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EVALUATION OF GENETIC CANCER PREDISPOSITION AND POTENTIAL CANCER GENES

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Evaluation of Genetic Cancer Predisposition and Potential Cancer Genes

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To families suffering from hereditary breast cancer syndromes

The magnificent shining stars only appear on the darkest night

POPULAR SCIENCE SUMMARY OF THE THESIS

Have you ever heard that a 20-year girl already had breast cancer and sarcoma? What is worse, she is waiting for a subsequent cancer. You may be curious why she developed several tumors at such a young age.

The reason is that she carried a germline *TP53* pathogenic variant. What is TP53? TP53 is a tumor suppressor called the guardian of the genome. TP53 responds to DNA damage and arrests cell growth to allow DNA repair. TP53 may also trigger cell death by apoptosis and thereby eliminate the tumor cells. But if the guardian is damaged, a tumor can occur and continue to grow within the body. *TP53* pathogenic variants are found in more than 50% of tumors.

What happens when the *TP53* pathogenic variant is present in germline and can be inherited? It will cause Li-Fraumeni syndrome, a cancer syndrome with increasing risk of developing mainly breast cancer, sarcoma, brain tumor, and adrenocortical carcinoma. In these families, about 15% of carriers develop childhood tumors before the age of 15, and up to 50% develop tumors before 30 years of age. Except for this severe phenotype, only hereditary breast cancer in adults is seen in some families. The underlying cause for this phenotypic variation between carriers of *TP53* variants remains unknown. **Paper I** and **Paper II** found that the reason can to some extent be assigned to different *TP53* variants by describing the clinical characteristics of these families and evaluating the genotype-phenotype correlation. There is no standard treatment for this disease, and these patients are treated in the same way as people with sporadic tumors caused by somatic pathogenic variants. Still, chemotherapy and radiotherapy can increase the risk of developing secondary tumors in patients with germline *TP53* pathogenic variants due to impaired DNA repair. Therefore, efficient preventive treatment would be important to these tumor-prone families. **Paper III** assessed the preventive effect of the molecule APR-246 in a mouse model for Li-Fraumeni syndrome and found that APR-246 may play a role in delaying tumor onset in male mice. We hope APR-246 can be tested for tumor prevention in these cancer-prone *TP53* mutated individuals in the future.

We often think of the famous actress Angelina Jolie when it comes to breast cancer. She underwent a prophylactic bilateral mastectomy to reduce her risk of developing breast cancer since she carried a mutated high-risk gene, *BRCA1* which is associated with hereditary breast cancer. *BRCA1* and *BRCA2* are the most prevalent genes for hereditary breast cancer, characterized by early-onset of breast cancer and co-occurrence with ovarian cancer or other cancer types. However, no disease-causing genes are found for most familial cases of hereditary breast cancer. **Paper IV** identified potential high-risk genes that could contribute to breast cancer in Swedish hereditary breast cancer families and provided additional genetic information for those without known breast cancer genes.

ABSTRACT

Germline pathogenic *TP53* variants are associated with a broad spectrum of hereditary cancers characterized from Li-Fraumeni syndrome (LFS) to hereditary breast cancer (HBC) outcomes, known as heritable *TP53*-related cancer (h*TP53*rc) syndrome. LFS is a rare inherited cancer syndrome characterized by premenopausal breast cancer, soft tissue sarcoma, brain tumor, osteosarcoma, and adrenocortical carcinoma. To identify carriers with high risk of LFS phenotype and explore stratifying clinical management for these carriers, we developed a phenotypic prediction model of LFS in relation to HBC risk based on predicted protein conformation changes for germline *TP53* missense variants in the international agency for research on cancer (IARC) *TP53* database and published papers in **Paper I**. This model was validated in our Swedish *TP53* cohort with more reliable pedigree and clinical information in **Paper II**. Our results indicated that this tool could be considered helpful in the genetic counseling of families with h*TP53*rc, in particular as a psychological relief for families with a predominance of HBC phenotype. Also, we summarized the clinical characterization of all known *TP53*-carriers in h*TP53*rc families in Sweden and explored genotype-phenotype correlations. Except for the very high lifetime risk of a broad spectrum of tumor types in LFS families, chemo- and radiotherapy can increase these patients' risk of secondary tumors. Thus, efficient preventive treatment would be important to these tumor-prone carriers. We have seen an indication of delayed tumor onset using the mutant TP53-targeting compound APR-246 in a mouse model of LFS with R172H (amino acid change at residue 172 from arginine to histidine) mutant *Trp53,* and it had the potential to be used in the clinical study in **Paper III**.

HBC is characterized by early-onset age of breast cancer, bilateral breast cancer, male breast cancer, and it can be accompanied by ovarian cancer or other cancer types. *BRCA1* and *BRCA2* are the most prevalent genes for HBC, however, most families are not associated with variants in any known breast cancer-related genes. We identified several potential high-risk genes that could contribute to breast cancer in three Swedish HBC families in **Paper IV**.

In summary, this thesis enriches the knowledge of genetic predisposition and prevention of hereditary breast cancer syndromes and potential cancer genes.

LIST OF SCIENTIFIC PAPERS

I. Association between predicted effects of *TP53* missense variants on protein conformation and their phenotypic presentation as Li-Fraumeni syndrome or hereditary breast cancer.
Yaxuan Liu[*], Olga Axell, Tom van Leeuwen, Robert Konrat, Pedram Kharaziha, Catharina Larsson, Anthony P H Wright, Svetlana Bajalica-Lagercrantz.
International Journal of Molecular Sciences. 2021 Jun 14; 22 (12), 6345.

II. Characterization of heritable *TP53*-related cancer syndrome in Sweden – a retrospective nationwide study of genotype-phenotype correlations in 98 families.
Yaxuan Liu[#], Meis Omran[#,*], Alexander Sun Zhang, Marie Stenmark-Askmalm, Anna Rosén, Anna-Lotta Hallbeck, Anna Poluha, Fredrik Persson, Hafdis T. Helgadottir, Emma Tham, Svetlana Bajalica-Lagercrantz.
Manuscript

III. Evaluation of the prophylactic use of APR-246 in a mouse model of the Li-Fraumeni syndrome with R172H mutant *Trp53*.
Yaxuan Liu[*], Charlotte Strandgren, Yajie Yang, Alexander Sun Zhang, Felix Haglund de Flon, Lennart Blomquist, Svetlana Bajalica-Lagercrantz, Klas G Wiman.
Manuscript

IV. Whole exome sequencing of germline variants in non-*BRCA* families with hereditary breast cancer.
Yaxuan Liu[*], Hafdis T. Helgadottir, Pedram Kharaziha, Jungmin Choi, Francesc Lopez-Giraldez, Shrikant M. Mane, Veronica Höiom, Carl Christofer Juhlin, Catharina Larsson, Svetlana Bajalica-Lagercrantz.
biomedicines. 2022 April 26; 10 (5), 1004.

[*]Corresponding author
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CONTENTS

LIST OF ABBREVIATIONS

ACMG/AMP	American College of Medical Genetics and Genomics/the Association for Molecular Pathology
APR-246	TP53 targeting molecule
ER	Estrogen receptor
ERN	European reference network
GENTURIS	Genetic tumor risk syndromes
HBC	Hereditary breast cancer
HER-2	Human epidermal growth factor receptor 2
H*TP53*RC	Heritable *TP53*-related cancer
IARC	The international agency for research on cancer
LFS	Li-Fraumeni syndrome
MMAF	Maximum minor allele frequency
MQ	Active methylene quinuclidinone
PHTS	PTEN Hamartoma Tumor Syndrome
PR	Progesterone receptor
R172H	Amino acid change at residue 172 from arginine to histidine
R181H	Amino acid change at residue 181 from arginine to histidine
SNPs	Single nucleotide polymorphisms
SWEA	The Swedish *BRCA1/2* Extended Analysis
SWEP53	The Swedish *TP53* study
TP53	Human/mouse tumor protein TP53
TP53	Human tumor protein TP53 gene
Trp53	Mouse tumor protein TP53 gene
WB-MRI	Whole-body magnetic resonance imaging
WES	Whole-exome sequencing
542G>A	Nucleotide substitution at position 542 from guanine to adenine

1 INTRODUCTION

1.1 TUMORIGENESIS AND HALLMARKS OF CANCER

Tumor development is known as a multistep process, triggered by changes in the genome where crucial alterations involve oncogenes, tumor suppressor genes, and DNA-repair genes. Oncogenes are characteristically altered to gain-of-function through amplifications, rearrangements or activating genetic alterations, while tumor suppressor genes lose their function through deletions or by inactivating genetic alterations. DNA-repair genes belong to a separate group of genes since their impairment result in an acceleration of general genetic alterations. Tumor suppressor genes and DNA-repair genes are characterized by the need for bi-allelic loss to lose the function of the gene on the cellular level. Hereditary cancer syndromes often involve inherited mono-allelic alterations of tumor suppressor genes or DNA-repair genes where the other allele is wild-type, whose function is later disrupted. Therefore, hereditary cancers are characterized by an earlier age of onset than sporadic tumors in agreement with the Knudson two-hit hypothesis (1). Less commonly hereditary cancer predisposition may also result from gain-of-function variants (2).

Weinberg and Hanahan proposed the concept of cancer hallmarks and cells with these acquired capabilities will lead to the tumorigenesis (3,4) (Figure 1). Six initial hallmarks were first described in 2000 (3). These include:

- *Sustaining proliferative signaling.* Normal cells are only stimulated by external growth factors to grow, while cancer cells acquire the capability to produce their own growth signals.

- *Evading growth suppressors*. Cancer cells cannot maintain a balance between proliferation and cell death like normal cells. They can escape the regulation of anti-growth signals and continue to divide and grow.

- *Activating invasion and metastasis*. Cancer cells with activated invasion capabilities can migrate from the primary site to distant sites in the body and continue to grow and survive without the limitation of space and nutrients.

- *Enabling replicative immortality.* Normal cells can only divide a certain number of times due to the shortening of telomere lengths, while cancer cells can activate telomerase to extend telomeres and become immortal.

- *Inducing angiogenesis.* Cells need oxygen and nutrients from the vasculature to survive and perform their function, and tumor cells are able to stimulate the establishment of new blood vessels.

- *Resisting cell death*. Apoptosis will be initiated in normal cells if DNA damages cannot be repaired but cancer cells can bypass this process.

Following these six hallmarks of cancer, Weinberg and Hanahan continued to propose two new hallmarks, and two emerging characteristics in 2011(4). These are:

- *Deregulating cellular energetics*. Cancer cells have also been shown to have the ability to reprogram the energy source by altering metabolic pathways.

- *Avoiding immune destruction*. Cancer cells have acquired the ability to avoid detection and elimination by the immune system.

- *Tumor-promoting inflammation*. Inflammation stimulates angiogenesis and immune response, then cancer cells can acquire the ability to create a suitable environment for proliferation and growth.

- *Genome instability*. A large number of variants and chromosomal rearrangements are found in tumor tissues. These aberrations are not thought to be essential for tumor establishment and development but are a result of an unstable genome.

With the increasing investigation of cancer characteristics, two emerging hallmarks, and two enabling characteristics were proposed by Hanahan in 2022 (5) (Figure 1). These are as follows.

- *Unlocking Phenotypic Plasticity.* Cancer cells have achieved abilities to avoid entering the state of terminal differentiation, including dedifferentiation from mature cells to progenitor cells, blocked differentiation from progenitor cells to mature cells, and transdifferentiation to different cell types.

- *Senescent cells*. Senescent cells promote tumor growth mainly due to the activation of the senescence-associated secretory phenotype involving the large release of chemokines, cytokines, and proteases to nearby tumor cells and other cells in the tumor microenvironment.

- *Nonmutational Epigenetic Reprogramming.* Except for genome instability, nonmutational epigenetic changes in microenvironmental mechanisms, epigenetic regulatory heterogeneity, and stromal cell types can result in tumor development and progression.

- *Polymorphic Microbiomes.* The gut microbiome proved to be associated with the pathogenesis of colon cancers. Microbiomes in other organs and intratumoral microbiota can also affect tumor development.

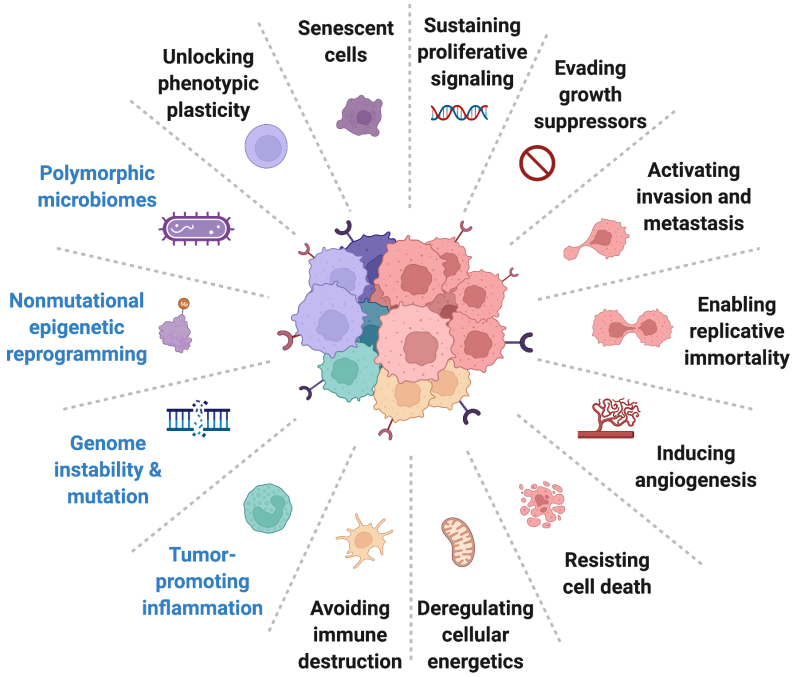

Figure 1. The hallmarks and characteristics of cancer. An illustration of the 10 hallmarks (black) and 4 enabling characteristics (blue) involved in the transformation of a cell into a cancer cell. Created with BioRender.com.

1.2 BREAST CANCER

Cancer is a common disease and every third human will develop a tumor during their lifetime. Breast cancer is the most prevalent malignancy among women worldwide. The breast cancer rate in Sweden is about 90 per 100,000 (6) meaning that around 9000 women are diagnosed yearly. Over the past decades, treatment for breast cancer has transformed from single surgery to comprehensive treatment including surgery, chemotherapy, radiotherapy, endocrine therapy, and targeted therapy, which has improved both survival and quality of life (7). However, breast cancer is recognized as a highly heterogeneous disease that brings many difficulties to treatment and clinical management. Five-year survival rates are above 80%, with better results at earlier diagnosis. Therefore, it is important to provide an adequate surveillance program for women with increased risk for breast cancer due to a genetic predisposition.

Early prevention is one effective method to reduce the burden of breast cancer. Risk factors for breast cancer are mainly age, family history of breast cancer, estrogen exposure, and lifestyle habits. Older age, early age of menarche, and late age at menopause are associated with an increased breast cancer risk. High estrogen exposure can promote estrogen receptor (ER)-positive breast cancer. Lifestyle-related risk factors are for example drinking alcohol, obesity, late pregnancy, no breastfeeding, and hormone replacement therapy, some of which are modifiable factors. Gene expression analysis subdivided breast cancer into four subtypes *i.e.* Luminal A, Luminal B, Basal-like, and human epidermal growth factor receptor 2 (HER2)-enriched involving the proliferation rate as well as the expression of ER, progesterone receptor (PR), and HER-2 (8).

1.3 HEREDITARY BREAST CANCER (HBC)

Hereditary breast cancer (HBC) accounts for about 5%-10% of the cases and is associated with early-onset age of breast cancer, often accompanied by ovarian cancer or other cancer types. Breast cancer is mainly a disease of the elderly woman with a median age of onset at 64 years, but if hereditary the age of onset for breast cancer tends to be much lower with an average age for tumor development starting from below 30 years and may affect up to every second woman in these families. Providing people from HBC families with risk assessment and genetic counseling is essential.

According to the European clinical guidelines for HBC, the specific screening criteria are (9)

- One case of breast cancer diagnosed under 40 years of age
- One case of triple-negative breast cancer diagnosed under 60 years of age
- Two cases of breast cancer diagnosed of which at least one under 50 years of age, including two tumors affecting one individual
- Three cases of breast cancer diagnosed of which at least one under 60 years of age, including two tumors affecting one individual
- Cases of breast and ovarian cancer in the family or in a single individual
- Male breast cancer (with at least one relative with breast or ovarian cancer)

HBC is most commonly associated with variants in *BRCA1* and *BRCA2* identified in 6% and 3%, respectively. Notably, almost 50% of all women with breast cancer who have been found to carry a *BRCA1* or *BRCA2* variant do not fulfill the screening criteria (10). One reason is thought to be paternal inheritance or small families with few women, and thus a risk of lack of family history of the disease. Other commonly mutated genes are *CHEK2*, *ATM, PALB2* and *TP53* detected in 3.5%, 1.5%, 0.78% and 0.75% of the families, respectively (The Swedish *BRCA1/2* Extended analysis (SWEA), personal communication). Together, these genes can only explain around 15% of the families with HBC, while in the remaining 85% of families a genetic cause has not been identified.

Early studies of hereditary cancer were mainly based on linkage analysis in large pedigrees and led to the discovery of several extremely rare variants in the high-risk genes (11,12). These variant carriers had about a ten-fold increased risk of breast cancer compared to women in the general population (13,14) (Figure 2). *BRCA1, BRCA2, PALB2,* and human tumor protein TP53 gene (*TP53*) are referred to as high-risk genes and they are associated with a high relative lifetime risk for breast cancer over 60% (15–17), respectively. Following genome-wide association studies in breast cancer families, some rare moderate-risk variants in incomplete penetrance genes have been identified, which are linked to a 2-4 times elevated risk of breast cancer (18,19). *CHEK2* and *ATM* are thought to be moderate-risk genes. The genes mentioned above are mostly linked to HBC (and ovarian cancer). Other genetic disorders, such as PTEN

Hamartoma Tumor Syndrome, are associated with an elevated risk of breast cancer. However, these syndromes possess a high risk of other tumors as well as an important characteristic of their phenotype. These syndromes are described in detail below in association with their corresponding genes. Single nucleotide polymorphisms (SNPs) with low-risk breast cancer susceptibility are common in the general population with an increased risk of developing breast cancer less than 1.5-fold (13) (Figure 2).

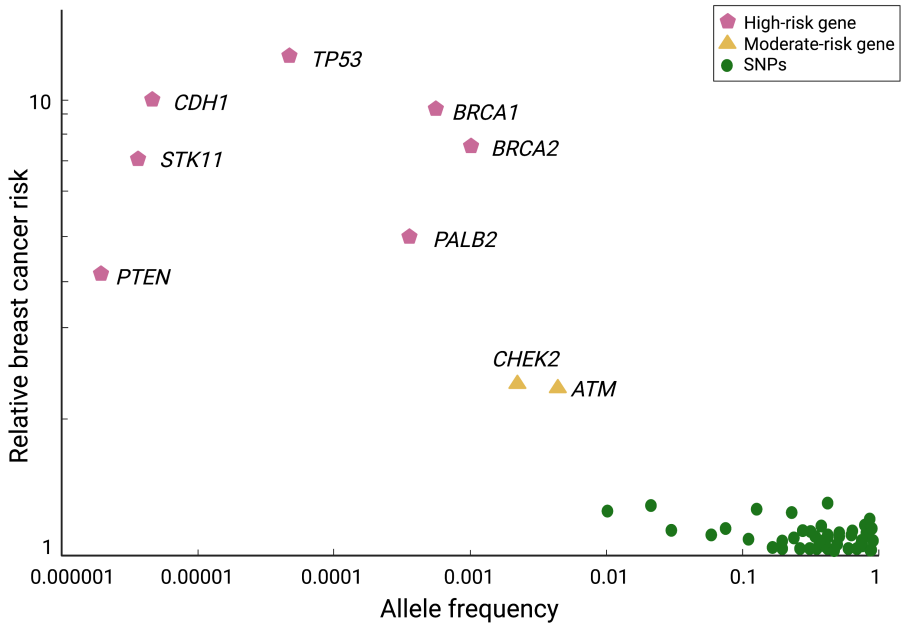

Figure 2. The relative risk and allele frequency of breast cancer-predisposing genes and SNPs. The selected genes are grouped into three categories: high-risk (pink), moderate-risk (yellow), and SNPs (green). Created with BioRender.com.

1.3.1 High-risk genes associated with increased breast cancer risk

1.3.1.1 BRCA1 and BRCA2

The most prevalent genes for HBC are *BRCA1* and *BRCA2* (20,21). The risk of developing breast cancer before 80 years of age is 72% in *BRCA1* variant carriers and 69% in *BRCA2* variant carriers (15). Meanwhile, the cumulative risk of ovarian cancer before 80 years of age was 44% for women with a *BRCA1* variant and 17% for women with a *BRCA2* variant (22). Both *BRCA1* and *BRCA2* are tumor suppressor genes and play a vital role in DNA repair maintaining the stability of the genome. If a variant occurs in *BRCA1* or *BRCA2*, DNA damage cannot be repaired correctly and more genetic alterations are required resulting in tumorigenesis. Protein truncating variants with a shortened coding sequence are predicted to result in loss-of-function, including nonsense variants, deletions, insertions, and splice-site variants. The variants 185AGdel in *BRCA1* and 5382insC in *BRCA2* are common disease-causing variants in most populations (23). Genetic testing for *BRCA1* and *BRCA2* is widely used in genetic counseling, yet these two genes can only explain about 3-4% of HBC (24).

1.3.1.2 TP53 – the guardian of the genome

Li-Fraumeni syndrome (LFS) is a rare inherited cancer syndrome mainly consisting of premenopausal breast cancer, soft tissue sarcoma, brain tumor, osteosarcoma, and adrenocortical carcinoma, which was first identified in 1969 (25) and subsequently shown to be associated with a germline *TP53* variant (26). In families with LFS, about 15% of the carriers develop childhood tumors before the age of 15, up to 50% of carriers develop tumors before 30 years of age, and major cancers will occur before the age of 60 (Figure 3). The Classic LFS criteria require a proband with sarcoma before the age of 45, who has a first-degree relative with cancer under 45 years and a first-degree or second-degree relative with cancer under 45 years or with sarcoma at any age (27). As more families with different types of cancers were reported, some of the families did not meet the Classic LFS criteria but were suggestive of LFS. A less restricted "Li Fraumeni like" (LFL) criteria was used to define these families (28). LFL criteria include Birch definition and Eeles definition. The Birch definition requires the proband to be diagnosed with any childhood cancer, sarcoma, brain tumor, or adrenocortical carcinoma before the age of 45, plus a first-degree or second-degree relative diagnosed with sarcoma, breast cancer, brain cancer, adrenocortical carcinoma, or leukemia at any age, and a first-degree or second-degree relative diagnosed with any cancer before age 60. Eeles definition requires two first-degree or second-degree relatives diagnosed with sarcoma, breast cancer, brain cancer, adrenocortical carcinoma, or leukemia at any age. Or it requires a proband with sarcoma at any age, with breast cancer before age 50 and/or brain tumor, leukemia, adrenocortical carcinoma, melanoma, prostate cancer, pancreatic cancer at before age 60, or a sarcoma at any age.

The LFS criteria have a 70% detection rate of germline *TP53* alterations while the LFL possesses a 20-40% detection rate (29), providing the possibility of a *TP53* gene test in more families and thus promoting cancer prevention. The Chompret criteria lead to the inclusion of even more families for *TP53* screening and therefore have a lower detection rate of 29% (30). The Chompret criteria require a proband with an LFS core tumor (e.g., breast cancer, soft-tissue sarcoma, osteosarcoma, brain tumor, adrenocortical carcinoma) before age 46 years and at least one first or second-degree relative with an LFS core tumor (except breast cancer) before age 56 years, or with multiple tumors; or a proband with multiple tumors (except multiple breast tumors), two of which belong to the LFS tumors and the first of which occurred before age 46 years; or a proband with adrenocortical carcinoma, choroid plexus tumor, or rhabdomyosarcoma of embryonal anaplastic subtype; or a female proband with breast cancer before age 31 years (31). The Chompret criteria are currently the most commonly used genetic screening criteria for LFS.

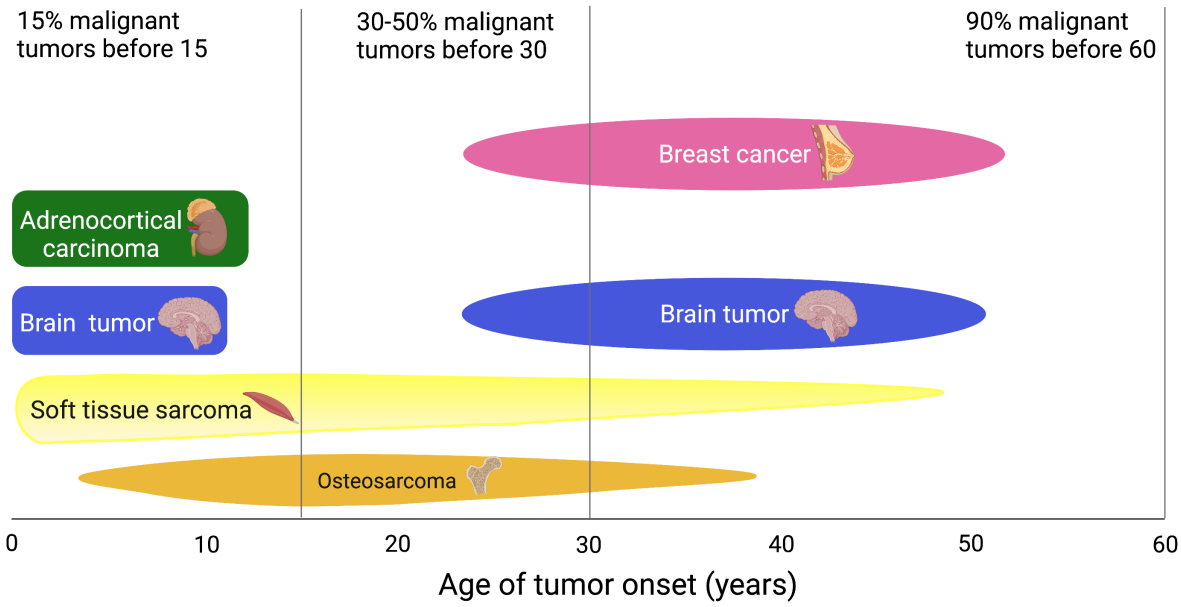

Figure 3. The age distribution for core tumor risk in Li-Fraumeni Syndrome. Adrenocortical carcinoma is more prevalent in children while adults are more likely to develop breast cancer. The age of onset for soft tissue sarcoma and osteosarcoma has a wide span and for brain tumor it is bi-phasic. Created with BioRender.com.

The *TP53* gene is a well-known tumor suppressor gene first discovered in 1979 (32). When a cell is exposed to DNA damage, oncogene activation, hypoxia, and other stressors, the level of TP53 protein will increase rapidly (33). Activation of TP53 can arrest the cell cycle to prevent the proliferation of the abnormal cell and initiate the DNA repair process. These normal cells can resume the cell cycle process after repairing genetic damages. If the damage within the cell is so severe that it cannot be repaired, TP53 may introduce apoptosis-related pathways and the cell will die. As widely studied, TP53 is involved in apoptosis, senescence, angiogenesis, autophagy, and metabolism (34) (Figure 4). Since TP53 plays a vital role in protecting DNA from damage caused by various stressors, it is also called the guardian of the genome.

The TP53 protein is a transcription factor that binds as a tetramer to DNA and activates a large number of genes involved in the aforementioned functions (35). Each monomer is composed of different structural and functional domains, including a transactivation domain, a proline-rich region, a DNA binding domain, an oligomerization domain, a nuclear localization signal, and a C-terminal regulatory domain (36). MDM2 specifically binds to the transactivation domain to promote TP53 degradation (37). The crystal structure of the DNA binding domain (residues 94-292), which consists of a beta-sandwich tertiary structure with two antiparallel beta-sheets, revealed the essential role of this domain (38). There are three main types of DNA-contacting residues. S241, R273, A276 and R283 bind to the backbone of DNA; C277, R280 and K120 bind to the major groove of DNA; and R248 bind to the minor groove of DNA. The oligomerization domain facilitates the formation of TP53 tetramers, associated with DNA binding and protein-protein interactions (39).

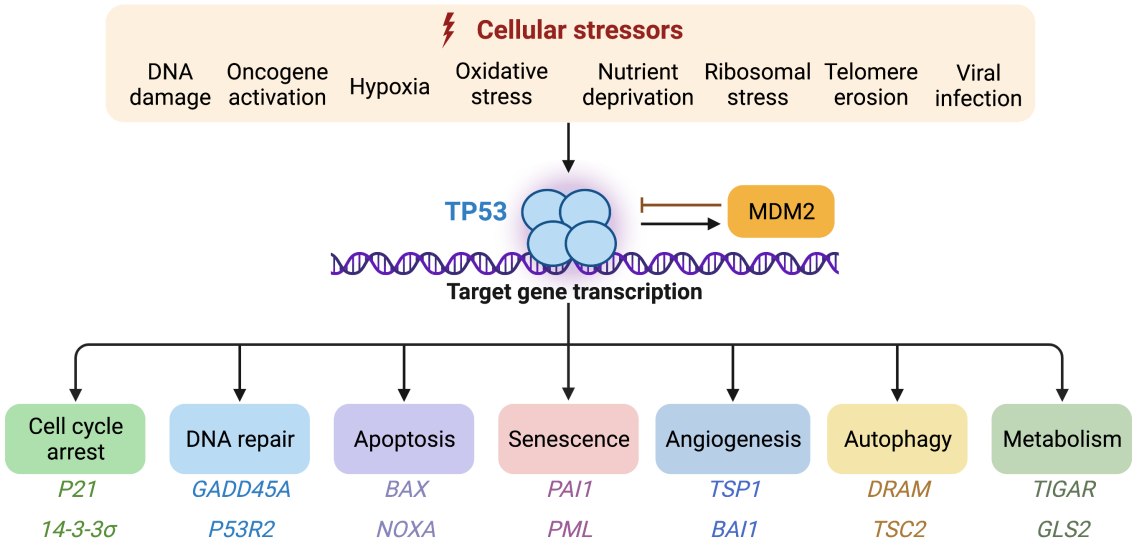

Figure 4. TP53 signaling pathways in response to cellular stressors. Differential cellular stressors (upper box) activate the TP53 protein that binds to DNA to initiate gene transcription. The MDM2 protein promotes TP53 degradation. Seven main TP53 pathways are indicated with colored boxes and representative TP53 target genes are respectively indicated below. Created with BioRender.com.

Somatic changes in *TP53* are the most frequently identified genetic alterations found in sporadic cancers (40,41), indicating a strong selective pressure for *TP53* inactivation during tumor development. About 70% of germline *TP53* variants are missense variants located in the DNA binding domain, *i.e.* the most highly conserved domain which is required for the sequence-specific DNA binding (42). Thus, these missense variants can impair the specification of DNA binding and transactivation of target genes. Missense variants can be classified as DNA-contact mutants and structural mutants (38). DNA-contact mutants, such as R248W and R273H, affect DNA-binding by loss of contacting residues with specific DNA sequences directly, while structural mutants disrupt the folding of the DNA binding domain such as R175H and Y220C and decrease the thermostability of the TP53 protein (43). According to Olivier et al. (44), missense variants located in the DNA-binding loop contacting the minor groove of DNA are associated with glioblastoma. In contrast, variants in the loops opposing the protein DNA interface are related to adrenocortical carcinoma.

LFS patients carry one allele with mutant *TP53* and one allele for wild-type *TP53* and are thus heterozygous. Since TP53 acts as a tetramer, the mutant monomers can, in some cases, bind with wild-type monomers as a hetero-tetramer, which exerts a dominant-negative effect (45). In addition, these variants can acquire more oncogenic abilities by forming hetero-oligomerization with p63 or other transcription factors, referred to as gain-of-function (46). Fifty-eight percent of carriers with R248W and 21% with R231Q develop cancer under 30 years of age, indicating the unequal gain-of-function effect of variants (47). Nonsense and frameshift variants, as well as gene deletion, cause loss-of-function of the TP53 (48). It has been reported that patients with missense variants have an earlier age of tumor onset (23.8 years)

than those with loss-of-function variants (28.5 years) (31). Heterogeneity of *TP53* variants affects the phenotype of the disease. The transcriptional activity of missense variants in downstream genes (*MDM2*, *WAF1*, *BAX*, *GADD45*, *AIP1*, *14-3-3σ*, *P53R2*, and *NOXA*) was evaluated using a yeast transcription assay system (49). Giacomeli et al. (50) developed a Z score to assess the loss of function and dominant-negative activities. Both were used for *TP53* specific American College of Medical Genetics and Genomics/the Association for Molecular Pathology (ACMG/AMP) classification of variants (51). In addition, BaysDel and Align-GVGD are variant functional prediction tools used to predict the pathogenicity of *TP53* missense variants (52,53). Researchers focus on the relationship between *TP53* variants and specific LFS tumor types and tumor onset age. In Brazil, for instance, the germline *TP53* variant R337H is a founder effect variant, and the carrier is more likely to develop adrenocortical carcinoma, later-onset cancer, and other cancer types, such as thyroid cancer, lung cancer, and renal cancer, compared to carriers of other germline *TP53* variants (54). Besides *TP53* variants, genetic modifiers are also crucial for tumor phenotypes, such as *MDM2* polymorphism (rs2279744), *TP53* polymorphism (PIN3), telomere length, copy number variations, and miRNAs (55). The polymorphism rs2279744 is located in the promoter of *MDM2*, which can enhance the degradation of TP53 by increasing the expression of *MDM2* (56). The *TP53* PIN3 polymorphism in intron 3 may be associated with the phenotypic variation of the LFS (57).

1.3.1.3 Other high-risk genes for breast cancer

PTEN Hamartoma Tumor Syndrome (PHTS), also known as Cowden syndrome, is a rare autosomal dominantly inherited disease including multiple benign hamartomas in various organs, which leads to the elevated risk of breast cancer, thyroid cancer, and other specific types of cancers. Benign breast diseases such as breast fibroadenoma and ductal hyperplasia are also accompanying symptoms (58). PHTS is associated with a disrupted *PTEN*, a tumor suppressor gene involved in cell cycle regulation, and is frequently lost in cancer. A variant in *PTEN* can result in unrestrained cell division, contributing to the development of tumors. The lifetime risk for *PTEN* variant carriers to develop breast cancer is about 50%-85%, and for thyroid cancer is 30%-40% (59,60). More truncated variants are found than missense variants, but there is no significant difference between them in cancer risk.

Peutz-Jeghers syndrome is a rare disorder in which the patients exhibit multiple benign polyps in the stomach and intestines and mucocutaneous pigmentation, with the additional risk of gastrointestinal, breast, and other cancer types. It follows an autosomal dominant inheritance pattern and is associated with variants of *STK11* (61). The *STK11* gene also acts as a tumor suppressor related to the cell cycle and apoptosis. The pathogenic *STK11* variants cannot control cell growth properly, leading to the formation of benign polyps and tumors. Women with a variant in *STK11* have a 24% to 54% lifetime risk of developing breast cancer (62). Protein-truncating variants are prevalent and up to one-third of pathogenic variants involve large deletions (63).

Hereditary diffuse gastric cancer is a rare genetic disease associated with germline *CDH1*

variant. Diffuse gastric cancer is a specific kind of stomach cancer affecting most of the stomach. The *CDH1* gene encodes a protein called epithelial cadherin (E-cadherin), which is located within the membrane surrounding epithelial cells and plays a vital role in cell adhesion, it belongs to a protein family named cadherins. *CDH1* variants lead to aberrant E-cadherin protein that cannot perform the function of cell adhesion properly, and increased cell motility property promotes tumor metastasis (64). The estimated risk for lobular breast cancer in females is about 39% to 52% by age 80 (65,66). Truncating variants are more common as compared to missense variants.

PALB2 is the partner and localizer of *BRCA2* and is involved in DNA repair. When faced with a double-strand DNA break, *PALB2* can accumulate *BRCA2* and interact with *BRCA1* in homologous recombination-mediated repair. Germline variants will affect the DNA repair function and lead to the development of several types of cancers, including breast cancer (67). The risk of developing breast cancer by 70 years of age is 33% to 58% in *PALB2* variant carriers (16). Many studies focus on loss-of-function variants of the types, nonsense, frameshift, or splicing variants. However, there is a lack of evidence to support a risk of cancer from missense variants.

1.3.2 Moderate-risk genes

The *CHEK2* gene encodes the protein checkpoint kinase 2, which is a cell cycle checkpoint that negatively regulates the progression of the cell cycle. It also phosphorylates *BRCA1* and *BRCA2* to promote DNA repair and phosphorylates *TP53* and *MDM4* to regulate cell apoptosis. The truncating *CHEK2* variant 1100delC was found in 5.1% of individuals with breast cancer from 718 families and the lifetime risk is about 25%-30% (68). The cancer risk also varies in the type of variant. The *CHEK2* missense variant I157T confers a 1.3-fold increased risk of breast cancer, much lower than the truncated variant (69). Other variants have not been further investigated. The protein encoded by *ATM* is a cell cycle checkpoint kinase, and it is a member of the PI3/PI4-kinase family. Except for cell cycle control, this protein can also interact with *BRCA1* and *TP53* to regulate DNA repair. The lifetime risk of developing breast cancer is 25% to 30% in *ATM* variant carriers (70), and most variants are missense. Notably, the missense variant V2424G in *ATM* confers an 11-fold increased risk of breast cancer (71) and is thus associated with a high risk of breast cancer. However, the vast majority of reports support *ATM* to be an intermediate risk gene for breast cancer.

1.3.3 Low-risk genetic susceptibility

More variants emerged subsequently during the past years from genome-wide association studies, some of which are SNPs with less than a 1.5-fold risk of developing breast cancer (72) (Figure 2). For instance, the Pro919Ser polymorphism in *BRIP1* was associated with an increased risk of breast cancer among premenopausal women (73). The Breast Cancer Association Consortium has performed several large-scale genotyping studies in more than 50 breast cancer case-control studies and identified 172 SNPs that account for approximately 18% of the risk of developing HBC (74).

1.4 HERITABLE *TP53*-RELATED CANCER (H*TP53*RC) SYNDROME

As mentioned above, germline pathogenic *TP53* variants are associated with LFS, comprising a high risk of malignant tumors. In some LFS families, a tumor spectrum with pediatric tumors appears more frequent, while in other, mainly breast cancer in adults is seen. The underlying cause for this phenotypic variation has to some extent been assigned to different *TP53* variants but remains largely unknown. Due to the significant genotype-phenotype variations in LFS, the syndrome is currently more often referred to as heritable *TP53*-related cancer (h*TP53*rc) syndrome (75).

1.4.1 Li-Fraumeni syndrome vs. Hereditary breast cancer

Screening of HBC families applying breast cancer-associated gene panels has increased the identification of families with a constitutional *TP53* variant. These families, however, show a large phenotypic variation in tumor development. LFS is associated with a 70-100% lifetime tumor risk, and five tumor types comprise 80% of all LFS tumors: premenopausal breast cancer (30%), brain tumor (14%), adrenocortical carcinoma (6%), osteosarcoma (13%), and soft tissue sarcoma (17%) (Figure 5). The median age of tumor onset is 25 years, with as much as a 15% risk of developing cancer before the age of 15. Interestingly, almost 1% of families with exclusively HBC have been shown to carry a germline *TP53* variant (76) (Figure 5). The wide range of phenotypic presentations associated with germline *TP53* variants makes tumor risk assessment difficult and genetic counseling challenging in these patients and families. Notably, 7%-20% of constitutional *TP53* variants are *de novo*. Therefore, a breast cancer patient carrying a *de novo TP53* variant is difficult to classify phenotypically as LFS or HBC due to the absence of family history, posing additional challenges for genetic counseling and clinical management.

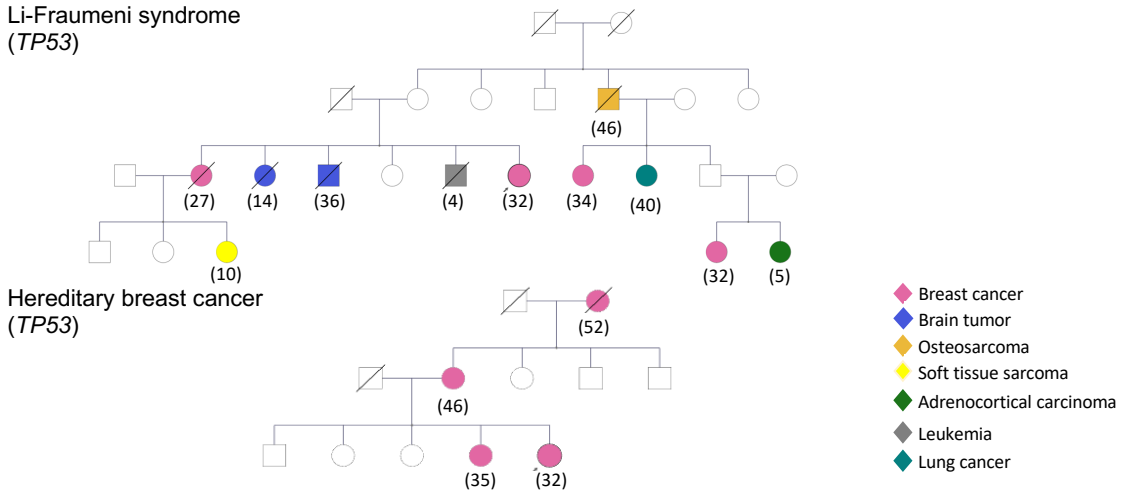

Figure 5. Examples of Li-Fraumeni syndrome (LFS) and hereditary breast cancer (HBC) pedigrees. The pedigrees indicate the autosomal dominant inheritance pattern of cancer predisposition related to *TP53* variants. The upper pedigree shows the occurrence of, among others, the five LFS core tumor types *i.e.* breast cancer, brain tumor, osteosarcoma, soft tissue sarcoma, and adrenocortical carcinoma. The lower pedigree shows HBC pedigree with breast cancer in adult women. Age of onset is indicated within brackets. Created with the PhenoTips software (77).

1.4.2 Surveillance program

For *TP53*-carriers, the screening for malignant tumors should be taken from childhood. The surveillance includes clinical examination and abdominal ultrasound every six months, whole-body magnetic resonance imaging (WB-MRI) and brain MRI annually. Adults are recommended annual clinical examination, whole-body MRI, breast MRI from 20 to 65 years (in women), and brain MRI to 50 years (75). An 11-year prospective study found a prolonged 5-year overall survival of *TP53*-carriers under surveillance in comparison with carriers who declined surveillance, and this prolonged survival was associated with early tumor detection through surveillance, indicating that *TP53*-carriers would benefit from the surveillance program (78). Until date, we do not have clinical tools to predict the phenotypic outcome in families with germline variants. Therefore, the European reference network (ERN) for genetic tumor risk syndromes (GENTURIS) has recently raised the need for improved surveillance for these families with hereditary *TP53* related cancers, suggesting yearly follow-up with whole-body MRI for all carriers, despite the fact that about 1/3 of these families present HBC (75).

In Sweden, all *TP53*-carriers are offered genetic counseling, clinical examination yearly, and breast surveillance (in women). Genetic counseling can empower people to understand and adjust to the medical, psychological, and familial effects of this genetic disease. One of the critical genetic counseling processes is risk assessment, based on family history and genetic testing. Peng et al. (79) proposed a model to predict the individual risk of being a *TP53*-carrier and developing any LFS-related tumor in the subsequent years based on family cancer history. However, the different *TP53* variants will also need to be taken into consideration when assessing the risk of developing tumors in these families with h*TP53*rc syndrome. A national Swedish *TP53* study (SWEP53) was initiated in 2017 to evaluate the effect of MRI and ultrasound in the *TP53*-carriers (80). Forty-two of 68 *TP53*-carriers in this study for whole-body MRI had a normal MRI scan, while 19 carriers with 30 lesions required further investigation, and finally, three carriers were diagnosed with malignant tumors (81). It added to the evidence supporting the use of whole-body MRI in surveillance. Within the SWEP53 study, national covering pedigree and genetic screening information was collected from the six hereditary cancer units in Sweden between January 2000 and March 2022. The prevalence of *TP53* variant carriers in the Western world is 1/5000-1/20000 (30), indicating that there are 450-1800 carriers in Sweden while only 98 families have yet been identified in our country. Four to thirteen percent of all women with breast cancer before 36 years of age have *TP53* variants, regardless of family history (82). Consequently, several *TP53* variant carriers are identified through a family history of breast cancer rather than on the basis of Classic LFS.

1.4.3 Preventive treatment

Surveillance with whole-body MRI in *TP53* carriers is, however, not reducing the risk of tumor development, but it is thought to increase the likelihood of early tumor detection and is therefore referred to as secondary prevention. Primary prevention strategies include risk-reducing surgery with the removal of risk organs and chemoprevention treatment such as the use of acetylsalicylic acid in hereditary colorectal cancer patients (83). Even though the

prophylactic mastectomy reduces the risk of developing breast cancer among female carriers, they have to suffer from surgical potential complications and psychological burdens (84), so it should be considered and discussed with these female carriers in the clinical practice with cautious. In HBC, chemoprevention has not been introduced despite ongoing studies evaluating Tamoxifen (85).

Around 25% of LFS patients surviving their first cancer diagnosis develop a second primary tumor, and 12.5% develop a third primary tumor (31). Chemotherapy and radiotherapy can increase the risk of developing secondary tumors in these patients due to impaired DNA repair, and the risk of sarcoma in the radiation field may be up to 30% (31,86). Therefore, efficient preventive treatment would be of enormous importance for these tumor-prone families.

1.4.3.1 APR-246

Many research groups focus on TP53-targeted anti-cancer therapy since *TP53* is the most frequently mutated gene in sporadic tumors and tumor cells often have a high level of mutant TP53 protein. Several small molecules can reactivate mutant TP53 and restore the function of TP53 (87–90). APR-246 is such a cysteine-binding compound that has been shown to reactivate the mutant TP53 (91) via conversion to the biologically active methylene quinuclidinone (MQ), a Michael acceptor that binds covalently to cysteines (C144 and C277) in the TP53 core domain (92). Additionally, MQ can deplete glutathione and inhibit thioredoxin reductase, so APR-246 increases oxidative stress and further induces cell death (92,93). Previous studies have shown that APR-246 can suppress the growth of xenografts of mutant TP53-expressing human tumors in immunodeficient SCID mice (87,91). APR-246 has been tested in humans as an anti-tumor drug in phase II clinical trials in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) combined with Azacytidine, and *TP53*-mutant MDS and AML demonstrated a high rate of overall response rate and complete remission (94,95). Moreover, APR-246 is currently being evaluated in combination with Venetoclax or Pembrolizumab for the treatment of chronic lymphocytic leukemia or solid malignant tumors, respectively. (see clinicaltrials.gov). It has been associated with only limited reversible side effects such as fatigue, nausea, dizziness, headache, confusion, ataxia, neutropenia and leukopenia (94,95).

1.4.3.2 LFS mouse models

Missense variants are the most commonly occurring variants in LFS patients, identified in about 70% of carriers, and they carry one mutant *TP53* and one wild-type *TP53* allele and are thus heterozygous. Research groups have tried to generate mouse models for LFS with these missense variants (96–98). In 2004, Lang et al. generated knock-in mouse models of LFS containing an amino acid change at residue 172 from arginine to histidine (R172H), which corresponds to the R175H hot-spot variant in human tumors (96). The heterozygous *Trp53*$^{172H/+}$ mice carrying one wild-type *Trp53* allele and one mutant *Trp53* allele and they start to develop tumors at around one year of age, most commonly sarcoma (53%), lymphoma (31.5%) and carcinoma (15.5%). Survival falls rapidly between 12 and 24 months of age (96). Tumors from the heterozygous *Trp53*$^{172H/+}$ mice are more prone to distant metastases compared to

homozygous *Trp53*$^{172H/H}$ mice even though they have a similar tumor spectrum. The homozygous *Trp53*$^{172H/H}$ mice, in which both *Trp53* alleles are mutant, develop mainly lymphoma (70%) and sarcoma (29%) tumors already at three months of age (96), thus earlier than heterozygous mice. Furthermore, Wang et al. (99) investigated the effect of metformin in these homozygous *Trp53*$^{172H/H}$ mice, which has shown a weak but statistically significant effect on preventing tumor onset via inhibiting mitochondrial respiration to prevent cancer cells from proliferation.

2 RESEARCH AIMS

The overall aim was to explore the genetic predisposition and prevention of Li-Fraumeni syndrome (LFS) and hereditary breast cancer (HBC) and increase the understanding of the phenotypic impact of *TP53* variants to facilitate the clinical handling of *TP53*-carriers and their families. The specific aims for each study were:

Paper I: To explore the impact of *TP53* missense variants on protein conformation and its correlation to genotype-phenotype and to develop a phenotypic prediction tool for LFS in relation to HBC for clinical use.

Paper II: To describe clinical characteristics of the Swedish constitutional *TP53* cohort and outline the phenotypic impact of the *TP53* variants. Also, to evaluate the prediction tool developed in **Paper I**.

Paper III: To evaluate the use of the TP53 targeting molecule APR-246 in a prophylactic setting to explore the possibility to delay, or even prevent, tumor development in an LFS-mouse model.

Paper IV: To identify new cancer risk genes by whole-exome sequencing in Swedish non-*BRCA* families with a pronounced history of HBC.

3 MATERIALS AND METHODS

Here is given an overview of the materials and methods used in this thesis. More detailed information is listed in each paper.

3.1 PATIENT COHORTS AND ETHICAL ASPECTS

3.1.1 The international agency for research on cancer (IARC) *TP53* database and TP53 protein structure analysis

The IARC *TP53* database has included *TP53* variant data in published papers or other public databases since 1989. Of 296 germline missense *TP53* variants reported in the IARC database, 62 variants were marked as LFS-class and we selected 24 unique variants from this class that did not show any overlap with other classes. The 24 exclusive HBC variants were selected both from the 117 variants reported in the IARC database not fulfilling any LFS criteria, from a meta-analysis of 41 germline missense *TP53* variants associated with hereditary breast cancer families (100), and from the study of germline missense *TP53* variants identified in the early Swedish *TP53* cohort (101). Notably, variants that were reported in both LFS and HBC families were excluded from the analysis in order to optimize the phenotypic groups. Finally, 24 variants from each group (LFS and HBC) were selected and used to build our phenotypic prediction model based on missense variants and their impact on protein conformation in **Paper I** (Figure 6).

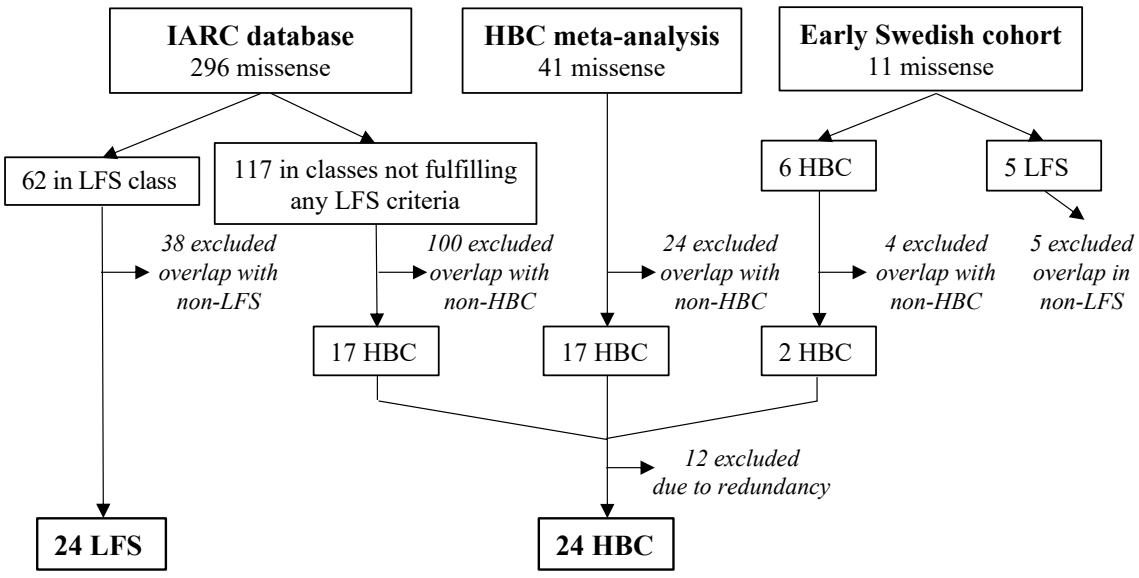

Figure 6. The selection of the *TP53* missense variants. Detailed overview of the selection process of the *TP53* missense variants uniquely reported as either LFS or HBC, 24 in each group, that were used to build the phenotypic prediction model presented in **Paper I**.

The TP53 protein conformation information of each variant was obtained from an X-ray crystallography structure of a TP53 tetramer complexed with the natural *P21* TP53-response element (102,103) by using PyMOL software (Schrödinger, https://pymol.org, 23 April 2019). This TP53 crystal structure included residues 94-292 in the DNA binding domain and residues 324-355 in the tetramerization domain. The alpha-helix, beta-sheet, or disordered regions elements from secondary structure, Surface or Buried locations elements from tertiary structure, and protein-protein interface or the DNA-protein interface elements from the quaternary structure were calculated in this structural context. Other concomitant prediction variables on protein conformation were obtained from Espritz, IUPred2A, Dynamine, and Meta-structure algorithm (104–108).

3.1.2 The Swedish h*TP53*rc syndrome cohort

Even though the IARC database is an excellent resource for LFS-associated research, it does not offer detailed pedigree and follow-up information on *TP53*-carriers. To improve these shortcomings, we have performed a Swedish constitutional *TP53* study including pedigree and genetic screening information through the six hereditary cancer units (Umeå, Uppsala, Stockholm, Linköping, Gothenburg, and Lund) and retrospectively identified in total 98 families with 188 *TP53*-carriers from January 2000 to March 2022 in **Paper II**. This not only described pedigree characteristics of families with h*TP53*rc syndrome to outline the genotype-phenotype correlation but also evaluated our previously published phenotype prediction model for *TP53* missense variants presented in **Paper I** to explore its possibility of clinical application.

3.1.3 The SWEA-negative families for whole-exome sequencing

The SWEA study was performed from 2012 to 2017, including nearly 4,000 families with HBC, to investigate the occurrence of HBC-predisposing genes in Sweden. The gene panel used in this study included 64 genes associated with breast cancer. We selected 17 SWEA-negative families with a striking pedigree of HBC at first and were able to collect blood samples for whole-exome sequencing for at least two generations in three families. The gene panel is a subset of disease-causing genes widely used in the clinical setting. Still, it may miss some crucial genetic information outside of the panel, and whole-exome sequencing (WES) compensates for this deficiency. WES is one of the next-generation sequencing techniques analysing all protein-coding regions known as exons, occurring in 1% of the human genome, which can be used to identify exonic variants in diseased individuals after comparing with the human reference genome. WES was used in **Paper IV** to identify genetic variants predisposing to hereditary breast cancer.

3.1.4 Ethical aspects

There are several psychological issues to consider when dealing with patients and families with a cancer risk syndrome, especially in the case of germline *TP53* variants, which increase the risk for tumors also in children and young adults. One crucial genetic counseling process is risk assessment, based on family history and genetic testing. In contrast to sporadic cancers, when

the initial focus is commonly on treatment and survival, diagnosis in families with inherited cancer risks is often preceded by long-term awareness of this risk, illness, and anticipation of reduced survival. These individuals have often witnessed the death of loved ones and/or seen several family members suffering from cancer simultaneously.

3.2 *IN VITRO* EXPERIMENTS

3.2.1 Primary lymphoma cell culture, growth suppression, and apoptosis assay

In **Paper III**, primary lymphoma cells were obtained from a homozygous *Trp53*$^{172H/H}$ mouse with thymic lymphoma. To confirm that APR-246 can reactivate R172H mutant TP53 and trigger tumor cell death in this genetic background, we first tested the cell growth suppression and apoptosis effect of increasing concentrations of APR-246 in these cells using the WST1 assay and Annexin V/Propidium iodide respectively. The cellular mitochondrial dehydrogenase generated by active cells can cleave the tetrazolium salt WST-1 to a soluble formazan, and the amount of formazan dye can be evaluated by a Tecan microplate reader. The measured absorbance is associated with the number of viable cells. The early apoptosis cells can bind Annexin V by the translocated phospholipid phosphatidylserine, while PI can penetrate cell membranes of late apoptosis cells into double-stranded DNA. The fluorescence difference is measured by flow cytometry, and the proportion of apoptotic cells is counted.

3.2.2 Breast cancer derived cell lines, Western blot, and Mass spectrometry

MDA-MB-231 and MCF7 breast cancer derived cell lines were used for functional analysis in **Paper IV**, and four potential breast cancer-predisposing genes were selected for validation through transfection and ectopic expression of wild-type and mutated constructs, followed by Western blot analyses and/or mass spectrometry profiling. Western blot is a widely used sensitive method to detect and quantify specific proteins. The isolated proteins from transfected cells are unfolded by a sodium dodecyl sulfate into linear chains and then are negatively charged. Proteins with different sizes are separated by gel electrophoresis and identified by specific antibodies. More proteins can be detected by mass spectrometry technology. Proteins are digested into peptides and become ion-peptides, then separated by the mass-to-charge ratio in the mass spectrometer. The measured peak will be aligned with the reference protein database to find the specific proteins. This is the primary method to investigate human proteomics.

3.3 *IN VIVO* EXPERIMENTS

In **Paper III** we used two different LFS mouse models with a germline missense *TP53* variant (altered at His172, which corresponds to the His175 hot-spot in human *TP53*): one homozygous *Trp53*$^{172H/H}$ and one heterozygous *Trp53*$^{172H/+}$ (96). We first tested the homozygous mice strain since these mice develop tumors earlier (from around the age of three

months) than the heterozygous mice strain that have a later tumor onset (from about the age of 12 months), allowing a more rapid evaluation of the potential preventive effect of the TP53 targeting molecule APR-246. Previous studies of *in vitro* and *in vivo* models have shown that APR-246 has an inhibitory effect on tumor development and has few side effects. Intraperitoneal injection (i.p.) of APR-246 was initiated at the age of 6 weeks and given during 4 consecutive days every third week (Figure 7). Mice were monitored daily by inspection and weekly by weighing and palpation. Mice that showed signs of illness and/or weight loss, reaching humane endpoints, were euthanized by an overdose of CO_2, a necropsy was performed, and tumors were collected for diagnosis.

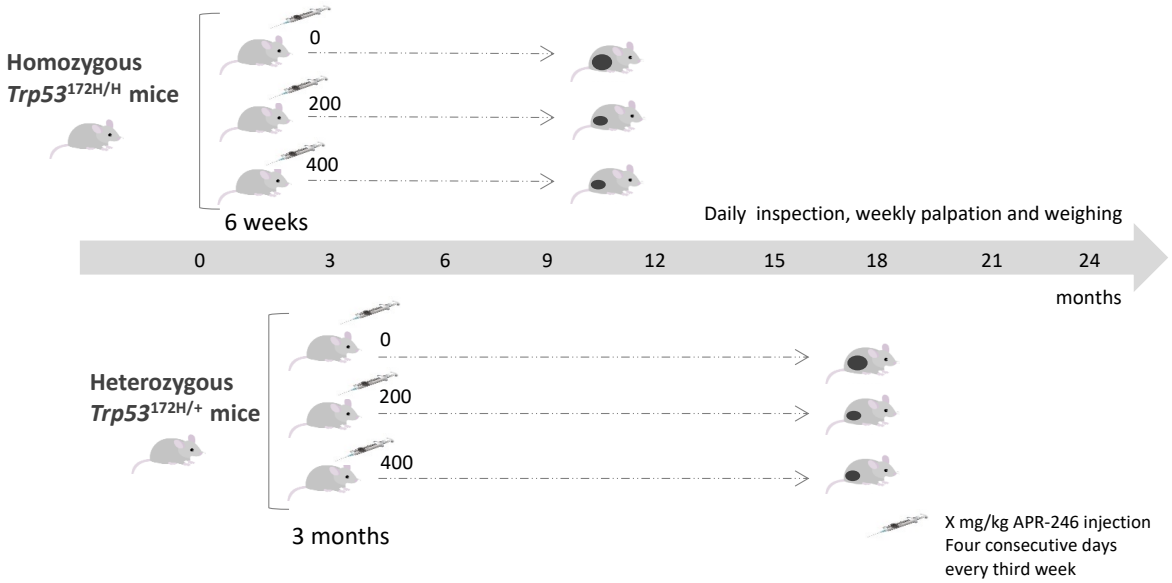

Figure 7. The study design of APR-246 used in LFS mouse models. Homozygous *Trp53*$^{172H/H}$ and heterozygous *Trp53*$^{172H/+}$ mice were randomly divided into three groups, *i.e.* control group (NaCl solution), single dose APR-246 group (200mg/kg), double dose APR-246 group (200mg/kg, twice daily). The treatment for all groups started at the age of 6 weeks in homozygous mice and the age of 3 months in heterozygous mice.

3.4 STATISTICAL ANALYSIS

The R software, version 3.6, was used for data collection and statistical analysis in **Paper I-III**.

4 RESULTS AND DISCUSSION

4.1 PAPER I. Association between predicted effects of *TP53* missense variants on protein conformation and their phenotypic presentation as Li-Fraumeni syndrome or hereditary breast cancer

Germline pathogenic *TP53* variants are associated with a wide spectrum of hereditary cancers characterized from the wider phenotype of LFS to the more limited HBC outcome. LFS is a rare inherited cancer risk syndrome associated with premenopausal breast cancer, soft tissue sarcoma, brain tumor, osteosarcoma, and adrenocortical carcinoma, commonly occurring during childhood and in young adults, while HBC is characterized by the increased risk of mainly breast cancer in adult women, causing genetic counseling and clinical management challenges. The focus of this project was to explore the relationship between germline *TP53* missense variants and their phenotypic impact concerning LFS and HBC based on conformational characteristics of the TP53 protein.

A total of 24 missense variants associated with LFS and 24 associated with HBC selected from the IARC *TP53* database, Fortuno et al. (100) and Kharaziha et al. (101) were included in this study. The majority of the amino acids corresponding to the wild-type of the missense variant were mapped to the DNA binding domain of the TP53 protein, and no apparent pattern was associated with either LFS or HBC. We examined the variant phenotypic association with secondary structure, tertiary structure, or quaternary structure aspects of TP53 using the Pymol software. We found that variant residues corresponding to LFS were preferably Buried in the core of the tertiary structure of TP53 (p=0.0014) (Figure 8).

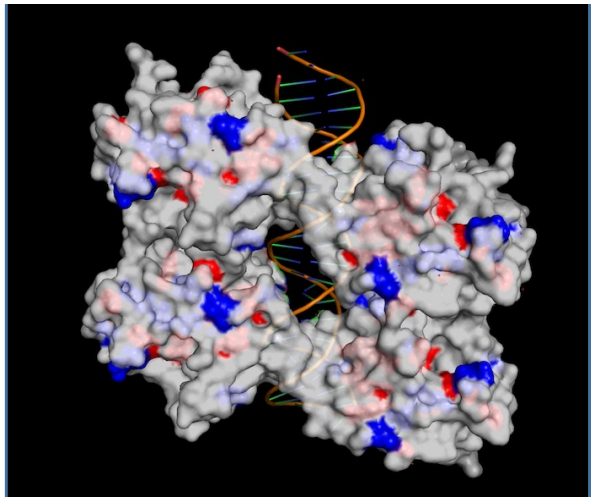

Figure 8. The crystal structure of the TP53 tetramer complex binding to DNA. The mutated residues corresponding to missense variants identified in LFS are indicated in red, and the corresponding residues identified in HBC variants are shown in blue. LFS residues were more likely to be Buried in the core of the tertiary structure (inside the protein structure).

The location of these variant LFS/HBC residues may change the folded conformation of TP53 or affect the tetramerization or interfere with the interactions between the TP53 protein and targeted DNA regions or proteins. We further developed logistic regression models to predict LFS or HBC phenotypic outcomes based on the Buried status for the corresponding residues using a series of protein conformation effect prediction variables obtained from Espritz, IUPred2A, Dynamine, and Meta-structure algorithm. Reduced models distinguished well between LFS and HBC (threshold 0.5) with a C-statistic of 0.78-0.84 and were well-calibrated to the known outcome. The favored logistic regression model included the Buried status of residues, protein compactness, and protein-protein interactions prediction variables.

We further estimated the potential clinical use of this model by building a nomogram using decision curve analysis. The Buried status of residues was the most crucial variable in the nomogram, followed by the protein compactness variable, with increased compactness favoring the LFS phenotype, and the protein-protein interaction variable, which also positively associated with the probability of the LFS phenotype. The decision curve indicated that using the nomogram to predict LFS adds more benefit than either the treat-all-patients as LFS or the treat-none as LFS if the threshold probability of an LFS patient was set to above 0.2.

This phenotypic prediction model indicated that variants that tend to strengthen the tertiary and quaternary structure of the TP53 tetramer would tend to have an LFS outcome. However, it needs to be validated in an independent cohort with more reliable pedigree and clinical information before it is used as a helpful tool in clinical practice.

4.2 PAPER II. Characterization of heritable *TP53*-related cancer syndrome in Sweden – a retrospective nationwide study of genotype-phenotype correlations in 98 families

The underlying cause for the large phenotypic variation in terms of LFS and HBC has to some extent been assigned to different *TP53* variants but remains largely unknown. Thus, the clinical handling and genetic counseling in these families is still challenging. Due to the wide range of genotype-phenotype variations between families with germline *TP53*, these are currently more often referred to as the heritable *TP53*-related cancer (h*TP53*rc) syndrome instead of LFS. The overall aim of this study was to outline the Swedish cohort of known families with h*TP53*rc syndrome and *TP53*-carriers in terms of tumor types, age of onset, etc, as well as the corresponding *TP53* variant, to evaluate the genotype-phenotype correlations. Also, the characteristics from this cohort were used to validate the phenotypic prediction model initially presented in **Paper I**.

We identified in total 98 families with 188 carriers, both healthy and diseased, of a clinically actionable (class 4 and class 5) *TP53* variant known in Sweden. Fourteen families (14.3%) fulfilled the stricter criteria for genetic screening as with Classic LFS, 43 (43.9%) the updated Chompret criteria, and 37 (37.8%) the screening criteria for hereditary breast cancer. Four (4.1%) "Other" families were identified through *TP53*-screening outside the above criteria, as the screening was performed due to a single case of childhood hypodiploid acute lymphatic leukemia, early-onset bilateral ductal breast carcinoma in situ (age 26), ovarian carcinoma (age 62), and leiomyosarcoma (age 47), respectively. Of the 188 *TP53* carriers, 121 (64.4%) developed at least one malignant tumor. Patients from HBC families (mean age 44) had later tumor onset compared to patients from Classic LFS (mean age 26, p=0.00037) and Chompret families (mean age 33, p=0.00042).

We found 47 different germline *TP53* variants consisting of 25 missense, 15 truncating, 5 splicing, and 2 in-frame deletion. Affected carriers with missense variants had a later tumor onset (mean age 40) than carriers with other variants (mean age 34, p=0.032). Patients with dominant-negative missense variants developed tumors earlier (mean age 38) than those with other missense variants (mean age 47, p=0.014).

Notably, the missense variant c.542G>A/p.R181H was identified in 19 families and represents the largest published cohort. It was previously reported in 8 families with this germline variant, of which one from Norway, one from Germany, one from France, and one from the UK, in close vicinity of Sweden. Therefore, we suggest that it may be a potential Swedish founder variant. However, this has to be further outlined by complementary haplotype analysis to ensure any founder effect. Of the 19 families with c.542G>A/p.R181H, 15 fulfilled the HBC-screening criteria, thus associated with a breast cancer phenotype.

Using our prediction model from **Paper I**, with the threshold of LFS set to 0.6, 15 of the 23 missense variants (65.2%) were phenotypically predicted correctly. Two of the 25 missense variants were considered as not informative since one was reported in 1 Classic LFS and 1 HBC family and the other was exclusively reported in the group of "Other". The suggested

Swedish founder c.542G>A/p.R181H was, in agreement with the cohort evaluation, predicted to be associated with an HBC phenotype as the predicted value of LFS was 0.33, thus low (Figure 9).

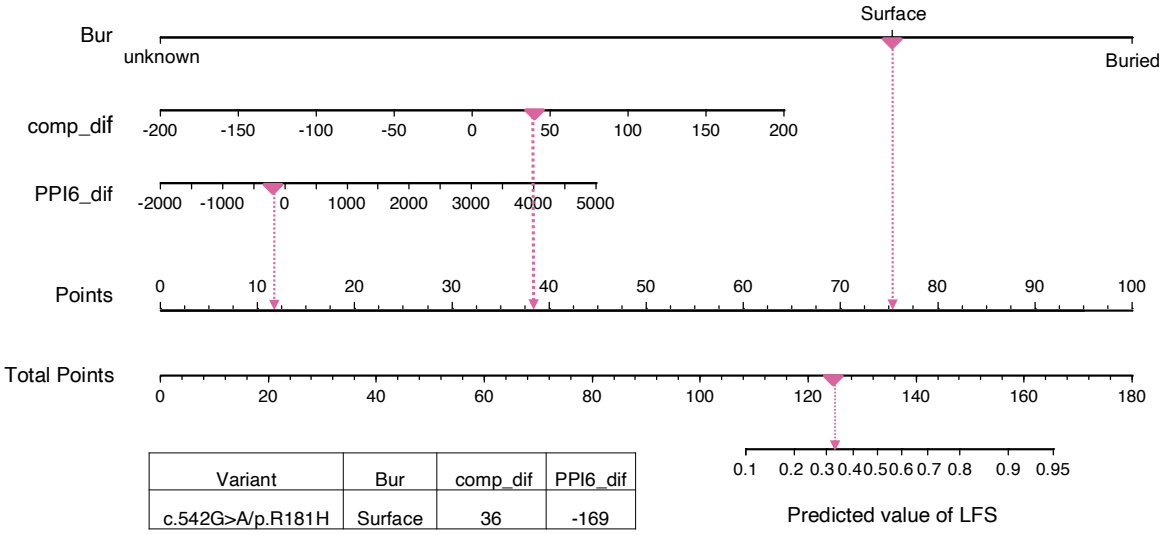

Figure 9. The nomogram for predicting the risk rate of LFS outcome. Three variables a) Bur, b) comp_dif, and c) PPI6_dif were used to estimate the risk of the phenotypic outcome of *TP53* missense variants. The variables for the potential Swedish founder variant c.542G>A/p.R181H were a) Surface residue, b) 36 and c) -169. Vertical lines were drawn from each variable axis to the 'Points' axis to get the corresponding value of 76, 38, and 12, respectively. The summated result in 'Total points' resulted in 126 that corresponds to a phenotypic LFS risk rate of 0.33 *i.e.* more likely to be associated with an HBC phenotype that was in agreement with the results in **Paper II**.

Several attempts have been made to explore genotype-phenotype correction and stratify the clinical management of LFS (47,101). However, it is still not defendable to stratify surveillance programs for different *TP53*-carriers, and all should be recommended extended surveillance, including whole-body MRI as indicated by the current recommendations (75). However, our prediction tool could be useful in the genetic counseling of families with h*TP53*rc syndrome, especially those with a preferably HBC phenotypic outcome

4.3 PAPER III. Evaluation of the prophylactic use of APR-246 in a mouse model of the Li-Fraumeni syndrome with R172H mutant *Trp53*

In Li-Fraumeni families, around 25% of patients surviving their first cancer diagnosis develop a second primary tumor. Up to 30% of breast cancer patients that have received adjuvant radiotherapy develop a sarcoma in the radiated field if they are germline *TP53*-carriers. Thus, radiotherapy, but also chemotherapy, that is used to cure the first cancer, may increase the risk of secondary tumors in these patients. Therefore, efficient preventive treatment would be of great importance for these tumor-prone families. The aim of this study is to investigate the ability of the mutant TP53-targeting compound APR-246 to delay or even prevent, tumor development in a mouse model of the Li-Fraumeni syndrome with R172H mutant *Trp53*.

We also established cell cultures from lymphoma that these mice developed and found that APR-246 inhibited cell growth and induced apoptosis. A total of 89 homozygous *Trp53*$^{172H/H}$ mice were randomly divided into three groups, *i.e.* 35 in the control group, 31 in the group for single-dose injections with APR-246 (200mg/kg), and the remaining 23 for double injection doses with APR-246 (200mg/kg, twice daily). Tumor development was monitored by daily observation and weekly palpation, and whole-body magnetic resonance imaging (WB-MRI) was performed on a subset of mice. Fifty-seven mice were sacrificed due to cancer, and the most frequent was lymphoma, occurring in 67% of the mice.

In the male cohort, prophylactic treatment with single dose APR-246 resulted in a statistically significant increase in survival by 47 days compared to the mice in the control (p=0.037) (Figure 10). No significant survival benefit of APR-246 was observed in the female cohort. The reason remains unknown, but it should be noted that female mice in the control group showed a large variation in cancer-free survival time.

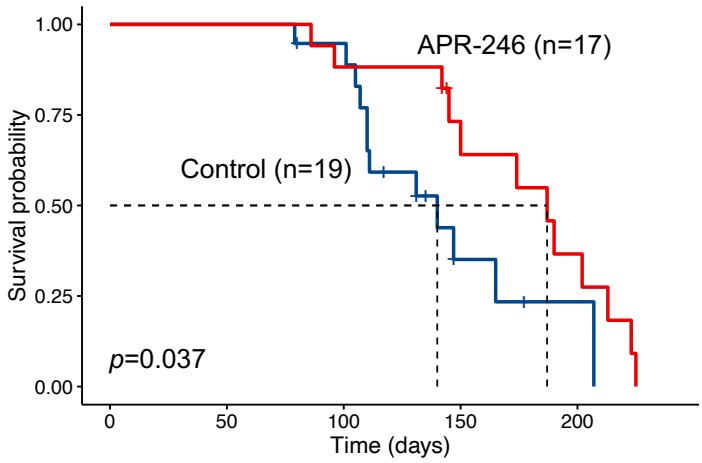

Figure 10. Kaplan-Meier analysis of cancer-free survival of male homozygous *Trp53*$^{172H/H}$ mice in the study. The male mice treated with APR-246 show an increased cancer-free survival with a median of 187 days compared to 140 days (p=0.037). There were 17 male mice in the single dose APR-246 group (red) and 19 male mice in the control group (blue).

One limitation of the present study is that the number of mice in our cohorts is relatively small. This is in part due to the fact that female *Trp53*$^{172H/H}$ mice do not breed well, then we have to utilize female *Trp53*$^{172H/+}$ mice as mothers. Furthermore, optimization of the treatment protocol, such as administration every other week, may be needed to enhance the efficacy of APR-246 in this model. It will also be interesting to evaluate APR-246 in combination with sulfasalazine, an inhibitor of the cystine-glutamate antiporter xCT that has shown robust anti-tumor efficacy in combination with APR-246 in a mouse PDX model (109), or in combination with metformin that has shown a weak but statistically significant effect on tumor onset in homozygous *Trp53*$^{172H/H}$ mice via inhibiting mitochondrial respiration to prevent cancer cells from proliferation (99).

4.4 PAPER IV. Whole exome sequencing of germline variants in non-*BRCA* families with hereditary breast cancer

Breast cancer is the most prevalent malignancy among women around the world, and hereditary breast cancer accounts for about 5%-10%. *BRCA1* and *BRCA2* are the most prevalent predisposition genes for HBC. In the majority of families, however, no breast cancer-related gene has been identified in spite of a remarkable family history of breast cancer. This aim of the study was to identify new predisposing genes for hereditary breast cancer by using whole-exome sequencing (WES).

Seventeen families with a striking pedigree of HBC, that were counseled through the clinical pipeline, and were no disease-causing gene had been identified in spite of an extended genetic screening within the Swedish *BRCA1/2* Extended Analysis (SWEA) study that included screening with a panel of 64 breast cancer-associated genes, were included in our WES-study. We were able to collect blood samples from patients (and from healthy individuals partly) from at least two generations in only three families, and they were therefore included for further analysis.

Among all the analysed individuals, both healthy and affected, 2,122 exonic variants with maximum minor allele frequency (MMAF) < 0.1% were identified. Considering the presence of incomplete penetrance in most hereditary cancer syndromes, we performed two filtering strategies in each family. Twenty-four variants in the family 1, characterized by three generations of bilateral breast cancer, 17 in the family 2 with two generations of breast cancer and lung cancer, and 35 in the family 3 with early-onset male breast cancer and renal cell cancer were found to be associated with disease by using combined annotation dependent depletion score >20.

Four potential breast cancer-predisposing genes (*UBASH3A*, *MYH13*, *UTP11L,* and *PAX7*) were further validated through transfection and ectopic expression of wild-type and mutated constructs in MDA-MB-231 and MCF-7 breast cancer cell lines followed by protein expression analysis, Western blot analyses and/or mass spectrometry profiling, however, no effects of cancer-related pathways were observed. We found 17-35 potential high-risk genes contributing to breast cancer in each family, but we could not identify any disease-causing genes with certainty. A series of low and moderate-risk variants may contribute to breast cancer susceptibility in these families instead of only one variant. Whole-exome sequencing may miss some critical intronic variants affecting splicing, therefore whole-genome sequencing could be performed to improve the detection of potential intronic variants associated with breast cancer in these families.

5 CONCLUSIONS

Paper I: This study presented a quantitative model to predict phenotypic outcome in terms of LFS or HBC according to the effects of germline *TP53* missense variants on protein conformation in an attempt to provide a valuable tool for genetic counseling.

Paper II: This study described the clinical characterization of all known *TP53*-carriers in heritable *TP53*-related cancer syndrome families in Sweden. The *TP53* missense variant c.542G>A/p.R181H was identified as a potential Swedish founder variant mainly associated with an HBC phenotype. Our calibrated phenotypic prediction model correctly predicted the phenotype in more than two-thirds of the families.

Paper III: This study presented preliminary indications that APR-246 may delay tumor onset in a mouse model with a germline *Trp53* pathogenic variant.

Paper IV: This study identified several potential high-risk genes contributing to breast cancer in three hereditary breast cancer families. A series of low- and moderate-risk variants may contribute to breast cancer susceptibility in these families.

6 POINTS OF PERSPECTIVE

Germline pathogenic *TP53* variants are associated with a broad spectrum of hereditary cancers characterized from LFS to HBC outcomes, known as heritable *TP53*-related cancer syndrome. **Paper I** explored the relationship between germline *TP53* missense variants and their phenotypic impact in terms of LFS and HBC based on conformational characteristics of the TP53 protein and presented a quantitative model to predict the phenotypic outcome in carriers of missense *TP53* variants. **Paper II** summarized the clinical characterization of all known *TP53*-carriers in Sweden and evaluated the genotype-phenotype correlation. We also identified the missense variant c.542G>A/p.R181H as a potential Swedish founder variant. In addition, our previously published model in **Paper I** was validated and correctly predicted the phenotypic outcome in more than two-thirds of the families and could potentially be helpful in the genetic counseling of families with h*TP53*rc syndrome.

The psychological burden associated with genetic testing and participation in surveillance programs may decrease if patients could be informed about the lower risk of LFS in relation to HBC. However, the knowledge of penetrance prediction factors is still limited and a substantial overlap of genotype-phenotype remains. It is therefore too early to individualize and stratify surveillance. All *TP53*-carriers should still be offered the same surveillance recommendations. Long follow-up time for *TP53*-carriers and prospective studies on our prediction model could potentially provide more evidence for future stratifying surveillance. **Paper III** investigated the ability of the mutant TP53-targeting compound APR-246 to delay tumor development in a mouse model of the Li-Fraumeni syndrome with R172H mutant *Trp53*. This may open possibilities for a clinical study of APR-246 in a tumor preventive setting for germline *TP53*-carriers.

However, a larger study cohort is needed to obtain more reliable survival data. The optimization of the treatment protocol, such as administration every other week, may be required to enhance the efficacy of APR-246 in this model. It will also be interesting to evaluate APR-246 in combination with sulfasalazine or metformin in this model. Hereditary breast cancer accounts for about 5%-10% of breast cancer. Except for known breast cancer-associated genes, **Paper IV** identified new predisposing genes for hereditary breast cancer by using whole-exome sequencing and explored the functional effects of these genes. However, we were not able to identify any disease-causing genes with certainty. A series of low- and moderate-risk variants may contribute the breast cancer susceptibility in these families instead of only one high risk variant. Whole-genome sequencing would be a further appropriate approach to enhance the chance of identifying disease-causing intronic variants or structural aberrations. A larger cohort would also improve such a study.

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