53, PTEN, STK11* and *CDH1*, genes with moderate or low penetrance are *ATM, CHEK2, PALB2, BRIP1, BARD1, RAD51C, RAD51D, NF1, NBN* and so-called "mismatch repair (MMR)" genes (Paduano et al. 2022, Angeli et al.2020, Shin et al. 2020, Guglielmi et al. 2021).

Another clinical example of a possible and indicated NGS multi-gene panel analysis is the genetic clarification of congenital colon rectal carcinoma and polyposis. Numerous pathogenic

germline mutations in various genes are associated with this clinical picture. Lynch syndrome (Hereditary Non-Polyposis Colorectal Cancer (HNPCC)) is the most common form of hereditary colon rectal carcinoma. It is caused by the MMR genes *MLH1, MSH2, MSH6*, and *PMS2* and is inherited in an autosomal dominant manner (Mao et al. 2023, Stern et al. 2019).

Hereditary forms of colon polyposis are familial adenomatous polyposis (FAP), which is associated with pathogenic variants of the *APC* gene, the *MUTYH*-associated polyposis (MAP), which is associated with pathogenic variants of the *APC* gene. MAP, which is characterized by biallelic pathogenic *MUTYH* variants, and polymerase proofreading-associated polyposis (PPAP), which is associated with pathogenic variants of the *POLE* or *POLD1* gene (Mao et al. 2021, Stern et al. 2019).

Due to the overlapping phenotypes in tumor syndromes with a limited sensitivity of clinical criteria for diagnosis, the genetic clarification of these clinical pictures using NGS is justified and efficient (Stern et al. 2019, Rohlin et al. 2017). This technology enables the detection of germline mutations associated with colon rectal carcinoma and polyposis with an increase in data diversity and a simultaneous decrease in costs, thereby expanding the phenotypic spectrum of these hereditary diseases. This leads to an increasing complexity in the characterization of these tumor syndromes (Stern et al. 2019, Tanakaya et al. 2019).

Table 2 contains important genes associated with hereditary colon rectal carcinoma and/or polyposis and recommended for NGS multi-gene panel analysis (Mao et al. 2021).

Table 2: Recommended target genes associated with hereditary colon rectal cancer and polyposis for multi-gene panel analysis.

Genes	**Phenotype**	**Inheritance**
High-risk genes		
APC	Familial adenomatous polyposis	Autosomal dominant
BMPR1A	Juvenile polyposis	Autosomal dominant
EPCAM	Lynch syndrome	Autosomal dominant
MLH1	Lynch syndrome	Autosomal dominant
MSH6	Lynch syndrome	Autosomal dominant
MUTYH	*MUTYH*-associated polyposis	Autosomal recessive
PMS2	Lynch syndrome	Autosomal dominant
PTEN	Cowden syndrome	Autosomal dominant
SMAD4	Juvenile polyposis	Autosomal dominant
STK11	Peutz-Jeghers syndrome	Autosomal dominant
High-risk genes		
GREM1	Hereditary mixed polyposis syndrome	Autosomal dominant
MLH3	Lynch syndrome	Autosomal dominant
NTHL1	Familial adenomatous polyposis	Autosomal recessive
POLD1	Polymerase proofreading-associated polyposis	Autosomal dominant
POLE	Polymerase proofreading-associated polyposis	Autosomal dominant

3.1.2 Hematology

The current importance of NGS diagnostics in the field of haematology lies in the initial diagnosis and relapse of myeloid neoplasms. Future prospects with improved sensitivity lie in the area of monitoring minimal residual disease (MRD).

The current limitations of NGS technology in hematological diagnostics lie in the sensitivity of MRD progression diagnostics in particular, but also in the expected low analysis time and cost efficiency. In order to guarantee a sufficient MRD level and to obtain therapy-relevant findings more quickly, NGS diagnostics is supplemented by other methods (e.g. digital PCR) in these areas.

The NGS multi-gene panel analysis of bone marrow and peripheral blood enables the simultaneous detection of single nucleotide variants (SNVs), insertions/deletions, copy number variants (CNVs) and translocations. Repeated NGS testing in patients with hematologic diseases allows the assessment of possible dynamic changes in genetic variants during the course of the disease.

The genetically examined tumor cells may contain germline mutations as well as somatic mutations and clonal hematopoietic DNA alterations (Kraft & Godley 2020). Molecular genetic analysis in hematologic diseases includes genetic variants of signal transducers, transcription factors, splicing factors and epigenetic regulators.

NGS diagnostics is particularly important in the detection of variants in AML, MDS, myeloproliferative neoplasms (MPN) and MDS/MPN overlap syndromes (Ferrone et al. 2021). The World Health Organization (WHO) classification of myeloid neoplasms and acute leukemias, revised in 2022, includes new genetic markers with crucial clinical, prognostic and therapeutic value (Khoury et al. 2022).

The target genes and costs of a commercially available multi-gene panel (OncomineTM Myeloid Assay GX: ThermoFisher, Waltham, MA, USA) for the detection of the special and heterogeneous molecular genetic map of clonal myeloid diseases are listed in Table 3 (Ferrone et al. 2021).

Table 3. Multi-gene panel analysis of myeloid diseases (Oncomine™ Myeloid Assay)

Hotspot genes (DNA					
ABL1	*DDX41*	*IDH1*	*MPL*	*PTPN11*	*SRSF2*
ANKRD26	*DNMT3A*	*IDH2*	*MYD88*	*SMC1A*	*U2AF1*
BRAF	*FLT3*	*JAK2*	*NPM1*	*SMC3*	*WT1*
CBL	*GATA2*	*KIT*	*NRAS*	*SETBP1*	
CSF3R	*HRAS*	*KRAS*	*PPM1D*	*SF3B1*	
Full genes (DNA)					
ASXL1	*CEBPA*	*IKZF1*	*PRPF8*	*SH2B3*	*TP53*
BCOR	*ETV6*	*NF1*	*RB1*	*STAG2*	*ZRSR2*
CALR	*EZH2*	*PHF6*	*RUNX1*	*TET2*	
Fusion driver genes (RNA)					
ABL1	*CREBBP*	*FUS*	*MET*	*NTRK3*	*RARA*
ALK	*EGFR*	*HMGA2*	*MLLT10*	*NUP98*	*RBM15*
BCL2	*ETV6*	*JAK2*	*MLLT3*	*NUP214*	*RUNX1*
BRAF	*FGFR1*	*KMT2A*	*MYBL1*	*PDGFRA*	*TCF3*
CCND1	*FGFR2*	*MECOM*	*MYH11*	*PDGFRB*	*TFE3*

This panel contains a total of 45 genes (DNA), 30 so-called fusion driver genes, including the key myeloid genes *CEBPA* and *FLT3*.

Mutations in the *CEBPA* and *FLT3* genes are among the most common genetic alterations in AML and have prognostic and therapeutic value. *CEBPA* codes for a hematopoietic transcription factor that is responsible for myeloid differentiation. AML patients with biallelic *CEBPA* mutations have a favourable prognosis (DiNardo & Cortes 2016). *FLT3* codes for a cytokine receptor. Internal tandem duplications (ITDs) of this protein *(FLT3*-ITD mutations)

occur more frequently in young adults with AML and are associated with a poorer prognosis. FLT3 also represents a target gene for customized inhibitor therapy (DiNardo & Cortes 2016).

The European LeukemiaNet (ELN) recommends molecular genetic risk stratification of AML with good (*NPM1* without *FLT3*-ITD; *RUNX1-RUNX1T1*; biallelic *CEBPA* mutations), intermediate (*NPM1* and *FLT3*-ITD) and poor (*RUNX1, TP53, ASXL1*, wild-type *NPM1* and *FLT3*-ITD, *BRC-ABL1*) prognosis (Dohner et al. 2017, Bhai et al. 2022).

The clinical implementation of NGS-based multi-gene panel diagnostics of acute and chronic myeloid diseases (AML, MDS, MPN, MDS/MPN, chronic myeloid leukemia) shows a high diagnostic yield, sensitivity and specificity in current studies (Bhai et al. 2022, Gargallo et al. 2022). Due to the diversity and heterogeneity of the assays, new challenges arise for routine clinical diagnostics.

National and international consensus recommendations from a network of reference laboratories indicate minimum requirements for a multi-gene panel testing strategy (Bhai et al. 2022, Dohner et al. 2022, Sargas et al. 2021, Haferlach 2020). Molecular genetic testing for *ASXL1, CEBPA, FLT3, IDH1, IDH2, NPM1, RUNX1,* and *TP53* mutations is mandatory for risk stratification and targeted therapy of AML (Bhai et al. 2022, Dohner et al. 2022, Sargas et al. 2021, Haferlach 2020). The criteria of the WHO classification and the ELN prognostic system of AML diagnostics can only be fulfilled by including the cytomorphological, immunophenotypic and molecular genetic test results (Haferlach 2020).

3.1.3 Oncology

Molecular genetic profiling of somatic mutations in tumor samples plays a crucial role in the clinical management of patients with underlying oncological diseases. Targeted NGS multi-gene panels contribute to better diagnostic and therapeutic stratification of these patients.

The human genome contains >20000 genes. To date, around 500 of these genes have been linked to cancer. With this limited and manageable number of genes, various NGS-based multi-gene panels covering the most important driver genes for solid tumors have been developed in recent years and brought to market for daily diagnostic routine.

One example is the OncomineTM Precision Assay GX (ThermoFisher, Waltham, MA, USA) (Nagahashi et al. 2019). The scope of this commercially available pan-cancer panel enables the simultaneous detection of hotspot mutations (substitutions, insertions, deletions), CNVs and gene fusions of a total of 50 oncological driver genes. This panel is listed in Table 4.

Table 4 Multi-gene panel analysis of oncological diseases with the OncomineTM Precision Assay GX (ThermoFisher, Waltham, MA, USA)

Oncological driver genes				
AKT1	*CHEK2*	*FGFR4*	*MAP2K1*	*PDGFRA*
AKT2	*CTNNB1*	*FLT3*	*MAP2K2*	*PIK3CA*
AKT3	*EGFR*	*GNA11*	*MET*	*PTEN*
ALK	*ERBB2*	*GNAQ*	*MTOR*	*RAF1*
AR	*ERBB3*	*GNAS*	*NRAS*	*RET*
ARAF	*ERBB4*	*HRAS*	*NRG1*	*ROS1*
BRAF	*ESR1*	*IDH1*	*NTRK1*	*RSPO2*
CDK4	*FGFR1*	*IDH2*	*NTRK2*	*RSPO3*
CDKN2A	*FGFR2*	*KIT*	*NTRK3*	*SMO*
CD274	*FGFR3*	*KRAS*	*NUTM1*	*TP53*

Other manufacturer-related or customized customer-oriented NGS multi-gene panels are similarly structured and contain the essential driver genes (Lee et al. 2018, de Biase et al. 2020. This creation of an NGS multi-gene panel in the oncological setting is based on the corresponding guidelines and requirements of individual specialist societies (Mosele et al.2020, Teixeira et al. 2022).

Among the various cancer entities, NSCLC is an example of molecular genetic precision medicine with a multi-gene panel testing strategy in clinical practice. Currently, targeted

therapeutics for *ALK-, BRAF-, EGFR-, MET-, NTRK-, RET-,* and *ROS1* alterations are used in patients with NSCLC (Teixeira et al. 2022, Yatabe et al. 2020). Current consensus recommendations suggest that NGS-based multi-gene panel testing should include not only genetic biomarkers of approved targeted therapies (*EGFR, ALK, ROS1, BRAF* and *MET*) but also the oncogenes *HER2* and *KRAS* (Mosele et al. 2020, Teixeira et al. 2022, Penault-Llorca et al. 2022).

The increasing number of available and meaningful genetic biomarkers and targeted therapies makes an NGS multi-gene panel testing strategy increasingly necessary in the clinical setting. This is particularly relevant for patients with advanced non-small cell lung cancer, where tissue samples are limited and NGS with multi-gene analysis can be expected to provide a significantly higher diagnostic and prognostic output compared to conventional sequential biomarker analysis (Yatabe et al. 2020, Penault-Llorca et al. 2022).

In a tertiary center, molecular genetic clarification of gastrointestinal tumors (colon rectal carcinomas: 80.3%; esophageal/stomach carcinomas: 7.5%; liver/gallbladder/pancreatic carcinomas: 6.5%; gastrointestinal stromal tumors: 3.2%; appendix carcinomas: 1.4%; small intestine carcinomas: 1. 1%) with formalin-fixed paraffin-embedded (FFPE) tissue samples using the Truseq Amplicon Cancer Panel (Illumina, San Diego, CA, USA), it was shown that *TP53* (45.9%) was the most frequently mutated gene, followed by *APC* (42.1%), *KRAS* (39.7%), *PIK3CA* (12.1%), *SMAD4* (7.6%), *BRAF* (6.2%) and *NRAS* (5.5%) (Bregni et al. 2020).

The current panel (AmpliSeqTM Cancer HotSpot Panel v2) from the same company covers approximately 2800 mutations in the hotspot regions of 50 oncogenes and tumor suppressor genes. The scope of this oncology panel is shown in Table 5.

Table 5. Multi-gene panel analysis of oncological diseases with the AmpliSeqTM Cancer HotSpot Panel v2 (Illumina, San Diego, CA, USA)

Oncogenes and tumor suppressor genes				
ABL1	*EGFR*	*GNAS*	*KRAS*	*PTPN11*
AKT1	*ERBB2*	*GNAQ*	*MET*	*RB1*
ALK	*ERBB4*	*HNF1A*	*MLH1*	*RET*
APC	*EZH2*	*HRAS*	*MPL*	*SMAD4*
ATM	*FBXW7*	*IDH1*	*NOTCH1*	*SMARCB1*
BRAF	*FGFR1*	*JAK2*	*NPM1*	*SMO*
CDH1	*FGFR2*	*JAK3*	*NRAS*	*SRC*
CDKN2A	*FGFR3*	*IDH2*	*PDGFRA*	*STK11*
CSF1R	*FLT3*	*KDR*	*PIK3CA*	*TP53*
CTNNB1	*GNA11*	*KIT*	*PTEN*	*VHL*

Another study with molecular genetic profiling of FFPE tissue samples from gastrointestinal tumors in another tertiary center led to similar results (*TP53*: 80.9%; *APC*: 46.3%; *KRAS*: 28.7%) (Moorcraft et al. 2018).

About a decade ago, colon rectal carcinoma was the first gastrointestinal malignancy where molecular genetic profiling had a decisive influence on therapy. Initially, it was observed that patients with a *KRAS* mutation did not respond to *EGFR*-targeted agents such as the monoclonal antibodies cetuximab and panitumumab (Mukherji et al. 2022). It was later shown that *BRAF, NRAS, PIK3CA* and *HER2* mutations also do not respond to *EGFR*-targeted therapeutics and are therefore associated with a poorer prognosis (Mukherji et al. 2022).

For the molecular genetic profiling of lung and colon rectal cancer, a single-gene approach to diagnostics is no longer appropriate or useful in today's patient setting. The exact molecular genetic characterization of these tumors is achieved by NGS-based multi-gene panel testing de Biase et al. 2022). With the increasing incidence of solid tumors combined with the

development of targeted therapies, the molecular genetic map and necessary testing strategy for these diseases is becoming increasingly complex (Chevrier et al. 2022).

In solid oncology, the importance of molecular tumor boards, which provide access to comprehensive data and studies, is steadily increasing. In the diagnosis and treatment of oncological patients, the complexity of treatment decisions for physicians is thus increasing. In addition to mutations, other factors covered by NGS multi-gene panels, such as tumor mutation burden (TMB), which measures the total number of somatic mutations per megabase of the genome under investigation, and microsatellite instability (MSI) in the area of repetitive DNA sequences, are playing an increasingly important role in solid oncology.

Liquid biopsy analysis using NGS, which detects mutations in tumor-derived nucleic acids in blood and other body fluids, is one of the most advanced diagnostic fields in modern oncology. This analysis is primarily used for the early detection of tumors, especially recurrent tumors, as well as the monitoring of possible residual tumor activity. Liquid biopsy is not yet a standard diagnostic method in either NGS testing or tumor monitoring. It is mainly used in studies or as part of molecular tumor boards when no tissue is available.

The implementation of an NGS-based multi-gene panel test strategy in routine clinical diagnostics opens up new diagnostic, prognostic and therapeutic possibilities. It is a powerful tool for the detection of already known and new mutations in the molecular genetic map of many types of cancer.

In particular, the molecular profiling of solid tumors in oncology (e.g. lung carcinoma, gastrointestinal malignancies), testing for germline mutations in hereditary tumor syndromes (e.g. hereditary breast and ovarian carcinoma syndrome, hereditary colon rectal carcinoma) and the use of multi-gene panels in variant detection of hotspot and driver genes in myeloid diseases (e.g. AML, MDS, MPN and MDS/MPN overlap syndromes) are just a few examples of useful applications in daily routine diagnostics.

The increasing complexity and data diversity of test results offer both opportunities and challenges for molecular genetic diagnostics in the coming years.

3.2 Interdisciplinary collaboration with the laboratory: Genetic diseases of iron metabolism

In the context of establishing NGS- based multi-gene panels in priority hospitals outside of human genetics centers, the interdisciplinarity of the different clinical disciplines, the institute of clinical chemistry and laboratory medicine and the institute of pathology is crucial.

The following example clearly shows that regarding the diagnosis of genetic diseases of iron deficiency or iron overload, the establishment of special NGS-panels, which are coordinated with clinicians could help to detect even rarer mutations in clinical practice, which are not established in most hospitals yet.

Disorders of the molecular regulation of iron homeostasis can have genetic causes. These include not only common but also rare forms of iron deficiency anemia and iron overload. In these cases, in addition to the conventional diagnostic markers of iron metabolism, such as ferritin or transferrin, NGS-based gene panels, for example, could make a secondary contribution to identifying the genetic causes of these special clinical pictures.

Although rare, iron deficiency may be due to a genetic cause. The rare picture of autosomal recessive inherited iron-refractory iron deficiency anemia (IRIDA) must be considered after all other possible causes of iron deficiency have been ruled out in cases of treatment refractory patients.

In addition to the long-known *HFE* mutations, excess iron can also be due to rarer genetic causes, such as *HJV, HAMP, TfR2* or *FPN1*-associated forms of hemochromatosis. The complexity of the physiology of the human iron balance is reflected in the diversity of the pathology of genetic diseases of this finely tuned regulatory system (Enko 2021).

3.2.1 Hereditary hemochromatosis

Hereditary hemochromatosis is often asymptomatic or with non-specific symptoms (Piperno et al. 2020). High serum iron levels, elevated transferrin saturation and elevated serum ferritin levels are usually determined in asymptomatic individuals. Non-specific symptoms include

fatigue, malaise and arthralgia. Hepatomegaly indicates a clinically relevant iron excess and is often associated with liver fibrosis on liver biopsy (Corradini et al. 2020).

In patients with haemochromatosis, the liver is the first organ of iron deposition, with other organs becoming involved later (Barton et al. 2010). The well-known classic picture of hereditary haemochromatosis with liver cirrhosis, diabetes mellitus and dark skin pigmentation, which is rather rare today, is mainly observed in patients with very high ferritin levels (Powell et al. 2016).

Despite the genetic heterogeneity of this disease, the C282Y mutation (= Cys282Tyr substitution) is the most common mutation in the population (Powell et al. 2016, Adams 2020). This mutation occurs in approximately one in ten people of Northern European origin. Consequently, approximately one individual in 200 people is a homozygous carrier of this mutation (Powell et al. 2016), In humans, hepcidin deficiency is associated with *HFE*-, hemojuvelin (*HJV*)-, hepcidin (*HAMP*)-, and transferrinreceptor-2 (TfR2) (*TfR2*)-associated hereditary hemochromatosis (Thomas 2020, Powell et al. 2016).

Hereditary hemochromatosis type 1 is an autosomal recessive disease that occurs clinically in Europe with a prevalence of 1:1000. Men are significantly more frequently affected than women (ratio 10:1) (Truckenbrodt 2021).

It is the most common congenital form of iron overload. In this form of mutation, guanine (G) is replaced by adenine (A) at nucleotide 845 of the *HFE* gene. This leads to an exchange of the amino acid cysteine for tyrosine at position 282 (C282Y mutation = Cys282Tyr) in the *HFE* protein (type 1A) (Kowdley et al. 2019, Katsarou et al. 2019, Gerhard et al. 2018, Barton et al. 2010).

Approximately 80-90% of the population in Northern Europe with diagnosed hemochromatosis are homozygous for C282Y (Powell et al. 2016, Barton et al. 2010). Hereditary hemochromatosis can manifest phenotypically with very different symptoms. The phenotype depends mainly on the extent of iron overload in the blood and tissues (Corradini et al. 2020).

Another type of mutation is the H63D mutation, which does not cause significant iron overload but is a cofactor for phenotypic expression, especially in combination with the C282Y mutation. This compound heterozygous C282Y/H63D genotype (type 1B) may occult with elevated transferrin saturation and blood ferritin levels.

HFE hemochromatosis type 1C has the mutation S65C. It is not associated with excessive iron storage in the parenchymal organs and can therefore be regarded as a genetic polymorphism without clinical significance (Powell et al. 2016, Kowdley et al. 2019). Most other mutations in the *HFE* gene are rare and only occur in certain families. They are usually not detected in conventional routine laboratories (Barton er al. 2012).

Hemojuvelin is a glykosyl-phosphatidyl-inositol (GPI)-anchored protein on the cell surface, which is jointly responsible for the regulation of hepcidin synthesis in hepatocytes. It is the product of the *HJV* gene, has 4265 base pairs (bp) and is localized on chromosome 1 (Chr1q21). The coding sequence consists of 3 exons (exon 2, 3, and 4). The *HJV* gene encodes a co-receptor for bone morphogenetic proteins (BMPs), which regulate the hepcidin concentrations circulating in the blood (Kong et al. 2019).

The hemojuvelin (*HJV*)-associated or juvenile form of hereditary hemochromatosis type 2A occurs very rarely and is characterized by an early and severe course of the disease (Katsarou et al. 2019). *HJV* mutations in patients with iron overload encode a functionally ineffective form of hemojuvelin ("loss-of-function" mutations) (Barton et al. 2012). The most common mutation form of the *HJV* gene is the G320V mutation (Barton et al. 2012, Corradini et al. 2020, Piperno et al. 2020).

Another very rare form of juvenile hereditary hemochromatosis is the autosomal recessive hepcidin (*HAMP*)-associated haemochromatosis type 2B. The *HAMP* gene (chromosome 19q13.12) codes for the 84 amino acid precursor peptide of hepcidin (Barton et al. 2012). Mutations in the *HAMP* gene lead to a hepcidin deficiency (Kowdley et al. 2019).

Transferrin receptors serve as the main route for iron entry into cells. Currently, two different subtypes are distinguished, the so-called transferrin receptor-1 (TfR1) and the transferrin receptor-2 (TfR2). While TfR1 is expressed on all proliferating cells, TfR2 is predominantly found on hepatocytes and on normal and neoplastic haematopoietic cells (Srai & Sharp 2012).

TfR2 (*TfR2*)-associated hemochromatosis (type 3) is a very rare autosomal recessive form of congenital iron overload (Piperno et al. 2020). The *TfR2* gene (chromosome 7q22) codes for the transmembrane receptor TfR2 (Gerhard et al. 2018). *TfR2* mutations lead to a reduction in hepcidin synthesis in the liver and thus to iron excess in the body (Kowdley et al. 2019, Brissot

et al. 2011). Mutation carriers can be identified by molecular genetic testing. Heterozygous *TfR2* mutation carriers usually do not exhibit a pathological phenotype (Barton et al. 2010).

The *FPN1* gene (2q32) codes for the regulatory protein ferroportin, which is critical for human iron homeostasis (Gerhard et al. 2018). Human ferroportin is particularly expressed at the basolateral membrane of duodenal enterocytes, macrophages and hepatocytes as well as in syncytio-trophoblasts of the placenta (Srai et al. 2012). It plays a crucial regulatory role with regard to the efflux of iron absorbed in enterocytes into the blood circulation. Furthermore, it plays also a role in the recycling of iron from aging erythrocytes, the mobilization of storage iron from the RHS and also the transfer of iron across the placenta into the developing fetus (Drakesmith et al 2015).

Mutations in the *FPN1* gene are the cause of a rare clinically and genetically heterogeneous group of autosomal-dominantly inherited diseases with iron overload (type 4) (Barton et al. 2012). Depending on the type of mutation, two different phenotypes of *FPN1* hemochromatosis are distinguished:

- Loss-of-function mutations are more frequent and lead to a reduced iron export activity of ferroportin with consecutive iron sequestration, particularly in the macrophages of the RHS in the spleen and liver (Kupffer cells). This form of mutation is referred to as ferroportin disease and was previously classified as hemochromatosis type 4A (Berton et al. 2012, Corradini et al. 2020, Ravasi et al. 2020, Pietrangelo 2017, Vlasveld et al. 2019, Bardou-Jacquet et al. 2014).

- Gain-of-function mutations result in hepcidin resistance of ferroportin. This form of mutation leads to increased intestinal iron absorption in the duodenal enterocytes and increased iron export from the macrophages of the RHS and was previously classified as hemochromatosis type 4B (Corradini et al. 2020, Kowdley et al 2019, Ravasi et al. 2020, Pietrangelo 2017).

The clinical symptoms of FPN1-associated hemochromatosis are not specific (Bardou-Jacquet et al. 2014). Many patients report uncharacteristic symptoms and have no physical abnormalities of iron overload.

Table 6 represents the most important genes of hereditary hemochromatosis suggested for an NGS-based multi-gene panel.

Table 6: Recommended target genes associated with hereditary hemochromatosis for NGS-based multi-gene panel analysis.

Gene	Protein	Inheritance	Hereditary hemochromatosis
HFE	HFE-protein	Autosomal recessive	Type 1
HJV	Hemojuvelin	Autosomal recessive	Type 2A
HAMP	Hepcidin	Autosomal recessive	Type 2B
TfR2	Transferrin receptor-2	Autosomal recessive	Type 3
FPN1	Ferroportin	Autosomal dominant	Type 4

3.2.2 Iron-refractory deficiency anemia

Iron-refractory iron deficiency anemia (IRIDA) is a rare genetic form of iron deficiency. The worldwide prevalence of this condition is estimated at < 1 per 100,000 people (Thangavelu et al. 2019). Due to this low prevalence rate, the medical literature on this genetic disease is limited. It is estimated that IRIDA is underdiagnosed and should always be considered when all other possible causes of iron deficiency can be ruled out (De Falco et al. 2013).

This iron-refractory form of iron deficiency anemia is an autosomal recessive disease caused by mutations in the *TMPRSS6* gene. This gene is located on the long arm of chromosome 22 (22q12-q13) and codes for matriptase-2, a negative regulator of hepcidin (De Falco et al. 2013, Bhatia et al. 2017). Mutations of the *TMPRSS6* gene lead to a reduced or completely absent protease activity of matriptase-2 (“loss-of-function” mutations) with resulting iron deficiency anemia. Patients with IRIDA phenotypes are either homozygous or compound heterozygous for *TMPRSS6* mutations (Barton et al. 2012).

It is easy to understand why this disease is underdiagnosed. At the same time, the possibility of *TMPRSS6* genetic testing and the clinical suspicion of a genetic cause of iron deficiency are very often lacking in everyday clinical practice (Bhatia et al. 2017). Another important aspect in the diagnosis and treatment of an IRIDA case is the screening of other siblings for this clinical picture (Bhatia et al. 2017).

IRIDA should be considered in the differential diagnosis of iron deficiency anemia that does not respond to oral iron therapy and is associated with normal or elevated serum ferritin levels. Molecular genetic testing for mutations in the *TMPRSS6* gene can confirm the diagnosis (Sal et al. 2016). In most patients with IRIDA, oral iron administration is ineffective. Intravenous iron administration is therefore indicated. The therapeutic response to parenteral iron can be variable, but in many cases leads to a progressive improvement in the blood hemoglobin concentration.

3.2.3 Congenital sideroblastic anemias

Congenital sideroblastic anaemias are a heterogeneous group of diseases characterized by iron deposition in the mitochondria of erythrocyte precursors (Abu-Zeinah & DeSancho 2020, Steinberg-Shemer & Tamary 2020).

The iron is usually deposited in the mitochondria as ferritin (Brissot et al. 2018). The dysfunction of mitochondrial iron metabolism is caused in particular by mutations in heme synthesis, in the biogenesis of iron-sulphur clusters and in general mitochondrial protein synthesis (Brissot et al. 2018, Steinberg-Shemer & Tamary 2020). Early detection and treatment of iron accumulation can prevent irreversible organ damage (Barton et al. 2010). Therefore, it would be of advantage for this special patient setting if diagnosis could be confirmed by genetic NGS-based analysis.

X-linked sideroblastic anemias are characterized by impaired mitochondrial iron metabolism with ring sideroblasts and increased erythropoiesis in the bone marrow and are among the most common forms of sideroblastic anemias (Barto et al. 2012). The *ALAS2* gene codes for 5-aminolevulinic acid synthase, which is found exclusively in the mitochondria of erythrocytic

cells. It is the first enzyme in heme synthesis and therefore plays an important role in this process (Brissot et al. 2018).

Mutations in the *ALAS2* gene lead to a defective heme synthesis in the red cell line (Brissot et al. 2018). Most mutations are missense mutations and result in a single amino acid substitution in 5-aminolevulinic acid synthase. *ALAS2* mutations have been described in Caucasians, Japanese, Chinese, and African Americans. Although some research laboratories offer genetic *ALAS2* mutation analysis, this testing method has not yet been introduced into routine laboratories (Barton et al. 2012, Barton et al. 2010). The diagnosis can be confirmed by identifying an *ALAS2* mutation (Brissot et al. 2018).

X-linked sideroblastic anemia with ataxia is caused by mutations in the *ABCB7* gene on chromosome X (Abu-Zeinah & DeSancho 2020). The exact pathophysiological mechanism of impaired neurological development or neurological damage caused by the *ABCB7* mutations is not known yet (Barton et al. 2012)

The *GLRX5* gene is located on the long arm of chromosome 5 (5q14) and encodes the glutaredoxin-5 protein (Barton et al. 2012, Abu-Zeinah & DeSancho 2020). An autosomal recessive inheritance of *GLRX5* mutations has been described (Barton et al. 2012, Barton et al. 2010).

The *SLC25A38* gene encodes an erythrocyte-specific mitochondrial glycine transport protein (Brissot et al. 2018, Abu-Zeinah & DeSancho 2020). Mutations in the *SLC25A38* gene are inherited in an autosomal recessive manner and lead to an impaired mitochondrial heme synthesis (Brissot et al. 2018). This subtype accounts for approximately 10% of all congenital sideroblastic anemias (Abu-Zeinah & DeSancho 2020).

Table 7 represents the most important genes of hereditary sideroblastic anemias and other rare diseases of iron metabolism suggested for an NGS-based multi-gene panel.

Table 7: Recommended NGS-based target genes associated with congenital sideroblastic anemias and other rare hereditary diseases of iron metabolism.

Congenital sideroblastic anemias		
Gene	**Protein**	**Type of sideroblastic anemia**
ALAS2	5-aminolevulinic acid synthase	X-linked sideroblastic anemia
ABCB7	ATP-binding cassette-subfamily-B-member-7 protein	X-linked sideroblastic anemia with ataxia
GLRX5	Glutaredoxin-5 protein	*GLRX5* sideroblastic anemia
SLC25A38	Erythrocyte-specific mitochondrial glycine transport protein	*SLC25A38* sideroblastic anemia
Iron-refractory iron deficiency anemia		
Gene	**Protein**	**Type of anemia**
TMPRSS6	Matriptase-2	Ineffective oral iron administration
Atransferrinemia		
Gene	**Protein**	**Type of anemia**
Tf	Transferrin	Severe hypochromic microcytic anemia
Aceruloplasminemia		
Gene	**Protein**	**Type of anemia**
Cp	Ceruloplasmin	Iron-restricted iron deficiency anemia
Divalent metal-ion transporter iron overload		
Gene	**Protein**	**Type of anemia**

DMT1 (SLC11A2)	Divalent metal-ion transporter	Severe hypochromic microcytic anemia with simultaneous iron overload

3.2.4 Atransferrinemia

Approximately 3-4 mg of the iron circulating in the blood serum is bound to transferrin (Srai & Sharp 2012). Each transferrin molecule can bind a maximum of two Fe^{3+} atoms and thus supplies the cells of the organism (Thomas 2020). The main physiological role of transferrin is to provide iron for the developing erythropoietic cells in the bone marrow, which require large quantities for the hemoglobin synthesis (Testa 2002).

Hereditary atransferrinemia is a very rare autosomal recessive inherited disorder characterized by a severe quantitative or functional deficiency of transferrin. Mutations occur in the *Tf* gene, which is located on the long arm of chromosome 3 (3q21) and comprises a total of 17 exons (Dabboubi R et al. 2020). *Tf* mutations are inherited either homozygous or compound heterozygous. Genetic polymorphism is widespread (Barton et al. 2012). Currently, more than ten point mutations in the *Tf* gene have been described (Dabboubi et al. 2020).

The diagnosis is confirmed by molecular genetic testing for possible mutations in the *Tf* gene (Chandra et al. 2017). Increased intestinal iron absorption and the presence of non-transferrin bound iron in the blood lead to massive iron overload of the liver, pancreas and heart. The iron overload can lead to liver fibrosis and cirrhosis, heart failure or diabetes mellitus (Chandra et al. 2017).

3.2.5 Aceruloplasminemia

In addition to the important role in copper metabolism, the glycoprotein ceruloplasmin is also an important enzyme in iron metabolism. This enzyme is active as a ferroxidase, which converts Fe^{2+} to Fe^{3+} (Pelucchi et al. 2018). This allows Fe^{3+} to bind to transferrin. At the same time,

ceruloplasmin stimulates the iron efflux from liver cells and thus plays a decisive role in the release of body storage iron for metabolic purposes (Srai et al. 2012).

Hereditary aceruloplasminemia is an extremely rare autosomal recessive disorder caused by mutations in the *Cp* gene (Barton et al. 2012). This gene is located on the long arm of chromosome 3 (3q23-24), comprises 20 exons, and codes for the metal regulatory protein ceruloplasmin, which consists of a total of 1046 amino acids (Barton et al. 2012, Cuenca et al. 2020).

The absence of this regulatory protein in the context of hereditary aceruloplasminemia leads to intracellular iron retention with progressive iron overload. At the same time, iron-restricted erythropoiesis occurs in the bone marrow with consecutive iron deficiency anemia (Piperno & Alessio 2018).

The clinical picture is characterized by genetic and phenotypic heterogeneity (Cuenca et al. 2020). Numerous different *CP* mutations can lead to similar phenotypic manifestations. Conversely, twins with the same genotype can have different phenotypes (Barton et al. 2012). The diagnosis can be confirmed by the additional detection of severely reduced ceruloplasmin concentrations in the blood and genetic testing of the affected patients and their families (Brissot et al. 2018). An NGS-based multi-gene panel approach could comprise also this rare genetic disease of iron metabolism in clinical routine.

3.2.6 Divalent metal-ion transporter iron overload

The divalent metal-ion transporter (DMT1), which is localized in the duodenum, is primarily responsible for the uptake of Fe2+ from the intestinal lumen into the cells (Mims & Prchal 2005).

A total of four different isoforms of DMT1 exist. These are created by alternative splicing in exon 16 or by the presence of 2 expression start sites in exon 1A and 1B (Srai & Sharp 2012). All four isoforms can ensure iron transport with the same efficiency. Iron depletion in the human body leads to an increase in DMT1 expression in the duodenum (Srai & Sharp 2012).

The *DMT1* gene (*SLC11A2*) is located on the long arm of chromosome 12 (12q13) (Barton et al. 2012). Mutations of this gene occur very rarely and are usually only available as case reports

in the medical literature (Barton et al. 2012, Priwitzerova et al. 2005). NGS-based diagnostic approach could include these rare mutations in clinical routine.

3.3 Cooperation with the Center for Human Genetics

In the previous subchapters of this doctoral thesis, useful applications of NGS-based multi-gene panels in the fields of hematology, oncology and congenital tumor syndromes were presented. The broad usage of this technology in clinical practice shows the advantages for the investigation of heterogenous genetic diseases (Schaflinger & Enko 2022, Enko et al. 2023).

Using the example of iron metabolism, it was shown that genetic diagnostics in particular are a valuable addition to conventional laboratory parameters such as ferritin and transferrin saturation. For this reason, good cooperation with the laboratories with regard to NGS-based diagnostics is essential.

In clinical practice, outside of human genetic reference centers, more and more NGS analyzers will be established in the laboratories of peripheral general hospitals in the future. This has the advantage that new expertise in the field of medical genetics will also emerge for these hospitals.

Nevertheless, special issues, such as the handling of scientific projects, should continue to be carried out in collaboration with university human genetics centers. In particular, the following examples of genetic diagnostics are considered to be useful collaborations with a human genetics center:

- Scientific projects
- Expertise with consanguineous diseases
- Disease genetics
- Causal relationships of rare diseases

The following examples of three scientific papers with original data from this sub-area (Schaflinger et al. 2022, Ali et al. 2020, Ahmed et al. 2019) were part of this doctoral thesis.

3.3.1 N-terminal frame shift mutation in *ZMPSTE24* in a consanguineous family

Mandibuloacral dysplasia with type B lipodystrophy is well known as a very rare disease, which is inherited in an autosomal recessive manner. The incidence of this disorder is < 1:1.000.000. This subtype of mandibuloacral dysplasia can be caused by mutations in the *ZMPSTE24* gene. The disease manifestation depends on the remaining enzyme activity of the ZMPSTE24 protein (Schaflinger et al. 2022).

In the present study we detected a novel homozygous N-terminal frame shift mutation in a consanguineous family, which was recruited in Pakistan. This family segregated with mandibuloacral dysplasia with type B lipodystrophy. WES was performed on a NovaSeq 6000 (Illumina, San Diego, California, USA).

The in-depth analysis of the mutated sequence showed a new downstream in-frame start codon. This genetic mechanism could be a possible explanation for the relatively mild clinical outcome of the investigated subjects. The findings of this study show, that N-terminal variants must be carefully interpreted regarding to have the potential for effecting translation initiation (Schaflinger et al. 2022).

3.3.2 A novel protein truncating mutation p.Asp98* in *XPC* in a consanguineous family

Xeroderma pigmentosum is also a rare genetic disease, which is inherited autosomal recessive. This disorder is characterized by severe sunlight sensitivity, sunburn and skin cancer. The *XPC* is one gene, which is associated with this skin disease.

In the present study, a consanguineous Pakistani family was recruited for molecular genetic characterization of xeroderma pigmentosum. Whole genome SNP genotyping was performed with the Infinium® Global Screening Array-24v1.0 (Illumina, San Diego, California, USA).

We identified a novel protein truncating mutation p.Asp98* in *XPC*, which leads to a premature stop codon at the amino acid position 98 (p.Asp98*). This study is a good example for special scientific questions, which should be answered in cooperation with a Center for Human Genetics. In this specific case, the knowledge of the mutational spectrum of *XPC* is extended. It is a valuable knowledge expansion regarding the genotype-phenotype correlation of xeroderma pigmentosum associated with *XPC*. These new scientific findings are a significant contribution to the genetic counseling of affected individuals and their families (Ali et al. 2019).

3.3.3 Genetic study of consanguineous families presenting microcephaly

The so-called primary microcephaly is a rare genetic disorder, which is inherited in an autosomal recessive manner. In the present study, a total of 15 patients (9 males and 6 females) from five consanguineous Pakistani families, presenting with primary microcephaly with intellectual disability, was investigated for the genetic factor, which is causing microcephaly (Ahmed et al. 2019).

All five families went through short tandem repeat (STR) markers genotyping and linkage analysis with subsequent Sanger sequencing of the *ASMP* gene. We identified *ASPM* gene mutations suggesting an involvement of these genes in families with primary hereditary microcephaly. Mutation analyses identified a missense mutation c.3978G>A (p.Trp1326*), a deletion mutation c.7782_7783delGA (p.(Lys259Serfs*6)), and a splice site defect c.2936+5G>A (Ahmed et al. 2019).

Table 8 represents the most important genes of the three studies with rare diseases in consanguineous Pakistani families.

Table 8: Suggested target genes associated with very rare disorders in studies with consanguineous individuals.

Gene	**Associated disorder**	**Inheritance**
ZMPSTE24	Mandibuloacral dysplasia with type B lipodystrophy	Autosomal recessive
XPC	Xeroderma pigmentosum	Autosomal recessive
ASPM	Primary hereditary microcephaly	Autosomal recessive

3.4 NGS and pharmacogenomics

Pharmacogenetics in the sense of personalized medicine has already found its way into many central and peripheral hospitals. This also increases the demands on the various laboratories in terms of testing the different pharmacogenes and their variants.

There are different approaches to the testing of pharmacogenes. Clinical laboratories can establish an NGS-based targeted multi-gene analysis as well as an NGS-based WES or a WGS analysis. The latter methods naturally have a far greater potential to discover new pharmacogenetic variants.

Another decisive factor in pharmacogenomics is the population in which the respective pharmacogenes are studied and described. The rapid technological development of the NGS method poses major challenges for clinical laboratories with regard to the interpretation of new variants. At the same time, clinical knowledge with regard to patient-oriented individualized medicine is being significantly expanded and implemented in clinical practice.

In the whole world, NGS methods are increasingly being used in routine clinical practice. This technology has become more and more popular during the last years and also the costs have decreased. The clinicians are aware that rare genetic variants may play an important role in many diseases and also in the response of the patients to their medication (Ramudo-Cela et al. 2021).

Pharmacotherapy is personalized. The aim is to give the right and individual dose of a drug to the individual, who needs it. There are inter-individual differences, which can be observed between different patients. The safety of the standard dose is not always given. It is known that data of NGS-based technologies are very helpful to characterize different populations with different frequencies of pharmacogenetic variants (Goljan et al. 2022).

The aim of pharmacogenomics is to reduce adverse drug effects and to optimize patients' medication (Ramudo-Cela et al. 2022). Pharmacogenetic testing should ideally be performed in laboratories accredited according to national regulations to ensure high analytical validity (Bousman et al. 2021).

Guidelines for performing pharmacogenomics testing from various organizations, such as the Canadian Pharmacogenomics Network for Drug Safety, the Dutch Pharmacogenomics Working Group, or the Clinical Pharmacogenetics Implementation Consortium, are now available and used in clinical practice (Ji et al. 2021, Caudle et al. 2017, Hippman et al. 2019).

The detection of new pharmacogenetic variants is more and more increasing. Most of them are detected with different sequencing methods, which can be divided into WGS, WES, and in targeted multi-gene panel sequencing (Funkunage et al. 2021). WGS includes also all the intergenic regions, while WES focuses exclusively on exons, and targeted sequencing focuses on specific pharmacogenes.

One of the advantages of WES and WGS is the high power to discover rare variants in pharmacogenomics. One disadvantage of both diagnostic approaches can be the discovery of variants of unknown significance. Furthermore, there may occur secondary genetic findings, which are unrelated to pharmacogenomics. Higher time requirement and more costs may also be disadvantages compared with targeted technologies (Hippman et al. 2019).

The discovery and detection of new variants in pharmacogenomics is very important because it increases the knowledge, gives new insights in the metabolism of drugs and is essential for the patients' outcome.

Many patients may have a benefit from clinical integration of this multidisciplinary field. Nevertheless, many studies have shown, that most of the investigated individuals are carriers of variants, which have not been routinely studied in most university and peripheral hospitals.

3.4.1 WGS in pharmacogenomics

WGS in pharmacogenomics is getting more and more important for clinicians. Table 9 represents WGS-based studies on different populations worldwide.

Table 9: WGS-based approach in pharmacogenomics.

Year	Study	Subjects	Analytical method
2019	Choi et al.	Anonymous individuals of 1,000 Genomes Project	Whole genome sequencing
2021	Chen et al.	Genetic testing reference material	Whole genome sequencing
2021	da Rocha et al.	Different populations in Africa	Whole genome sequencing
2021	Park et al.	Individuals with depression in Korea	Whole genome sequencing
2020	Caspar et al.	Subjects with rare disorders	Whole genome sequencing and whole exome sequencing
2022	Zhou et al.	Different human populations	Whole genome sequencing and whole exome sequencing

The interest in the clinical use of WGS for testing rare and new variants in pharmacogenomics increases rapidly (Caspar et al. 2021). WGS has the advantage, that it overcomes the limitations of WES. This technology enables adequate coverage, detection of copy number variants, and the sequencing of the non-coding regions (Caspar et al. 2021, Meienberg et al. 2016).

The genetic profiling of cytochrome P450 2D6 (*CYP2D6*) is one important example of the use of WGS in pharmacogenomics. *CYP2D6* is responsible for encoding one of the most important enzymes in the drug metabolism. This gene is highly polymorphic and therefore it is a great challenge for pharmacogenetic profiling (Ji et al. 2021, Caspar et al. 2021, Chen et al. 2021, Caspar et al. 2020).

In their study, Chen et al. used the new bioinformatic method Cyrus. They presented accurate *CYP2D6* genotyping with ninety-six reference (Genetic Testing Reference Material) blood samples (Chen et al. 2021).

The cytochrome P450 genes are responsible for metabolizing drugs. Recently, Zhiu et al. comprised about twelve study populations with > 140.000 individuals without relation. In these study populations, the authors examined all the data of the majority cytochrome P450 genes with WES and WGS (Zhou et al. 2022). They found that rare and uncharacterized alleles are responsible for 1.5-17.5% of the total genetically variability (Zhou et al. 2022). All these data are a very valuable contribution in pharmacogenomics. They have the potential to optimize precision medicine (Zhou et al. 2022).

In another study, Choi et al. performed WGS as a part of the 1.000 Genomes Project, which comprised about 1.000 anonymous study participants (Choi et al. 2019). They detected more than 69.000 genetic variants, when they investigated different well-established genes in pharmacogenomics (Choi et al. 2019).

Of all these variants, more than 8.000 showed a strong linkage disequilibrium with known variants in pharmacogenomics. These variants may have the potential of causative mutations, which are responsible for special drug effect phenotypes. Therefore, the identification and also the characterization of such causal variants enables an accurate and more precise drug administration and treatment (Choi et al. 2019).

Another published study used eleven suggested pharmacogenes of the Dutch Pharmacogenomics Working Group and compared data of these pharmacogenes with WGS and WES (Caspar et al. 2020). The authors represented both, common variants, and also new variants. The investigated individuals suffered mainly from rare connective tissue diseases and cardiovascular diseases (Caspar et al. 2020).

In this study WGS showed a better performance in identifying promotor or intron variants proposed by the Dutch Pharmacogenomics Working Group compared to WES. This might be one reason, why WES based methods may be not have the potential for special treatment recommendations, which are given in guidelines (Caspar et al. 2020).

In further published studies, it was shown, that WGS had a higher accuracy regarding the identification of variants in coding regions compared to WES, even with the same coverage (Belkadi et al. 2015, Meynert et al. 2014). Nevertheless, WES is not so cost-intensive compared to WGS. Therefore, this technique is widely used in many laboratories.

In particular psychiatric patients are often treated with multiple medications. Clinicians often use consensus guidelines, which are well-established in pharmacogenomics for this patient setting (Bousman et al. 2021, Hicks et al. 2015, van Westrhenen et al. 2020). These guidelines provide clinical recommendations for a wide range of psychotropic drugs based on functional *CYP2D6* and *CYP2C19* variants (Bousman et al. 2021, Hicks et al. 2015, van Westrhenen et al. 2020). Further studies with WGS technology are needed in order to get more and more knowledge and to expand guidelines for clinicians. These consensus guidelines should support their daily work, especially in the implementation of pharmacogenomics (van Westrhenen et al. 2020).

In a recent study, Park et al. performed WGS, and they detected a new variant with loss-of-function (rs3213755) of the keratin-associated protein 1-1 (*KRTAP1-1*) (Park et al. 2021). The study population consisted of individuals with major depressive disorder. The patients were treated with selective serotonin reuptake inhibitors (Park et al. 2021). It was shown that there is a link between this loss-of-function variant and the remission after the treatment with the antidepressants (Park et al. 2021). Such studies demonstrate, that WGS has the potential to identify valuable disease-causing as well as predictive variants in pharmacogenomics. This is very optimal for the patient because the clinicians have much more information before drug therapy. This technique allows to represent a complete profile in pharmacogenomics of one individual (Caspar et al. 2021).

One recent study by Rocha JEB et al. comprised eight study populations in Africa (da Rocha et al. 2021). The authors used collected WGS data of African Genome Variant Project and the 1.000 Genomes Project. The aim was to investigate *DPYD* mutations in the sub-Saharan region (da Rocha et al. 2021). The authors found special twenty-nine coding variants of *DPYD*. One special variant, which is specific for Africa showed distinct enzyme function impairment, which was linked with severe toxicity (da Rocha et al. 2021). These data demonstrate that variants in pharmacogenomics may have great relevance for specific regions in the world and these

relevant data should also be considered to be implemented in accurate guidelines (da Rocha et al. 2021).

This subchapter highlights the current NGS-based diagnostic WGS approach in pharmacogenomics. The next subchapter represents WES in pharmacogenomics.

3.4.2 WES in pharmacogenomics

In this subchapter, systematic studies in pharmacogenomics with WES approach are illustrated in Table 10.

Table 10: WES-based approach in pharmacogenomics.

Year	Study	Subjects	Analytical method
2016	Neroldova et al.	Subjects with statin-related myopathy	Whole exome sequencing
2022	Lanillos et al.	Spanish or Latin Americans	Whole exome sequencing
2022	Silgado-Guzman et al.	Columbian population	Whole exome sequencing
2021	Liu et al.	Patients with acute coronary syndrome	Whole exome sequencing
2021	Hutchcraft et al.	Patients with cancer	Whole exome sequencing
2021	Aboul-Soud et al.	Patients with tumor	Whole exome sequencing
2019	Floyd et al.	Subjects with and without statin-related myopathy	Whole exome sequencing
2020	Caspar et al.	Subjects with rare disorders	Whole genome sequencing and whole exome sequencing
2022	Zhou et al.	Different human populations	Whole genome sequencing and whole exome sequencing

WES can be used by laboratories as one possible strategy for genetic testing in pharmacogenomics. Although this method does not have low costs, it is a diagnostic approach for laboratories to optimize their testing concept at regular intervals (Katragadda et al., 2021).

WES and WGS have greater potential to identify secondary pharmacogenomic findings that may be predictive of pharmacotherapy outcome compared to highly focused NGS multigene panels (Fukunaga et al. 2021, Hicks et al. 2018).

A recently published study with a Columbian study population detected rare loss-of function variants, which were present in >60% of all the pharmacogenes, which were analyzed (Silgado-Guzman et al. 2022). The author, which performed WES with an absorption, distribution, metabolism, and excretion panel of different genes, observed that the detected variants were responsible for different drug responses and individual variabilities between the study participants (Silgado-Guzman et al. 2022).

Especially in individuals with cancer, WES is reported to be used as a relevant method identifying relevant pharmacogenomic germline mutations (Hutchcraft et al. 2021, Aboul-Soud et al. 2021). Based on genetic variants, the Clinical Pharmacogenetics Implementation Consortium recommends dosing changes for six anticancer drugs (Relling & Klein 2011):

- irinotecan and *UGT1A1*
- 5-fluorouracil and capecitabine and dihydropyrimidine dehydrogenase (*DPYD)*
- 6-mercaptopurine and thioguanine and thiopurine methyltransferase (*TPMT)*
- tamoxifen and *CYP2D6* (Relling & Klein 2011).

All these studies in pharmacogenomics with WES data enable deep insight in the outcome of treatment and also in the progression of disease. All further interventions of clinicians are determined by their results (Aboul-Soud et al. 2021).

One published study with WES technology comprised individuals with and without statin-related myopathy (Floyd et al. 2019). The authors could not observe possible significant associations between statin-related myopathy and rare coding variants (Floyd et al. 2019). In contrast, the study based on WES published by Neroldova et al. identified a rare mutation in individuals with mild statin-related myopathy, which was related to this adverse drug effect (Neroldova et al. 2016).

Different study designs with different populations, different phenotypes, different sample sizes and different methods of analysis may cause various study results regarding the identification of new and rare variants in pharmacogenomics (Floyd et al. 2019).

A recently published WES report in pharmacogenomics detected eight new variants in a study population of patients with an acute coronary syndrome (Liu et al. 2021). During an eighteen-months follow-up period, these variants were linked with major adverse cardiovascular events (Liu et al. 2021).

These results suggest that WES in pharmacogenomics can help clinicians to better understand pathogenesis and make decisions about therapeutic intervention in medical conditions.

Another systematic WES report performed by Lamillos J et al., comprised 280 alleles in eleven pharmacogenes listed in the Pharmacogenetics Implementation Consortium guidelines in Latino or Hispanic subjects (Lanillos et al. 2022). The study group found many (>190) novel variants, partly with loss-of-function (Lanillos et al. 2022). All these data obtained from WES expand the knowledge, which can be used in pharmacogenomics.

3.4.3 NGS- based multi-gene panels in pharmacogenomics

Compared to a single gene test, a testing strategy in pharmacogenomics with NGS-based multi-gene panels can be beneficial for patients on multiple medications and especially in preventive medicine. The information on multiple genes makes it possible to identify current and future medication problems. Currently, different laboratories vary considerably in terms of the alleles included in the different pharmacogenetic gene panels (Nicholson et al. 2021).

If a laboratory plans to implement NGS-based multi-gene panels in pharmacogenetic testing some considerations are recommended (Haidar et al. 2022):

- Which pharmacogenes for which clinical question are required.
- Before routinely usage, the gene panel must be validated.
- Test costs must be calculated.
- Acceptance criteria for the turn-around-time must be defined for each laboratory.

- How should results be reported.
- Which variants for which gene should be reported.

Recently, Katragadda et al. reported that a targeted multi-gene panel strategy with NGS technology has the potential to be cost-effective and is more cost-efficient compared with WES and WGS (Katragadda et al. 2021). If a laboratory chooses the right panel for pharmacogenomics or rare diseases combined with the observation of the frequency of each laboratory's test orders, this strategy could be cost-optimal (Katragadda et al. 2021).

Various pharmacogenomics genes have been described by several working groups, comprising the Clinical Pharmacogenetics Implementation Consortium (Relling & Klein 2011), the Canadian PharmacogenomicsNetwork for Drug Safety Clinical Recommendations Group (Drogemoller et al. 2019), the European Medicines Agency and the Dutch Pharmacogenomics Working Group (Swen et al. 2011). These working groups summarize the current knowledge of studies in pharmacogenomics.

Table 11 summarizes important multi-gene panels in pharmacogenomics with NGS approach.

Table 11: Multi-gene panel approach based on NGS in pharmacogenomics.

Year	Study	Subjects	Number of pharmacogenes
2017	Han et al.	Korean population	114
2019	Klein et al.	Caucasians	340
2021	Ramudo-Cela et al.	Patients with breast cancer	18
2021	Runcharoen et al.	Subjects of different countries	100
2021	Fukanaga et al.	Subjects with different oncologic and psychiatric disorders	100
2022	Goljan et al.	Saudians	8
2022	Wen et al.	Hmong individuals	286

NGS multigene panel strategy enables the characterization of pharmacogenes that cannot be detected by conventional genotyping platforms. In general, it is estimated that approximately 90-95% of individuals have a usable genotype for at least one pharmacogene (Haidar et al. 2022).

A recent study of patients with breast cancer, which underwent a neoadjuvant therapy, reported genetic variants not previously described in the literature and also a high prevalence of relevant variants in pharmacogenomics, which have clinical impact (Ramudo-Cela et al. 2021). The authors established a panel of clinically relevant pharmacogenes, which are frequently addressed in guidelines (Ramudo et al. 2021, Relling & Klein 2011, Drogemoller et al. 2019, Swen et al. 2011).

Another indication for a single- or multi-gene-based test strategy is assessing the individual response to anticancer drugs. They have typically a narrow therapeutic range. Important gene-drug pairs include:

- *UGT1A1*-irinotecan
- Thiopurine methyltransferase *TPMT* and nudix hydrolase 15 (*NUDT15*)-thiopurines
- *DPYD*-fluoropyrimidines

If a patient occurs with a reduced activity or with a loss-of-function mutation in one of these genes, he is at risk of presenting an adverse drug reaction. It is recommended that these individuals, which are at high risk, should be detected before the get a therapy (Mhandire & Goey 2022, Nicholson et al. 2021).

There are further examples of establishing targeted NGS in the laboratory. Fukunage et al. reported fourteen variants and ten new haplotypes of *CYP2D6,* when they investigated a Japanese study population with 100 genes, which have a relevance in pharmacogenomics (Fukunage et al. 2021). NGS-based multi-gene panel assays are considered to have a better detection performance than single-gene investigations (Hippman & Nislow 2019).

It is important to mention that elderly people often take many drugs (polypharmacy). This patient setting is at high risk for multiple drug interactions. Patients with multiple medications should be tested with multiple-gene panels of pharmacogenomics. These panels will allow to identify of drug-gene interactions and as a consequence, the hospitalization of this patient setting can be reduced (Hayashi et al. 2022).

One study group established a NGS gene panel comprising >300 genes, which contribute to the metabolism of drugs, such as absorption, distribution and excretion (Klein et al. 2019). The authors of this study investigated Caucasians, who underwent liver donation. They identified germline variants in pharmacogenomics, which were not described in the literature yet (Klein et al. 2019). Han et al. performed a study with two NGS multi-gene panels and reported similar results (Han et al. 2017).

In order to identify clinically relevant mutations in pharmacogenomics, it is necessary that thorough investigations of unique study populations are initiated. Data with implications for phenotype prediction accuracy are needed to in order to improve diagnostic and therapeutic strategies of clinicians (Wen et al. 2022, Klein et al. 2019). Using a targeted NGS-based multi-gene panel with 286 absorption, distribution, metabolism and excretion genes, Wen YF et al. identified novel *CYP2D*6 variants in Hmong subjects that may have an impact on therapeutic response to drugs (Wen et al. 2022).

An NGS panel of eight relevant pharmacogenes recommended by the Clinical Pharmacogenetics Implementation Consortium, was conducted in a big Saudi population (Goljan et al. 2022). Overall, 99.2% of the study participants were observed with one pharmacogenetic variant that need to be experimentally validated for potential functional effects on drug response (Goljan et al. 2022).

A multi-gene panel approach, which is based on NGS technology and is performed in different laboratories (e.g. research laboratories, clinical laboratories), may comprise various actionable pharmacogenes and may also consist of genes, which are associated with diseases or disease risk (e.g. mental disorders, colorectal cancer, breast cancer) (Haidar et al 2022).

In their study, Runcharoen et al. used a panel of 100 pharmacogenes (Runcharoen et al. 2021). The multi-gene panel consisted of Flavin-containing mono-oxygenase genes, uridine diphosphate glucuronosyl transferase genes, genes for drug transporters, cytochrome P450-enzyme genes, and also other genes (Runcharoen et al. 2021). The authors reported frequency data of all these pharmacogenes from nine various countries. Such study designs are needed because they can be the basis for further studies on pharmacogenomics, which investigate possible effects of various genetic variants (Runcharoen et al. 2021).

4. Conclusions

NGS is a high-throughput sequencing method, that will increasingly be used in peripheral hospitals in the future and will replace other genetic methods. In particular, its use in oncology, hematology and in the diagnosis of hereditary tumor syndromes are examples of a useful application of this technology in peripheral hospitals.

Multi-gene panels could be established in peripheral hospitals also to clarify specific clinical pictures, such as genetic disorders of iron metabolism. Another useful application is in pharmacogenomics. In the sense of individualized medicine, patients in peripheral hospitals would also benefit from this.

For special topics, such as the determination of the causes of rare diseases, expertise in consanguineous diseases, or the implementation of scientific projects, collaboration with a university center, such as the Institute of Human Genetics, is necessary.

The establishment of NGS technology in conjunction with appropriate human genetic counseling in peripheral hospitals would secure the expertise and competence at the relevant locations and further expand it accordingly. Young doctors would also benefit, particularly in terms of knowledge gathering and interdisciplinary collaboration.

The introduction of NGS technology in peripheral hospitals will have a decisive impact on both the diagnostic options and the therapeutic consequences derived from them in the coming years and will raise them to a new level of knowledge.

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