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# *ATM* mutations in familial pancreatic cancer

# ATM Mutationen bei familiärem Pankreaskarzinom

Inaugural-Dissertation

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## List of abbreviations

| | |
|---|---|
| APC | Adenomatous-Polyposis-Coli |
| AT | Ataxia Telangiectasia |
| ATM | Ataxia Telangiectasia Mutated (gene) |
| BMT | Bone Marrow Transplantation |
| CF | Cystic Fibrosis |
| CFTR | Cystic Fibrosis Transmembrane Regulator |
| CNV | Copy Number Variations |
| FAP | Familial Adenomatous Polyposis |
| FaPaCa | National case collection for **Fa**milial **Pa**ncreatic **Ca**ncer in Germany |
| FPC | Familial Pancreatic Cancer |
| GWAS | Genome-wide Association Studies |
| HBOC | Hereditary Breast-Ovarian Cancer |
| HGMD | Human Gene Mutation Database |
| HP | Hereditary Pancreatitis |
| IPMN | Intraductal Papillary Mucinous Neoplasm |
| INDEL | INsertion/DELetion |
| LFS | Li-Fraumeni Syndrome |
| LS | Lynch Syndrome |
| MCN | Mucinous Cystic Neoplasm |
| MLPA | Multiplex Ligation-dependent Probe Amplification |
| NCBI | National Center for Biotechnology Information |
| NR | Non-Redundant |
| PanIN | Pancreatic Intraepithelial Neoplasia |
| PC | Pancreatic Cancer |
| PCMS | Pancreatic Cancer Melanoma Syndrome |
| PCR | Polymerase Chain Reaction |
| PDAC | Pancreatic Ductal Adenocarcinoma |
| PJ | Peutz-Jeghers Syndrome |
| PNET | Pancreatic Neuroendocrine Tumour |
| PROVEAN | Protein Variation Effect Analyzer |
| PSI-BLAST | Position-Specific Iterative Basic Local Alignment Search Tool |
| PTV | Premature Truncating Variants |
| SIFT | Sorting Intolerant From Tolerant |
| SNP | Single Nucleotide Polymorphism |
| WGS | Whole Genome Sequencing |

# 1 Introduction

## *1.1 Epidemiology*

Cancer-related death is increasing in its importance in the developed world. In 2017, the 'Statistisches Bundesamt' documented 932,263 disease-related cases of death in Germany, 24.4% being the lethal result of malignant neoplasms. Only cardiovascular diseases surpassed this with more disease-related deaths (38,5%) (Statistisches Bundesamt 2020).

The 'Top 5' locations of these lethal malignancies in Germany in 2017 were lung/bronchia (19.1%), mammary gland (7.9%), pancreas (7.6%), colon (6.7%) and prostate (male: 11.0%). Pancreatic neoplasms present with a low prevalence, but a deflating relative 5-year survival rate of 9%, which is by far the lowest rate of all cancers (Statistisches Bundesamt 2020). By 2030, pancreatic ductal adenocarcinoma (PDAC) is expected to become the second leading cause of cancer-related death, after non-small cell lung carcinoma (Valle et al. 2018).

Up to 10% of PDAC cases have a hereditary background including **familial pancreatic cancer** (FPC), **hereditary tumour predisposition syndromes** and **tumour syndromes with chronic inflammation dysfunction of the pancreas** (Bartsch, Gress, and Langer 2012). Members of FPC families have an at least 5- to 10-fold increased risk of PC, but the causative germline mutations have been identified in only 10% to 15% of the FPC families (Slater et al. 2014).

## *1.2 Pancreatic Ductal Adenocarcinoma (PDAC)*

### 1.2.1 Anatomy

The pancreas is a combined exocrine and endocrine gland, located secondary retroperitoneal in the epigastric abdominal area, near to the upper lumbar vertebrae. The pancreas is macroscopically classified into head, neck, body and tail.

The exocrine part of the pancreas, consisting of pyramidal acinar cells surrounded by connective tissue, producing an all serous digestion-secretion, dominates with over 90% of the volume of the gland. Vagus nerve stimulation or cholecystokinin secretion by enteroendocrine cells of duodenum and jejunum result in the secretion of the pancreatic juice, which contains several proteatic proenzymes (Trypsin, Chymotrypsin, Carboxypeptidase A and B) and Phospholipase A. The pancreatic juice enzymes are needed for digestion of proteins, lipids and carbohydrates in the small intestine. The secretion flows through the intercalated duct into the intra- and interlobular excretory duct. These end at the duct of Wirsung (main pancreatic duct) where the secretion from the pancreatic tail and the pancreatic neck flows through the ampulla of Vater (hepatopancreatic ampulla) from the lower part of pancreatic head finally through the major papilla into the duodenum. The upper part of the pancreatic head may drain the produced pancreatic juice through the accessory duct of Santorini and the minor papilla into the duodenum.

The ductal epithelial layer of the proximal intralobular duct consists of cuboidal cells developing into columnar cells as they approach the distal interlobular duct. Furthermore, there are some isolated mucus-producing goblet cells and enterochromaffin cells found in between the epithelial cells. The intralobular duct epithelial cells have a highly concentrated cytosolic carboanhydrase and, basolaterally, a very active Na/K-ATPase which allows these cells to secrete bicarbonate in response to the hormone secretin from the small intestinal mucosa. The ductal epithelial cells may have apical microvilli and cilia that aid in fluid flow.

Thus, the pancreatic ductal system produces an alkaline fluid, containing bicarbonate and mucus, that transport the acinar cell pro-enzymes into the

duodenum where the acidic gastric juice can be neutralized and the inactive enzymes can be activated (Zilles and Tillmann 2010).

The endocrine part of pancreas consists of about 1-2 million isolated cell aggregations, called islets of Langerhans. Each islet is 100-200 µm in diameter and contains about 2,000 to 3,000 cells producing different hormones (Zilles and Tillmann 2010):

- 70 % B-cells producing insulin
- 20 % A-cells producing glucagon
- 5 % D-cells producing somatostatin
- < 5 % PP-cells producing the pancreatic polypeptide.

Tumours developing from the endocrine part of pancreas are called Pancreatic Neuroendocrine Tumours (PNETs), but they are relatively rare in comparison to PDAC (Lee, Kim, and Kim 2017).

### 1.2.2 Aetiology and risk factors

The majority of pancreatic neoplasms are localized to the pancreatic head (80%) (Siewert, Rothmund, and Schumpelick 2010). The principal histologic subtype of pancreatic cancer is ductal adenocarcinoma, the second most prevalent subtype, but even much lower, are pancreatic neuroendocrine tumours. PDAC is described as a disease with a higher incidence in the western and industrial world due to life style, but there is also a difference in the incidence resulting from geographical and ethnic origin (Adler et al. 2007).

The most important factor for predisposition is age, as PDAC is diagnosed with an average diagnose-age of 67 years for male and accordingly 74 for female patients (Seufferlein and Adler 2009). Additional risk factors for PDAC include diabetes mellitus, chronic pancreatitis, obesity, a family history of pancreatic cancer and a history of smoking or alcohol consumption (Kamata et al. 2017).

### 1.2.3 Carcinogenesis

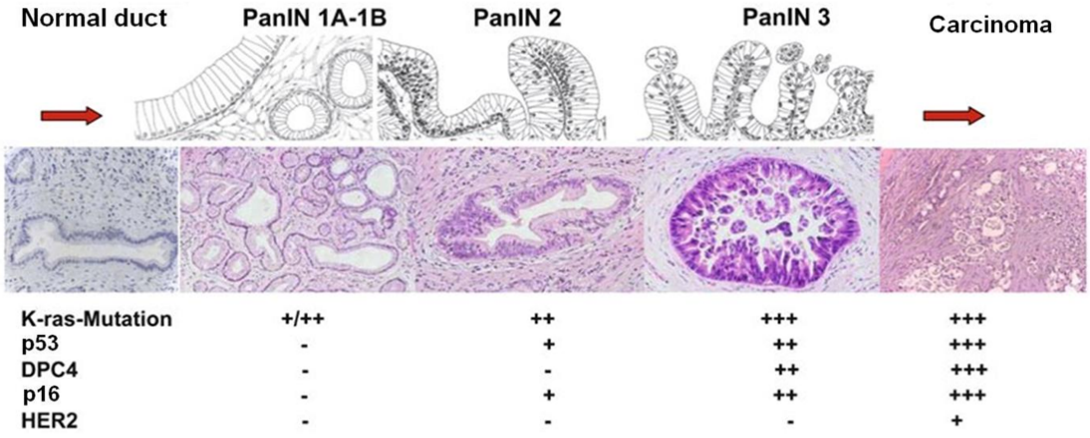
PDAC, the most common type of pancreatic cancer, originates in cells of the exocrine pancreas through a malignant transformation (Seufferlein and Adler

2009). The ductal differentiation of the carcinoma led to the theory that the carcinoma develops from terminally differentiated ductal cells as opposed to undifferentiated pancreatic stem-cells (Valle et al. 2018).

Similar to other epithelial carcinomas, PDAC develops from precursor lesions, called pancreatic intraepithelial neoplasia (PanIN). Intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN) can also develop into PDAC in rare cases (Brand et al. 2007). The PanIN classification divides lesions into benign neoplasia (PanIN 1), papillary lesions with low grade dysplasia (PanIN 2) and non-invasive carcinoma (PanIN 3/ Carcinoma in situ) with high grade dysplasia.

This histological classification is accompanied by typical mutations: ***K-ras*** often mutates early and remains in a permanently active state, mostly due to a missense point mutation, which is estimated to be an initial event of pancreatic cancerogenesis. Another important event is the inactivation of the tumour suppressor-gene, ***CDKN2***, encoding the INK-protein p16 that is important for cell-cycle regulation in the G1-phase. Inactivation of the tumour suppressor **p53**, important for initiation of DNA-repair and apoptosis, is also a very important and frequently detected event in carcinogenesis (Siewert, Rothmund, and Schumpelick 2010).

**Figure 1: Model of tumour progression: Adenoma-carcinoma sequence, with PanIN classification and several mutated genes**




Source: Siewert, Rothmund, and Schumpelick 2010

### 1.2.4 Hereditary cancer syndromes with increased PDAC risk

**Hereditary breast-ovarian cancer** (HBOC) **syndrome** is thought to be caused mainly by mutations in ***BRCA2*** and ***BRCA1*** genes, especially when the cancer is diagnosed at a young age (Ford et al. 1998). The PDAC risk of HBOC patients with *BRCA1* germline mutations is controversial, BRCA2 mutations are described with an increased relative risk in the range of 2.3 to 7 (Carrera et al. 2017). ***PALB2*** germline mutations also go along with a 5- to 9-times increased risk of breast cancer (Antoniou et al. 2014), truncating variants are also detected in PDAC patients, assuming to be causing there pancreatic carcinoma (Slater, Langer, Niemczyk, et al. 2010).

**Pancreatic cancer melanoma syndrome** (PCMS; Familial atypical multiple mole melanoma, FAMMM) is caused mainly by ***CDKN2A*** germline mutations, although other genes such as ***CDK4*** and ***BAP1*** have been associated with this syndrome (Carrera et al. 2017). The PDAC risk rises up to 17% (at p16-Leiden mutation: 19bp deletion in exon 2 of CDKN2A) depending on the pathogenetic variant of the *CDKN2A* mutation (Vasen et al. 2000).

**Peutz-Jeghers syndrome** (PJ) results from mutations in the ***STK11/LKB1*** gene. Phenotypically, it is characterized by mucocutaneous pigmentation, and pathognomonic intestinal hamartomatous polyps. PJ-patients have a cumulative lifetime risk for PC of 11% and there is also an increased risk for cancers of the colon, stomach, small intestine and breast (Carrera et al. 2017).

**Familial adenomatous polyposis** (FAP) is an autosomal dominant entity characterized by hundreds to thousands of adenomas throughout the colon. FAP patients have a risk of developing colorectal carcinoma by the fourth decade of life that is nearly 100% (Waller, Findeis, and Lee 2016). FAP syndrome is caused by mutations in the ***APC*** (adenomatous-polyposis-coli) gene. Mutations in this tumour suppressor gene result in a PDAC relative risk that is 4.5 times higher than for the general population (Moussata et al. 2015).

**Lynch syndrome** (LS, Hereditary non polyposis colorectal cancer) develops due to germline mutations in mismatch repair system genes (***MLH1***, ***MSH2***, ***MSH6***, ***PMS2***) and it represents the most common cause of hereditary colorectal cancer (Carrera et al. 2017). There is a 9-fold increased PDAC risk for mutation carriers (Kastrinos et al. 2009).

**Li-Fraumeni syndrome** (LFS) is an autosomal dominant cancer predisposition condition characterized by the development of a wide spectrum of malignancies (most frequently breast cancer, sarcomas, brain tumours and leukemia). LFS is caused by germline mutations in ***p53*** gene (Carrera et al. 2017). It is estimated that approximately 50% of the individuals with LFS will develop cancer by the age of 30 years (Correa 2016). The relative risk for developing PDAC is increased nearly 7-fold (Ruijs et al. 2010).

### 1.2.5 Hereditary pancreatic dysfunction syndromes with increased PDAC risk

**Hereditary pancreatitis** (HP) is an extremely rare diagnosis and comes with a 50- to 70-fold increased relative risk for PDAC as compared to the general population (Howes, Lerch, et al. 2004). Approximately 80% of HP patients host pathogenic variants in ***PRSS1*** gene (encoding cationic trypsinogen) that leads to an activation of trypsinogen inside the pancreas with auto digestion of the gland, thus causing an inflammation (Howes, Greenhalf, et al. 2004). ***SPINK1*** (serine peptidase inhibitor) gene encodes a trypsin inhibitor that is secreted by the pancreatic acinar cells and mutations in *SPINK1* are also associated with an increased HP risk (Carrera et al. 2017).

**Cystic fibrosis** (CF) is caused by mutations in the cystic fibrosis transmembrane regulator (***CFTR***) gene, that disrupt the localization and function of the cAMP-mediated chloride channel, resulting in an obstruction of pancreatic ducts by mucus secretions (Fendrich, Langer, and Bartsch 2014). It is estimated that 1.5% of all CF patients will suffer pancreatitis, potentially increasing the risk of PDAC development (Carrera et al. 2017).

## *1.3 Familial pancreatic cancer (FPC) syndrome*

### 1.3.1 Definition

FPC syndrome describes an established entity of inherited PDAC (Zhen et al. 2015). Families with FPC have two or more first-degree relatives (sibling-sibling or parent-child) with PDAC (Bartsch et al. 2004) and do not fulfil the criteria for another inherited syndrome (Slater, Langer, Niemczyk, et al. 2010). FPC is a rare but established inherited tumour predisposition syndrome (Zhen et al. 2015). Members of FPC families carry a 2.3- to 32-fold increased PDAC risk, depending upon the number of affected family members (Klein et al. 2004).

In the following, the term ‘hereditary pancreatic cancer’ will be used to refer to a familial accumulation of pancreatic cancer. The genetically background therefore may be based on the above defined FPC syndrome, other hereditary tumour syndromes or other hereditary dysfunction syndromes of the pancreas, both not fulfilling the definition of FPC syndrome.

### 1.3.2 ‘Nationale Fallsammlung Familiäres Pankreaskarzinom der Deutschen Krebshilfe’ – FaPaCa

The ‘FaPaCa’ case registry for FPC and PCMS patients, as well as their relatives, was established in 1999 at the Universitätsklinikum Marburg/ Philipps-Universität Marburg, Germany (Bartsch et al. 2001). FaPaCa collects and evaluates the phenotype of FPC families, perform a board-approved clinical screening program for the early detection of PDAC and its high grade precursors and aims to identify additional FPC susceptibility genes.

For a family to be included in the study, there must be at least two first-degree relatives with PC (FPC), or one PC and one melanoma in the family (PCMS), without fulfilling the criteria for other hereditary tumour predispositions syndromes such as HBOC, according to the FPC definition in section 1.3.1. Three PDAC patients in one family, independent of the relationships, or a detected *BRCA1/2* mutation with at least one PDAC in the family, also qualifies for enrollment in the FaPaCa registry.

The FaPaCa registry contains over 200 families with over 500 affected persons. The known pathogenic variants in some families include the genes *BRCA2, BRCA1, PALB2, CDKN2A* and *CHEK2*.

High risk relatives are offered annual screening in a controlled study in FPC centres starting at an age ten years earlier than the youngest PDAC diagnosis in the family or at 40 years of age (Langer et al. 2009) (Bartsch et al. 2016).

### 1.3.3 Susceptibility genes

Five genes have yet been established as low-penetrance FPC susceptibility genes. These include:

- ***BRCA1*** (Al-Sukhni et al. 2008)
- ***BRCA2*** (Murphy et al. 2002) (Hahn et al. 2003)
- ***CDKN2A*** (Slater, Langer, Fendrich, et al. 2010) (Zhen et al. 2015)
- ***PALB2*** (Slater, Langer, Niemczyk, et al. 2010)
- ***CHEK2*** (Bartsch et al. 2006)

Few other genes have been reported to be potential FPC predisposition genes:

The Pancreatic Cancer Genetic Epidemiology (PACGENE) Study (ClinicalTrials.gov Identifier: NCT00526578) described a prevalence of 8% of all pathogenic variants among the 521 included FPC patients in previously identified FPC susceptibility genes (*BRCA1*, 1.2%; *BRCA2*, 3.7%; *PALB2*, 0.6% and *CDKN2A* 2.5%) (Zhen et al. 2015).

A whole genome sequencing project from Roberts et al., using germline DNA of 638 FPC patients from 593 families, analysed 87 genes that included all susceptibility genes for hereditary pancreatic cancer (Roberts et al. 2016). The previously reported FPC susceptibility genes frequently contained deleterious mutations, which underscores their importance. Furthermore, they detected a large number of private heterozygous Premature Truncating Variants (PTV), whose role in the heterogenic origin of FPC needs to be confirmed (Roberts et al. 2016). As new candidate genes are named *ATM, BUB1B, CPA1, FANCC, FANCG, POLN, POLQ* and *APC*, which contain more deleterious variants in

FPC patients than in controls. The frequency of detected mutations in the known genes associated with hereditary pancreatic cancer is difficult because patients with known deleterious variants in the common FPC susceptibility genes *BRCA1, BRCA2, CDKN2A* and *PALB2* were excluded to maximize the opportunity to discover novel susceptibility genes (Roberts et al. 2016).

In 2018, Chaffee et al. published their results of sequencing from 25 cancer susceptibility genes in lymphocyte DNA from 185 FPC patients (Chaffee et al. 2018). Of these 185 patients, 25 (14%) carried deleterious mutations in genes known to be associated with PDAC: *BRCA2* 4.3%, *BRCA1* 1.1%, *CDKN2A* 2.2%, *ATM* 3.2%, *PALB2* 0.5%, *MSH* 0.5%, *PMS2* 0.5%, *CHEK2* 0.5% and in genes not known to be associated with PDAC: *BARD1* 0.5%, *NBN* 0.5%) (Chaffee et al. 2018).

The results from a Japanese study are quite similar to those of the Western countries. They published the findings of eight deleterious mutations found in 54 FPC patients (14.5%): *BRCA2* (3 mutations), *PALB2* (2), *ATM* (2) and *MLH1* (1).

*MLH1, MSH2, MSH6* (all known from HNPCC), *PMS2* (HNPCC), *PRSS1* (HP), *STK11* (PJS), and *p53* (LFS) are also named as possible PC susceptibility genes (Roberts et al. 2016), but only analysed in patients with pancreatic cancer based on hereditary tumour syndromes, not fitting to the FPC definition.

The genetic basis underlying disease susceptibility in the remaining 80 – 90% of FPC patients is unknown (Roberts et al. 2016).

Identifying further FPC susceptibility genes that are responsible for the inheritance of increased PDAC risk is important for many reasons: screening of family members carrying the mutation(s) might allow the detection of suspect pancreatic neoplasms in an earlier state when preventive resection of precursor lesions or therapeutic options with curative potential are still available (Bartsch et al. 2016). A newly published multi-analytic blood test offers the possibility of early detection of the rapidly growing PC (Cohen et al. 2018). This test combines protein biomarkers with genetic biomarkers and is able to detect the presence of some common solid tumour types (e.g. PDAC). Unfortunately, it was only tested on diagnosed tumour patients and cannot be safely applied for the detection of the less severe and potentially rapidly

growing precursor lesions like PanIN 2-3 (Cohen et al. 2018). Cohen recommends using additional cancer biomarkers, such as metabolites, mRNA transcripts, miRNAs, or methylated DNA sequences to increase sensitivity and localization of cancer site. Knowing FPC susceptibility genes is important, because FPC susceptibility genes also increase risk for extra pancreatic neoplasm, which then might be screened as well (Wang et al. 2009). In addition, in some FPC families PDAC develops earlier in subsequent generations because of the effect of anticipation (McFaul et al. 2006). Furthermore, the knowledge of the existing mutation might also lead to a personalized therapy, as described below in section 1.4.6.

Genetic susceptibility also plays an important role in sporadic PDAC risk. Genome-wide association studies (GWAS) with over 10,000 PC patients have been performed with the aim of identifying additional common pancreatic cancer risk loci in western and Asian populations (Klein et al. 2018). Because of the high number of analysed PC patients and the strong association of mutations to PC cases, the newly identified genes (*NOCL2, TNS3, HNF4G, HNF1B* and *GRP*) that seem to increase PDAC risk, might also be analysed in FPC patients.

## *1.4 Ataxia telangiectasia mutated (ATM) gene*

### 1.4.1 *ATM* as a target gene

The *ATM* gene does not actually belong to the routinely tested susceptibility genes in FPC patients of the 'FaPaCa' case registry. However, Roberts published a role of pathogenic variants in *ATM* gene as possibly a new FPC susceptibility gene (Roberts et al. 2012, Roberts et al. 2016). Roberts described the analysis of *ATM* mutations in 166 FPC subjects (Roberts et al. 2012). Considering only the more severely affected families with more than 3 FPC patients per family, the rate of deleterious variants in *ATM* gene in FPC patients even increases from 2.4% (4/166) up to 4.6% (4/87), underlying the possible importance of these pathogenic variants in increasing the PDAC risk in these families. Besides, the families with *ATM*-mutations detected by

Roberts not only include PC patients, but also malignancies in lung, colon, uterus, prostate and ovaries and lymphoma and malignant melanoma.

The fact that the tumour suppressor *ATM* is known to be a cancer predisposition gene (Rahman 2014) raises the question if analysing the *ATM* gene in all 'FaPaCa' patients would detect the genetic background for the increased PDAC-risk in some 'FaPaCa' case registry families.

### 1.4.2 Human *ATM* gene

The human *ATM* gene (NCBI Reference Sequence for gDNA: NC_000011.10) is made up of 66 exons, of these 62 protein coding (Uziel et al. 1996), is located on chromosome 11 q22-q23 (UCSC Genome Browser 2016) and spans 150 kb of genomic DNA. The gene encodes a 350 kDa kinase, consisting of 3065 amino acids (Weizmann Institute of Science 2018). The mRNA sequence NM_000051.3 is the reference standard in the NCBI Reference sequence (RefSeq) project, containing of 13147nt with the coding sequence from 386-9556, describing the full length protein as it has been used for our analyses. The ATG start site is located in exon 2 (386- 388) and the stop codon is located in exon 63 (9554-9556).

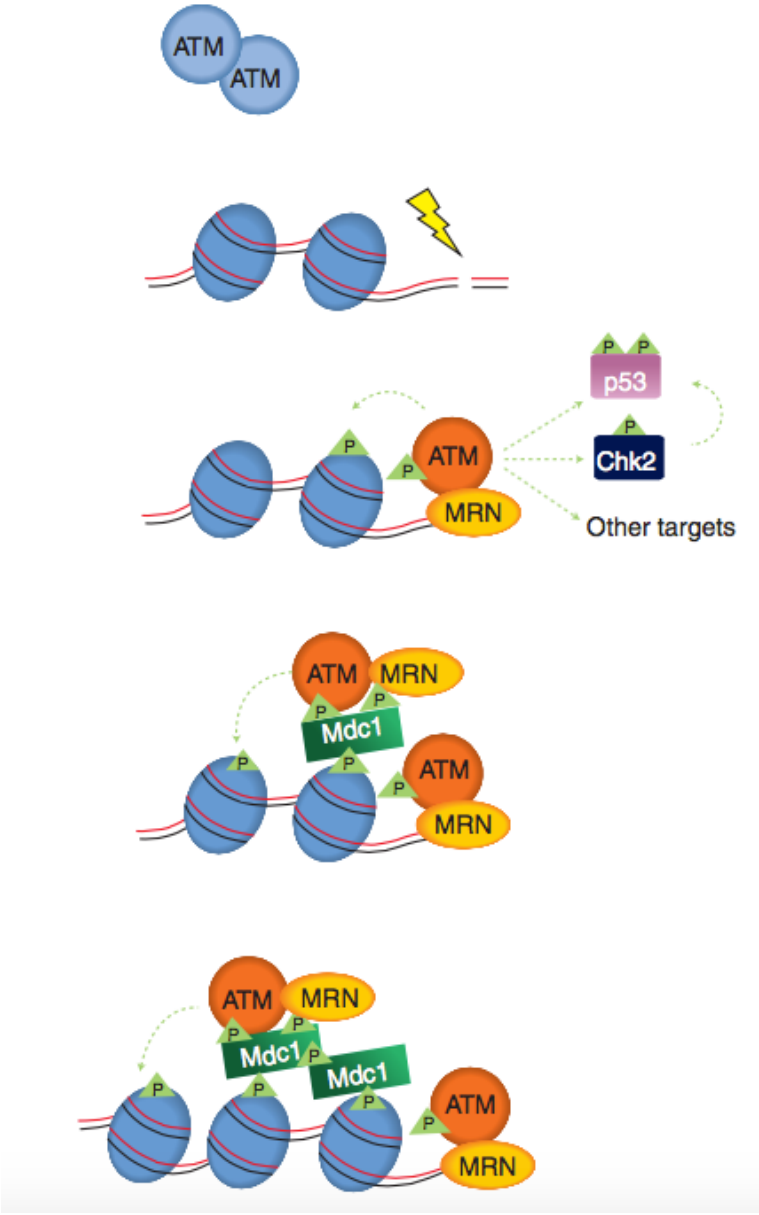
The *ATM* gene is named after Ataxia telangiectasia (AT), also called Louis-Bar syndrome, an inherited neurological disease, first described and named in 1941 (Louis-Bar 1941). In 1995, Savitsky detected the gene that harbours deleterious germline mutations in cases of AT (Savitsky et al. 1995).

### 1.4.3 ATM kinase

The protein encoded by the *ATM* gene is a serine/threonine-kinase, belonging to the PI3/PI4-kinase-like kinase (PIKK) family and primarily located in the nucleus (Tripathi et al. 2016). ATM kinase plays an important role in DNA double-strand-break (DSB) repair and replication stress management (Maréchal and Zou 2013). DSBs represent the most deleterious form of DNA lesions that can arise spontaneously during DNA replication or as a response to certain exogenous hazards such as ionizing radiation (Larsen and Stucki 2016).

ATM kinase phosphorylates proteins that contain Serine (S) or Threonine (T) residues that are followed by Glutamine (Q) (SQ or TQ motifs) (Awasthi, Foiani, and Kumar 2016).

**Figure 2: ATM activation at DSBs**



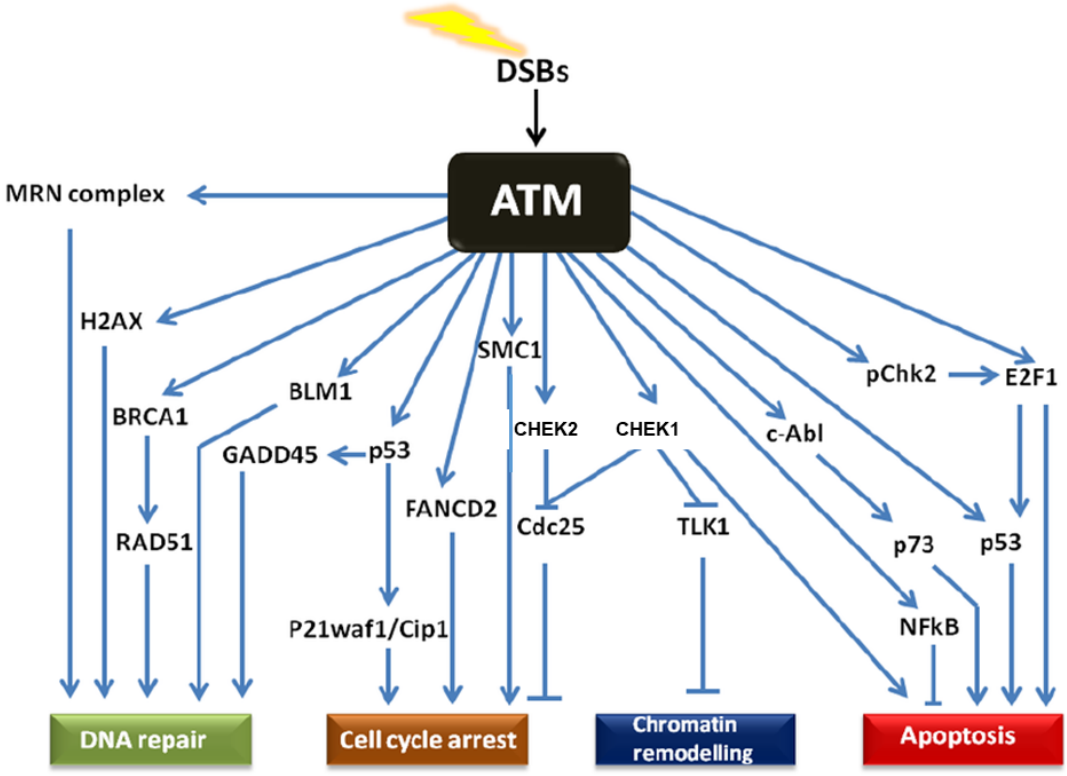

Source: Maréchal and Zou 2013

Awasthi et al. (Awasthi, Foiani, and Kumar 2016) reviewed ATM activation in 2016 and described an inactive ATM existing in the form of a homodimer or multimer. Awasthi reports about a MRE11– RAD50–NBS1 (MRN) complex acting as a sensor for DSBs and binding the dsDNA at a detected DSB. ATM activation then becomes stimulated by MRN complex by ATM binding MRN.

This contact leads to ATM auto-phosphorylation resulting in the simultaneous dissociation of the ATM homodimers. Research on the detailed molecular mechanism is still in progress. The ATM monomers are catalytically active that leads to a widespread phosphorylation of downstream-acting proteins which is the main part of activated ATM (Khalil et al. 2012).

One of the key processes is the phosphorylation of the histone variant H2AX in the DSB flanking region by ATM, leading to an mdc1-mediated chromatin relaxation and binding from several DSB repair complexes (Polo et al. 2010). Furthermore, ATM phosphorylates checkpoint kinase 2 (CHEK2, Checkpoint arrest in G1 phase), p53 and many others substrates, which is required for accumulation of numerous DNA repair proteins and chromatin-remodelling complexes around the occurred DSB (Maréchal and Zou 2013), cell cycle arrest, chromatin remodelling or apoptosis (Khalil et al. 2012).

**Figure 3: ATM signalling pathway**




Source: adapted to Khalil et al. 2012

Thus, the absence of the ATM protein leads to a failure in the signalling network responding to DSBs and other types of genotoxic stress. The resulting genomic instability can cause the development of cancers (Shiloh 2003).

### 1.4.4 Ataxia telangiectasia/ Louis-Bar-Syndrome

Ataxia telangiectasia (AT) is a rare autosomal recessive disorder with a world-wide prevalence between 1/40,000 and 1/100,000 live births. AT is often referred to as a DNA damage response syndrome where the main characteristics are **progressive cerebellar degeneration** (ataxia, postural instability) without mental retardation, **oculomotor apraxia**, **telangiectasia** (on bulbar conjunctiva and sclera), **hypersensitivity to ionizing radiation**, **immunodeficiency** (frequent sinopulmonary infections) and **cancer susceptibility** (particularly of lymphoid organs). The syndrome displays great variability in the severity of its symptoms and the age of their manifestation - the onset is most often in early childhood, but some neurological features may arise later (Rothblum-Oviatt et al. 2016).

AT is usually diagnosed based on clinical appearance, supported by specific laboratory abnormalities (e.g. IgA deficiency, lymphopenia especially affecting T-lymphocytes and increased alpha-fetoprotein levels). Furthermore the AT diagnosis can be confirmed by identification of bi-allelic deleterious mutations in the *ATM* gene or finding an absence or deficiency of ATM protein or its kinase activity in cultured cell lines (Rothblum-Oviatt et al. 2016).

AT patients show severe bi-allelic mutations in *ATM* gene, commonly they have compounded heterozygous mutations (Hassin-Baer et al. 1999). There is no area of the *ATM* gene especially susceptible to mutations; nonsense mutations, frame shift mutations caused by insertions and deletions, but also missense and leaky splice-site mutations have been described all over the gene (Telatar et al. 1998).

AT patients have an increased incidence (about 25% lifetime risk) of cancers (Rothblum-Oviatt et al. 2016), most often **lymphoma**, **leukemia**, and **breast cancer** (Reiman et al. 2011). A variety of other solid tumours including liver,

gastric and oesophageal carcinomas known from unpublished observations are also described by Rothblum-Oviatt et al. (Rothblum-Oviatt et al. 2016).

### 1.4.5 Diseases with increased prevalence at *ATM* germline mutations

Relatives of AT patients, who have a heterozygous mutation in the *ATM* gene, are generally healthy (Rothblum-Oviatt et al. 2016). The reported mono-allelic carrier frequency of pathogenic *ATM* variants in the population is relatively common with 0.5 - 1% (Taylor and Byrd 2005) or 1.4 - 2.2 % (Khalil et al. 2012).

There is an increased life time risk for heterozygous ATM mutation carriers to develop cancer, confirmed for several entities:

- Breast cancer (Buys et al. 2017, Athma, Rappaport, and Swift 1996)
- Leukemia (Bullrich et al. 1999)
- Lymphoma (Oguchi et al. 2003)

However, a meta-analysis published in 2016 could only show that heterozygous carriers of a pathogenic *ATM* mutation have a decreased life expectancy and an increased risk of developing **breast cancer**, **diabetes**, and **cardiovascular** and **neurodegenerative diseases**. The possibility of developing cancer of the gastrointestinal tract could not be verified clearly and needs to be further investigated (van Os et al. 2016).

### 1.4.6 Treating *ATM* mutated cancer with targeted therapy

Thus, deleterious *ATM* mutations decrease the ATM kinase functionality, which leads to genomic instability through loss of DSB DNA repair. Stalled replication forks or unrepaired single-strand breaks (SSB) convert into DSBs that cannot be repaired. Based on this rationale, inhibition of other kinases involved in SSB DNA repair has been considered a potential mechanism for synthetic lethality in cancers hosting deleterious *ATM* mutations (Choi, Kipps, and Kurzrock 2016).

Poly(ADP-Ribose)polymerase-1 (PARP-1) is involved in repair of single-stranded DNA breaks. When these breaks are encountered during DNA replication, the replication fork stalls, and double-strand DNA (dsDNA) breaks accumulate (Schultz et al. 2003). A study in ATM-deficient lymphoid tumour cells showed an increased sensitivity to **PARP-1 inhibition** with 'Olaparib' (Weston et al. 2010), indicating that PARP-1 inhibitors may be effective in ATM-deficient pancreatic tumours (Bakker and de Winter 2012).

The ATR (ataxia telangiectasia and Rad3-related protein) - CHEK1 (checkpoint kinase 1) pathway is another potential target for therapy in cancers containing deleterious ATM variants, as it is a primary sensor and mediator of SSB DNA repair (Choi, Kipps, and Kurzrock 2016). A synthetic lethal siRNA screen confirmed that mantle cell lymphoma cells with loss of ATM function have increased sensitivity to **ATR inhibition** (VE821, VE822, and AZD6738) (Menezes et al. 2015). CHEK1 is a serine/threonine-kinase and an important downstream substrate which gets phosphorylated by activated ATR. Thus, **CHEK1 inhibitors** might have the same DNA-repair blocking effect in ATM-deficient cancer cells as ATR inhibitors have, which might lead as well to an increased DNA damage and cell death (Choi, Kipps, and Kurzrock 2016).

## *1.5 The aim of the thesis*

It was the aim of this thesis to analyse the ATM gene in germline DNA from German FPC patients of the FaPaCa case registry in order to detect possible pathogenic variants that might be responsible for the increased PDAC risk in some of these FPC families. WGS, Sanger sequencing and MLPA were to identify different mutations and then it was necessary to identify the deleteriousnsess to distinguish pathogenic and non-pathogenic variants. The question finally to be answered was whether or not 'FaPaCa' patients should be screened regularly for pathogenic variants in the ATM gene, as is presently performed with the BRCA2, CDKN2A and PALB2 genes.

# 2 Materials and methods

## *2.1 Materials*

The materials and appliances which were used are listed in the table below.

**Table 1: Appliances**

| Name | Manufacturer |
|---|---|
| Centrifuge 5430 R | Eppendorf, Hamburg, DE |
| Dri Block DB 2A | Techne, Oxford Cambridge, UK |
| Eppendorf Mastercycler ep gradient S Thermal Cycler | Eppendorf, Hamburg, DE |
| Eppendorf Research Plus Pipette; 1000µl,200µl , 100µl, 20µl, 10µl, 2,5µl | Eppendorf, Hamburg, DE |
| Heraeus Biofuge Pico | Thermo Fisher Scientific,Waltham,US |
| Nanodrop Lite | Thermo Fisher Scientific,Waltham,US |

**Table 2: Chemicals and Enzymes**

| Name | Manufacturer |
|---|---|
| Alkaline Phosphatase | New England Biolabs, Frankfurt am Main, DE |
| Ethanol >99.8% | Sigma Aldrich, Steinheim, DE |
| Exonuclease I | New England Biolabs, Frankfurt am Main, DE |
| GeneRuler 50 bp DNA Ladder | Thermo Fisher Scientific, Osterode, DE |
| GeneScan™ 500 LIZ™ dye Size Standard | Thermo Fisher Scientific, Osterode, DE |
| peqGOLD Universal Agarose | Peqlab Biotechnologie GmbH, Erlangen, DE |
| peqGREEN DNA/RNA Dye | Peqlab Biotechnologie GmbH, Erlangen, DE |
| TBE buffer<br>10.8g/L Tris(hydroxymethyl)aminoethane, 99+%<br>5.5g/L Boric acid<br>0.82g/L EDTA | <br>ACROS Organics, Geel, BE<br>Fisher Scientific UK, Leicestershire, UK<br>ACROS Organics, Geel, BE |
| 6x DNA Loading Dye | Thermo Fisher Scientific, Osterode, DE |

**Table 3: Consumables**

| Name | Manufacturer |
|---|---|
| Biosphere Filter Tips 1250µl extra long | Sarstedt, Nürnbrecht, DE |
| Biosphere Filter Tip 200, 100 | Sarstedt, Nürnbrecht, DE |
| Biosphere Filter Tip 2,5 farblos | Sarstedt, Nürnbrecht, DE |
| Multiply-Pro Gefäß 0,2 ml | Sarstedt, Nürnbrecht, DE |
| Reagiergefäß 1,5ml Safe Seal | Sarstedt, Nürnbrecht, DE |
| SafeSeal SurPhob Spitzen, 10µl, extra lang, steril | Biozym, Hessisch Oldendorf, DE |

**Table 4: Ready to use systems (Kits)**

| Name | Manufacturer |
|---|---|
| DNeasy Blood & Tissue Kit (250) | Qiagen, Hilden, DE |
| ReadyMix™ Taq PCR Reaction Mix | Sigma Aldrich, Steinheim, DE |
| SALSA MLPA EK1 reagent kit 100 rxn (6 vials) - FAM | MRC-Holland bv, Amsterdam, NL |
| SALSA MLPA P041 ATM-1 probemix | MRC-Holland bv, Amsterdam, NL |
| SALSA MLPA P042 ATM-2 probemix | MRC-Holland bv, Amsterdam, NL |

**Table 5: *ATM* Exon Sequencing Primer**

| | | | |
|---|---|---|---|
| cDNA | sequence: | NM_000051 | uc001pkb.1 |
| Genomic | sequence: | | hg19_chr11:108092558-108240826 |

| NAME | LEFT PRIMER | NAME | RIGHT PRIMER | PRODUCT SIZE (bp) |
|---|---|---|---|---|
| ATM-E02-03F | CTTTGACCAGAATGTGCCTC | ATM-E02-03R | GCCAAATTCATATGCAAGGC | 585 |
| ATM-E04F | TTTGTGATGGCATGAACAGC | ATM-E04R | CAAACTTATGCAACAGTTAAGTCC | 336 |
| ATM-E05F | CTTGGGGCCATAATTTGC | ATM-E05R | AGACAGAGTGCTTTCTTTGGTG | 387 |
| ATM-E06F | TCTGTATGGGATTATGGAATATTTAAG | ATM-E06R | CTGAGTCTAAAACATGGTCTTGC | 336 |
| ATM-E07F | TCAGCATACCACTTCATAACTGTTC | ATM-E07R | TCAACCAGAGAAATCCAGAGG | 438 |
| ATM-E08F | AATTTTGTGGGAGCTAGCAG | ATM-E08R | TTTTAAAAGCCCAAAATGCC | 314 |
| ATM-E09F | TCCAACCTGGGCAACAAC | ATM-E09R | GGTTGAGATGAAAGGATTCCAC | 340 |
| ATM-E10F | CTATGGAAATGATGGTGATTCTC | ATM-E10R | TGATCAGGGATATGTGAGTGTG | 569 |
| ATM-E11F | CCTTAACTAAATGTATGTGCCAGG | ATM-E11R | GGTTAGATCTGGGAAATTCTGC | 413 |
| ATM-E12F | GGTTGTCCTCCTTAAATTGTCC | ATM-E12R | AAAACAACCTCTTCCCTGGC | 428 |
| ATM-E13F | ATAAGTAGGTCTCAAAGTCCGAAG | ATM-E13R | CTGCCCCTATTTCTCCTTCC | 487 |
| ATM-E14F | TCCAGGATATGCCACCTTTAAC | ATM-E14R | AAAAGAGAAAGGGTTAACCTGC | 381 |
| ATM-E15F | TGCTTATACTGTATGACTACGTGGAAC | ATM-E15R | CTGGAAACCAGGAGGTCAAG | 409 |
| ATM-E16F | GAAGCAGCATATATATTGGCCC | ATM-E16R | CACCTGGCCTTAATTTCCAC | 364 |

| NAME | LEFT PRIMER | NAME | RIGHT PRIMER | PRODUCT SIZE (bp) |
|---|---|---|---|---|
| ATM-E17F | CACATGAACCTTGAATTGTTTG | ATM-E17R | AAGAAAGGAACAATCCTAAAAGG | 720 |
| ATM-E18F | GAAGAGGAGGAAATTTGAGTTAATATG | ATM-E18R | TCTTCAAAGACACCATGTGATTC | 382 |
| ATM-E19-20F | AAATATTTACAGCTGTGTGTGTGTG | ATM-E19-20R | GTTCTTCAAGCGACTGAGGG | 939 |
| ATM-E19-20F_seq | TTTTTCCCTCCTACCATCTTAGTATC | ATM-E19-20R_seq | GTCCTTGAGCATCCCTTGTG | 337 |
| ATM-E21-22F | GTTTAGCACAGAAAGACATATTGG | ATM-E21-22R | TTGCAACTGTGAGCTGTTACTATG | 516 |
| ATM-E23F | TGCTTTGGAAAGTAGGGTTTG | ATM-E23R | AATCAATTCATACAGTTGTTTTAGAGC | 337 |
| ATM-E24F | AAAATCTGGAGTTCAGTTGGG | ATM-E24R | AGTGCCACTCAGAAAATCTAGC | 349 |
| ATM-E25F | TCAAGAAAAGTTGAATGAATGTTG | ATM-E25R | TGGTATGTGTGTTGCTGGTG | 377 |
| ATM-E26F | CTTTAATGCTGATGGTATTAAAACAG | ATM-E26R | GCCAATCAGAGGGAGACAAC | 535 |
| ATM-E27F | AGTCTACAGGTTGGCTGCATAG | ATM-E27R | TGTGGGGAGACTATGGTAAAAG | 493 |
| ATM-E28F | CAGTAGAAAAATGGTTTTTGAATTTG | ATM-E28R | TGAATGTTTTCCTTTTTAATTATGAG | 383 |
| ATM-E29F | TTGTAGCCGAGTATCTAATTAAACAAG | ATM-E29R | GCGGACAGAGTGAGTCTTTG | 360 |
| ATM-E30F | CTGCCTATTCTGCATTTTGC | ATM-E30R | AACATAAAACACTCAAATCCTTCTAAC | 432 |
| ATM-E31F | CACAGAAACTAAAAGCTGGGTATC | ATM-E31R | ATAGGCATGAGCCAATGTGC | 469 |
| ATM-E32F | CATTGTAGGGTTTGCAGTGG | ATM-E32R | TGACAATGAAACCAAGAGCAAG | 391 |
| ATM-E33F | TTGTCACATATTGCTAATCACTTTC | ATM-E33R | ATTGGGCAATTCTGTCATTG | 528 |
| ATM-E34F | TGGGATTACGGGCCTGC | ATM-E34R | TTTAACAGGGACCTTGTCTGG | 679 |
| ATM-E35F | GGAATTGCAGTTGCAGTGATTAG | ATM-E35R | TCTGAGCTTTTCCACACTGC | 552 |
| ATM-E36F | TGTTATACTCATTTTGTGTAGGAAAGG | ATM-E36R | TTTACAACAGTTTGAGTGGGG | 372 |

| NAME | LEFT PRIMER | NAME | RIGHT PRIMER | PRODUCT SIZE (bp) |
|---|---|---|---|---|
| ATM-E37F | GAAATTTAATATGTCAACGGGG | ATM-E37R | TCCTGTAAAGTGCTTTTAGTGGG | 372 |
| ATM-E38F | ATAGCATAGTGGGAGACAGACAC | ATM-E38R | TCATGTTAAAATTCAGCCGATAG | 258 |
| ATM-E39F | CAGATTCTCTGATAGGTTTGAGGG | ATM-E39R | TAAATGGGGAACAGGAGGC | 489 |
| ATM-E40F | TCACAAATTCCTTTTCCATCC | ATM-E40R | TGATGCTTAGTCCAGTAAGTAAATTC | 389 |
| ATM-E41-42F | TTGGGAGTTACATATTGGTAATGATAC | ATM-E41-42R | GGAGTATCCCTGAATGTTTAGCC | 547 |
| ATM-E43F | CACCCAGCTGATATTTTGGG | ATM-E43R | TGCTTATTGCTATCTTACTGAAGG | 565 |
| ATM-E43F_seq | TTAGGCCTTGCAGAATTTGG | ATM-E43R_seq | TACCTGACGGAAGTGCAATG | 156 |
| ATM-E44F | AAATTTTGTCCTTTGGTGAAGC | ATM-E44R | TCTCTTCATCAATGCAAATCC | 300 |
| ATM-E45F | TTTTGTTTCCACTGCTATTTTG | ATM-E45R | AGTGATCCACCCACTTCAGC | 424 |
| ATM-E46F | TCTGACCGCATAGCATTTTG | ATM-E46R | GCCCAAGGCTTAAAAGTCAG | 611 |
| ATM-E47F | GACAGATAGGCAGACGTGGG | ATM-E47R | CGACCACATGATGGACTGATAG | 561 |
| ATM-E48F | GTACATGAAGGGCAGTTGGG | ATM-E48R | CACTCCACCCTAGAGACTATACAGAG | 341 |
| ATM-E49F | GTGGGTTGGACAAGTTTGC | ATM-E49R | AAAGACCAAGTCACTCTTTCTATGC | 455 |
| ATM-E50F | TTCCCTTTATAATCCTTAGAAGTTTG | ATM-E50R | TGTGTAGAGCACTGGACCAAG | 473 |
| ATM-E51F | CCACTTGTGCTAATAGAGGAGC | ATM-E51R | GAGGCCTATGAGGAATTAGAGAG | 397 |
| ATM-E52F | CGCTCTACCCACTGCAGTATC | ATM-E52R | CTTGAACCGATTTTAGATGGC | 386 |
| ATM-E53F | TGACTGTTTTGTTTGTATCTGAGG | ATM-E53R | GTAACCAGGGAATGCTGAAG | 443 |
| ATM-E54F | GGCCAGTGGTATCTGCTGAC | ATM-E54R | GCCAATATTTAACCAATTTTGACC | 276 |
| ATM-E55F | TCACATCGTCATTTGTTTCTCTG | ATM-E55R | AAGACAAAATCCCAAATAAAGCAG | 350 |

| NAME | LEFT PRIMER | NAME | RIGHT PRIMER | PRODUCT SIZE (bp) |
|---|---|---|---|---|
| ATM-E56F | TGAGTGCCCTTTGCTATTCTC | ATM-E56R | ATATGGGCATGAGCCACTTC | 285 |
| ATM-E57F | AAAATGCTTTGCACTGACTCTG | ATM-E57R | TTGACAATTACCTGATGAAATTAAAG | 360 |
| ATM-E58F | TTCCCTGTCCAGACTGTTAGC | ATM-E58R | TGCCAAACAACAAAGTGCTC | 363 |
| ATM-E59F | ACATGGCCGGTTATGCAC | ATM-E59R | TTGGTAGGCAAACAACATTCC | 313 |
| ATM-E60F | TTGCCTTTGTAAAGTTCACATTC | ATM-E60R | AAAGAACCATGCCCAGCC | 271 |
| ATM-E61F | AGCATAGGCTCAGCATACTACAC | ATM-E61R | TGAAGCAGTGCTCTTCACATC | 259 |
| ATM-E62-63F | TGTGCATGATGTTTGTTCCC | ATM-E62-63R | AAGAGTGAAAGCAGAGATGTTCC | 766 |
| ATM-E62F | TGACTGGCTTATTTGTATGATACTGG | ATM-E62R | GAACAGTTTAAAGGCCTTGGG | 305 |
| ATM-E63F | GATGACCAAGAATGCAAACG | ATM-E63R | AAGAGTGAAAGCAGAGATGTTCC | 523 |

**Table 6: SRY-Amplicon Primer**

| | |
|---|---|
| SRY-for: | GAGTGAAGCGACCCATGAACG |
| SRY-rev: | CTTGAGTGTGTGGCTTTCGTACAG |
| Product_size: | 349bp |
| Producer: | Eurofins Genomics, Braunfels, Germany |

## 2.2 Samples

Germline DNA from affected PDAC patients from the FaPaCa registry has been used for Whole Genome Sequencing, Sanger Sequencing and MLPA.

All coding ATM exons have been subjected to Sanger Sequencing and MLPA, as there is no area of the gene that is especially susceptible to mutation. Mutations have been identified throughout the coding region and splice sites (Rothblum-Oviatt et al. 2016).

The patients chosen were taken from FPC families with the most PDAC cases per family. This should increase the probability of detecting a genetic reason causing the hereditary background as found in another studies (Roberts et al. 2012).

All these patients and their family members are part of the FaPaCa registry and gave informed consent for the genetic analysis of their blood samples (Etic vote: “Klinische und genetische Untersuchung des familiären exokrinen Pankreaskarzinoms mit Aufbau einer nationalen Fallsammlung”; Studie 36/97, printed in the appendix in section 8.1). The EDTA-treated blood tubes had been stored at -80°C until DNA extraction.

## 2.3 DNA extraction

DNA for the mutation analyses was extracted out of leucocytes from venous blood of the FPC patients using the ‘DNeasy® blood and tissue kit’. In contrast to the manufacturer’s protocol of the kit, we used 200µl of the anticoagulant-treated blood (from EDTA-treated tube) to increase DNA yield and 20µl proteinase K taken from the used kit and added to a 1.5ml Eppendorf tube. PBS from the kit was not added at this step, according to the manufacturer's instructions. Then DNA extraction was performed as recommended in the ‘DNeasy® Blood & Tissue Handbook’. The concentration of the DNA (eluted in buffer AE) was identified using NanoDrop lite by Thermo Fisher Scientific and stored in 1.5ml Eppendorf tubes at -20°C until further use.

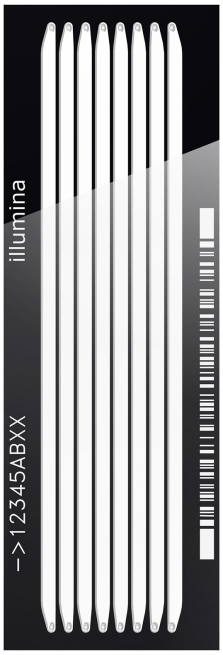
## *2.4 Whole Genome Sequencing (WGS)*

WGS was performed at the DKFZ (Deutsches Krebsforschungszentrum) in Heidelberg initially with 3 families (13 FPC cases and 17 healthy family members) to identify new FPC susceptibility genes candidates. The 3 families had been selected because of their particularly high number of PDAC patients without a known mutation in FPC susceptibility genes. Later, 12 more FPC cases from different families were also sequenced. The data has been analysed in cooperation with the DKFZ Heidelberg and the Helmholtz Zentrum München.

The 'HiSeq X ten system' from Illumina was used for WGS, as a 'Sequencing by Synthesis'-method with a four-steps workflow:

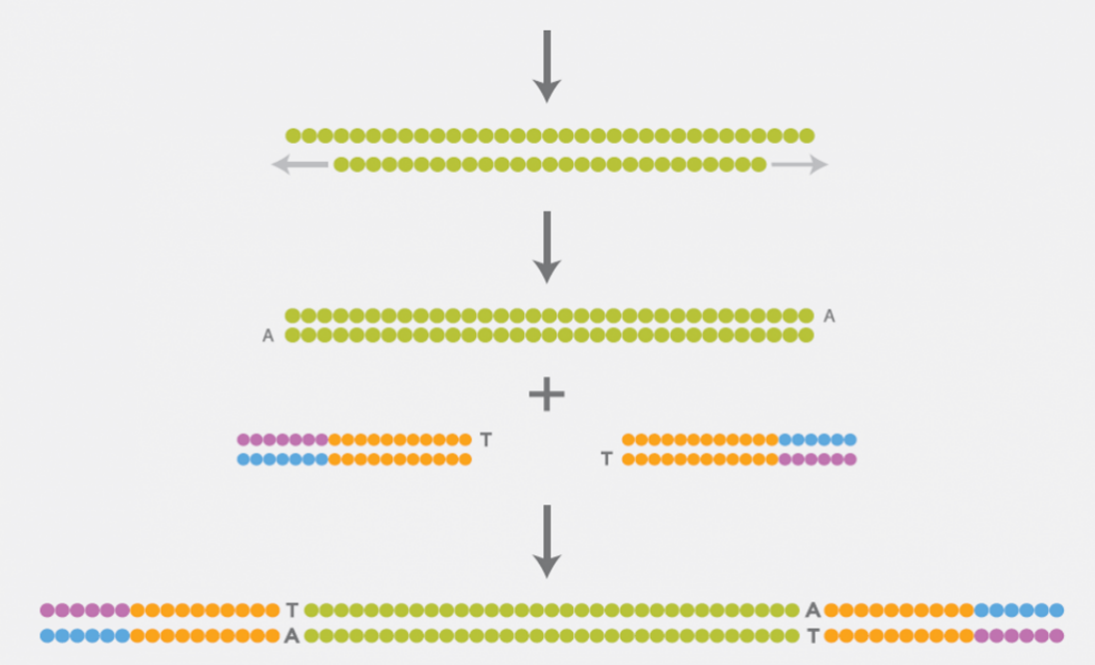
The '**Sample Preparation'** started with extracted DNA that is fragmented. Nucleic-adaptor molecules need to be ligated to the end of the DNA fragments. The adaptors contain motives including primer binding sites and regions complementary to the flow cell oligonucleotides. The flow cell is the in Figure 4 shown glass slide with lanes; each lane binds two types of oligos at its surface.

**Figure 4: Illumina Flow Cell**




Source: Illumina 2018a

**Figure 5: Sample Preparation**

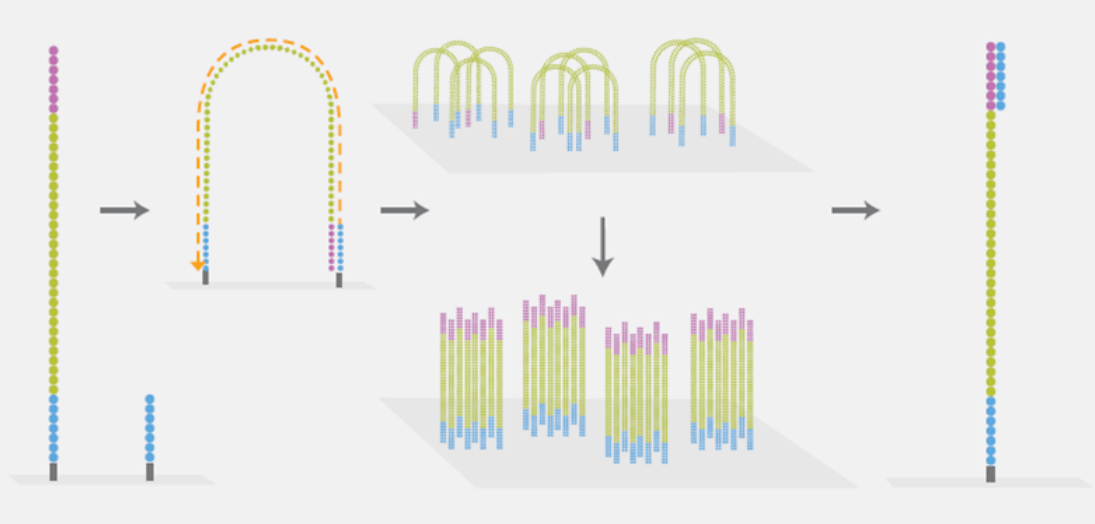



Source: CeGaT 2018a

**Cluster Generation**: Each fragment molecule binds to the first type of the two oligonucleotides on the flow cell surface by hybridization. This oligo is complementary to the adaptor region on one side of the strand. Then a polymerase creates a complement of one of the hybridized fragments. The double stranded molecule is denatured and the original template is washed away.

The strands are clonally amplified by so-called bridge amplification: In this process, the strand falls over and the adaptor region on the other side of the oligo hybridizes to the second type of oligo on the flow cell. Polymerases generate the complementary strand, forming a double stranded bridge. This bridge is denatured, resulting in two single stranded copies of the molecules that are arising from the flow cell. The process is then repeated over and over again and occurs simultaneously for millions of clusters resulting in the clonal amplification of all the fragments. After amplification, the reverse strands are cleaved and washed off, leaving only the forward strands. The 3'-ends are blocked to prevent unwanted priming.

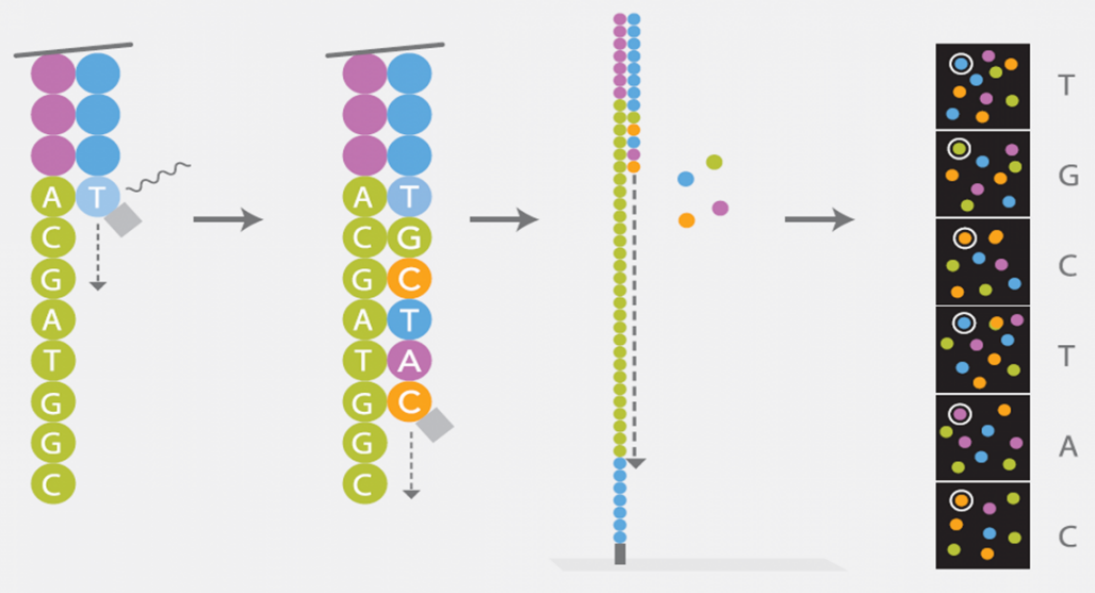
**Figure 6: Cluster Generation on Flow Cell**



Source: CeGaT 2018b

**Sequencing** begins with the forward strand (Read 1) by binding and extension of the forward primer. With each cycle, fluorescently tacked nucleotides compete for addition of the growing chain. Only one is incorporated, based on the sequence of the template. After the addition of each nucleotide, the clusters are excited by a light source and a characteristically fluorescence signal is emitted. The number of cycles determinates the lengths of the read. The emission wavelength along with the signal intensity determinates the identified base sequence. For a given cluster, all identical strands are read simultaneously. The Illumina HiSeq system is able to perform 5.3 - 6 billion reads per run. Thus, millions of clusters are sequenced at the same time. After the completion of the forward strand (Read 1), the complete read-product is washed away, the 3'-ends are deprotected and the template strand is again falling over and binding the second oligo on the flow cell. Polymerases extend the second flow cell oligo, forming a double stranded bridge, which then gets linearized and the 3'-ends are blocked. The original forward strand is cleaved off and washed away, leaving only the reverse strand. The sequencing of the reverse strand (Read 2) begins with the introduction of the reverse primer, as with Read 1. Sequencing steps are repeated and the desired read length is achieved.

**Figure 7: Sequencing**




Source: CeGaT 2018b

**Data Analysis**: This sequencing process generates billions of reads, representing all the fragments. Sequences from the whole process are collected and compared. Reads with similar overlapping base sequence, are locally clustered and paired creating continuous sequences, which are aligned back to the reference genome for variant identification. Source: (Illumina 2018b, c)

To identify pathogenic variants in new FPC susceptibility genes out of thousands of detected genetic variants, the sequencing results have been sorted by using the following algorithm:

i. Removing old controls (If older healthy family members carry a mutation, disease causing can be assumed as unlikely to be)
ii. Only missense/splice site mutations or stop-gains/losses as more severe mutations
iii. Mutation is present in PC patients
iv. CADD(PHRED)-Score > 10 (defining a strong segregation of mutation with the disease)
v. Low population frequency

By using this algorithm, several candidate genes with initially unknown significance have been identified.

Another 12 FPC patients from different families have been included in the WGS-project to increase the number of candidate genes. As only one patient per family was sequenced, it was not possible to use the algorithm explained above because it is not possible to compare the detected variants without other sequenced family members. Thus, the sequencing data has been compared with reference sequences in particular from cancer driver genes and potential FPC susceptible genes known from earlier studies and literature to identify SNPs or INDELs with disease causing potential (*BRCA1/2, PALB2, CDKN2A, CHEK2, ATM, TP53, STK11, MLH1, MSH2, MSH6, PMS2, PRSS1, PRSS2*).

## *2.5 Deleterious tests*

Missense variants are tested by MutationTaster, PolyPhen-2, SIFT and PROVEAN to identify their disease causing potential; at least 3 tools, which are based on different data, should be positive to expect deleteriousness.

### 2.5.1 MutationTaster

MutationTaster uses a ‘Bayes classifier’ to eventually predict the disease potential of an alteration. The ‘Bayes classifier’ is fed with the outcome of all tests and the features of the alterations and calculates probabilities for the alteration to be either a disease causing mutation or a harmless polymorphism. For this prediction, the frequencies of all single features for known disease mutations/polymorphisms were studied in a large training set composed of >390,000 known disease mutations from Human Gene Mutation Database (HGMD) and >6,800,000 harmless Single Nucleotide Polymorphism (SNPs) and Insertions/Deletions (INDEL) from the 1000 Genomes Project.

The **probability value** is the probability of the prediction, a value close to 1 indicates a high 'security' of the prediction.

(Schwarz et al. 2014)

### 2.5.2 PolyPhen-2

PolyPhen-2 (Polymorphism Phenotyping v2) is a tool similar to ‘MutationTaster’, which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations.

(Adzhubei et al. 2010)

### 2.5.3 SIFT

SIFT (Sorting Intolerant From Tolerant) predicts whether an amino acid substitution affects protein function. SIFT prediction is based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences, collected through 'Position-Specific Iterative Basic Local Alignment Search Tool' (PSI-BLAST).

SIFT takes a query sequence and uses multiple alignment information to predict tolerated and deleterious substitutions for every position of the query sequence. SIFT is a multistep procedure that searches for similar sequences, chooses closely related sequences that may share similar function to the query sequence, obtains the alignment of these chosen sequences, and calculates normalized probabilities for all possible substitutions from the alignment.

SIFT-Score: Ranges from 0 to 1. The amino acid substitution is predicted damaging if the score is ≤ 0.05, and tolerated if the score is > 0.05.

Median Info: Ranges from 0 to 4.32, ideally the value would be between 2.75 and 3.5. This is used to measure the diversity of the sequences used for prediction.

(J. Craig Venter Institute 2018b)

### 2.5.4 PROVEAN v1.1

PROVEAN (Protein Variation Effect Analyzer) was developed as an advancement of SIFT and is also used to predict whether a protein sequence variation affects protein function. This tool is able to provide predictions for any

type of protein sequence variations including single or multiple amino acid substitutions, insertions or deletions.

PROVEAN introduces a ‘delta alignment’ score based on the reference and variant versions of a protein query sequence with respect to sequence homologs collected from the ‘National Center for Biotechnologiy Information’ (NCBI) non redundant protein database through BLAST. The PROVEAN score distribution of a set of 58,000 ‘UniProt’ human protein variants with known functional outcome. For maximum separation of the deleterious and neutral variants, the cut-off is currently set at -2.5 (deleterious vs. neutral).

(J. Craig Venter Institute 2018a)

## *2.6 Exon Polymerase Chain Reaction (PCR) Amplification*

A Polymerase chain reaction (PCR) was used for specific amplification of ATM exons and surrounding regions including the splice sites for following sequence analyses. Each sample contained the listed ingredients.

**Table 7: PCR Samples**

| **PCR samples** (in 0.2ml PCR tubes) | Volume |
|---|---|
| PCR Mastermix | 10µl |
| Primer forward | 1µl |
| Primer reverse | 1µl |
| DNA | 30ng |
| $dH_2O$ | add to 20µl |

At 95°C DNA double strands are denaturing, so that the hybridization of the named primers (2.1, Table 5) can occur at lower temperature on complemented sequences at the 3’-end of each strand. Stable hydrogen bonds between complementary bases are formed only when the primer sequence matches the template sequence. The DNA-Taq-polymerase binds

to the primer-template hybrid and begins DNA elongation at 72°C in 5'-to-3'-direction. The process then repeats, starting with denaturation of the template DNA and the synthesized DNA. After 35 cycles, the final elongation lasts 5 minutes to complete the polymerization and in the end the cycler cools down to 4°C to hold ready for storage.

**Table 8: PCR Cycler Adjustment**

| **PCR cycler adjustments** | Time | Temperature |
|---|---|---|
| 1. Denaturation | 5 min | 94°C |
| 2. Denaturation | 30 sec | 94°C |
| 3. Annealing | 30 sec | Depending on the structure of the used primers |
| 4. Elongation | 30 sec | 72°C |
| Repeat steps 2 – 4 for 35x | | |
| 5. Elongation | 5 min | 72°C |
| 6. Pause | ∞ | 4°C |

The PCR amplification success was tested before sequencing by running an agarose gel (2% agarose in 100ml TBE buffer, 7µl peqGREEN) for 40 minutes, using the 50bp GeneRuler as a size marker to control the length of the amplicon and 5µl of each PCR product sample, including a negative control (no DNA), a positive control (amplicon of wildtype DNA) and the amplified amplicons of interest, each sample being mixed with 1µl of 6x DNA Loading Dye.

## *2.7 Sanger Sequencing*

Sanger Sequencing was performed to detect potentially existent smaller DNA mutations, such as point mutations or INDELs of a few nucleotides in the amplified DNA from exons and splice sites flanking the exonic region. In total, 30 FPC patients have been analysed by Sanger Sequencing.

Sanger sequencing was performed by the Helmholtz Institut in München and Seqlab Microsynth (Göttingen, Germany) with 'Barcode Economy Run'. The

synthesized amplicon of interest (5µl), was treated in a 200µl tube, with 1µl alkaline Phosphatase and 1µl Exonuclease I and incubated in a PCR thermal cycler using the 'Rapid PCR Cleanup' program (5 minutes at 37°C, 10 minutes at 80°C, finally cooling down to 4°C). The product of the clean-up process was transferred to a 1.5ml tube, containing 6.5µl $dH_2O$ and 1.5µl forward primer (c= 20pMol/µl), which had been used for the PCR amplification.

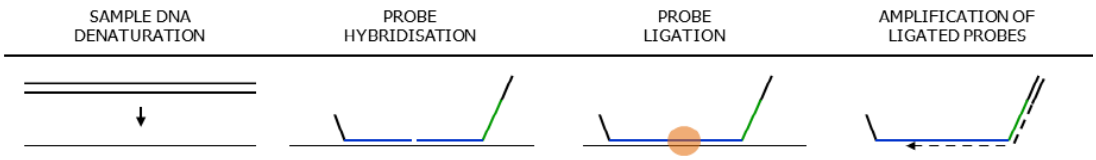
The sequencing method, developed in 1977 by Frederick Sanger and colleagues (Sanger, Nicklen, and Coulson 1977), is based on the incorporation of chain-terminating dideoxynucleotidetriphosphates (ddNTPs) by the DNA Polymerase during in vitro DNA replication. Here it was chosen to validate the Whole Genome Sequencing (WGS) results and search for further mutations in exonic DNA of the ATM gene as it is a reliable and moderately priced method.

The sample contained the DNA template/amplicon to be sequenced, the DNA Polymerase, a DNA primer, normal deoxynucleosidetriphosphates (dNTPs) and fluorescently labelled ddNTPs, each of which emit light at different wavelengths. When dNTPs are incorporated to extend the synthesized DNA strand, the DNA-polymerase continues working. Whenever ddNTPs are incorporated, DNA-polymerase stops working because of the missing 3'-OH group required for the formation of a phosphodiester bond between two nucleotides. Because ddNTPs get incorporated randomly, the synthesized DNA strand can be stopped after each nucleotide, and the strands produced can be arranged by their size in a capillary electrophoresis. Due to the differentially labelled four ddNTPs, the fluorescence detector can distinguish the wavelengths of the length-sorted chains. In the generated chromatogram, the DNA sequence is readable and can be analysed using the free chromatogram viewer 'Chromas' (Technelysium DNA Sequencing Software 2018).

## *2.8 Multiplex ligation-dependent Probe Amplification (MLPA)*

MLPA was performed to identify copy number variations (CNV) such as deletions or duplications e.g. of whole exons. We used an MLPA Kit from MRC-Holland with ATM-specific probe mixes, as listed in section 2.1.

**Figure 8: MLPA Reaction**




Source: MRC-Holland 2018

For the MLPA we used about 100ng of DNA, extracted from the patient's blood as described in section 2.3 and then proceeded according to the instructions in the 'MLPA® General Protocol' from MRC-Holland.

The frequency of ATM deletions or duplications in AT patients is around 2-5% (Podralska et al. 2014, Cavalieri et al. 2008), whereas in breast cancer it is less than 0.1% (Susswein et al. 2016).

The principle of MLPA as described in the MRC-Holland Protocol is based on a PCR amplification of up to 60 probes, each of which detects a specific DNA sequence of approximately 60nt in length. Starting with DNA denaturation of the sample, a mixture of MLPA probes is added next. Each MLPA probe consists of two oligonucleotides that must hybridise to immediately adjacent target sequences in the analysed region in order to be ligated into a single probe. Each probe in an MLPA probe mix has a unique amplicon length (green part of the oligonucleotide in Figure 8 illustrates the part with variable length), typically the whole amplicon ranges between 130-500nt. In the following PCR reaction, all ligated probes are amplified simultaneously using the same PCR primer pair (primer binding sites are marked black at the oligonucleotide in Figure 8). One PCR primer is fluorescently labelled, enabling the amplification products to be visualised during fragment separation through capillary electrophoresis. The relative height of each individual probe peak (blue) in the electropherogram, as compared to the relative probe peak (orange) height in various reference DNA samples, reflects the relative copy number of the corresponding target sequence in the sample. Thus, a deletion of one or more target sequences becomes apparent as a relative decrease in peak height, an increased relative peak height reflects an amplification of the target sequence.

The capillary electrophoresis was performed in ABI-Prism 310 Genetic Analyser, using FAM (6-fluorescein amidite) primer dye and a 47cm capillary with the following injection mixture (Table 9):

**Table 9: Injection mixture**

| **Injection mixture** | volume |
|---|---|
| PCR reaction | 0.75µl |
| dH2O | 0.75µl |
| GS 500 LIZ size standard | 0.5µl |
| HiDi (highly deionized) formamide | 13.5µl |

Before capillary electrophoresis, it is essential to heat the injection mixture for 3 minutes at 86 °C and then cool for 2 minutes at 4°C. The initial settings for capillary electrophoresis are as follows, injection voltage: 1.6 kV, injection time: 15seconds, filter set: D, polymer: POP4.

Source: (MRC-Holland 2018)

For the analysis of the MLPA capillary electrophoresis results, GeneMapper v4.0 has been used to analyse the measured data as described in section 3.4.

# 3 Results

## *3.1 ATM-analyzed families*

In total there have been used 35 families for analysing the *ATM* gene concerning deleterious variants. All chosen families correspond to the above-named FPC definition. In 12 families are no further tumour entities known than PDAC, the additional tumour entities to PDAC of all other families are listed in the Table 10. Family 02-5-0382 contains 4 PDAC cases, but 2 of them are a married couple without a genetic relationship, therefore the number of PDAC cases in family is listed as 3. Table 10 also contains the tests, which have been executed at the PDAC index patients out of the 35 analyzed families.

**Table 10: ATM-analyzed families**

| | Family ID | Patient number | Number of PDAC cases in family | Other tumours in family | WGS | Sanger Sequencing | MLPA |
|---|---|---|---|---|---|---|---|
| 1 | 02-5-0382 | 181 | 3(4) | Breast CA, Colorectal CA, Malignant Melanoma, Leukemia | 1 | 0 | 0 |
| 2 | 09-2-0552 | 185 | 2 | | 1 | 0 | 0 |
| 3 | 25-1-000091 | 503 | 3 | Gastric CA | 1 | 1 | 1 |
| 4 | 25-1-000172 | 696 | 3 | | 0 | 1 | 1 |
| 5 | 25-1-000176 | 704 | 3 | Head/Neck Squamous Cell CA (SCC) | 0 | 1 | 1 |
| 6 | 25-1-000204 | 768 | 3 | | 0 | 1 | 1 |
| 7 | 25-2-000014 | 153 | 3 | Colorectal CA | 1 | 1 | 1 |
| 8 | 25-2-000129 | 605 | 3 | Prostate CA | 1 | 1 | 1 |
| 9 | 25-2-000193 | 748 | 2 | Breast CA, Oesophageal CA | 0 | 1 | 1 |
| 10 | 25-2-000203 | 767 | 3 | Colorectal CA | 0 | 1 | 1 |
| 11 | 25-2-000213 | 792 | 2 | Colorectal CA | 0 | 1 | 1 |
| 12 | 25-3-000181 | 715 | 3 | Breast CA, Colorectal CA | 0 | 1 | 1 |
| 13 | 25-3-000190 | 735 | 3 | Breast CA, Malignant Melanoma | 0 | 1 | 1 |
| 14 | 25-4-000005 | 31 | 4 | Renal CA | 0 | 1 | 1 |
| 15 | 25-4-000046 | 299 | 3 | Colorectal CA, Oesophageal CA | 1 | 0 | 0 |
| 16 | 25-4-000088 | 498 | 2 | | 1 | 1 | 1 |

| | Family ID | Patient number | Number of PDAC cases in family | Other tumours in family | WGS | Sanger Sequencing | MLPA |
|---|---|---|---|---|---|---|---|
| 17 | 25-4-000106 | 571 | 3 | Colorectal CA, Malignant Melanoma | 1 | 1 | 1 |
| 18 | 25-4-000175 | 701 | 3 | Breast CA, Colorectal CA | 0 | 1 | 1 |
| 19 | 25-5-000067 | 427 | 4 | Breast CA | 1 | 0 | 0 |
| 20 | 25-5-000196 | 756 | 3 | Breast CA, Prostate CA | 0 | 1 | 1 |
| 21 | 25-5-000205 | 774 | 3 | | 0 | 1 | 1 |
| 22 | 25-6-000069 | 432 | 2 | Colorectal CA, Malignant Melanoma | 0 | 1 | 1 |
| 23 | 25-6-000078 | 481 | 3 | Breast CA | 1 | 1 | 1 |
| 24 | 25-7-000100 | 523 | 4 | | 1 | 1 | 1 |
| 25 | 25-7-000170 | 54 | 5 | Cervial CA, Colorectal CA | 1 | 1 | 1 |
| 26 | 25-7-000212 | 788 | 3 | | 0 | 1 | 1 |
| 27 | 25-8-000168 | 685 | 6 | Malignant Melanoma | 1 | 1 | 1 |
| 28 | 25-8-000178 | 709 | 2 | Colorectal CA, Lung CA, Prostate CA | 0 | 1 | 1 |
| 29 | 25-8-000183 | 718 | 4 | | 0 | 1 | 1 |
| 30 | 25-9-000044 | 298 | 3 | Malignant Melanoma | 1 | 0 | 0 |
| 31 | 25-9-000056 | 363 | 5 | Breast CA | 0 | 1 | 1 |
| 32 | 25-9-000113 | 562 | 4 | BreastCA, ColorectalCA, RenalCA, SCC | 1 | 1 | 1 |
| 33 | 25-9-000182 | 717 | 3 | | 0 | 1 | 1 |
| 34 | 25-9-000185 | 721 | 3 | | 0 | 1 | 1 |
| 35 | 25-9-000186 | 723 | 4 | | 0 | 1 | 1 |
| | | | | | **15** | **30** | **30** |

## *3.2 Whole Genome/Exome Sequencing (WGS/WES)*

In total, genomic DNA from PC patients of 15 different FPC families have been sequenced. These patients were selected for WGS because they show the highest number of first-degree relative PDAC patients among the families with high total numbers of PDAC cases. The sequencing data has been compared to reference sequences of cancer driver genes and FPC susceptibility genes to identify possible pathogenic variants.

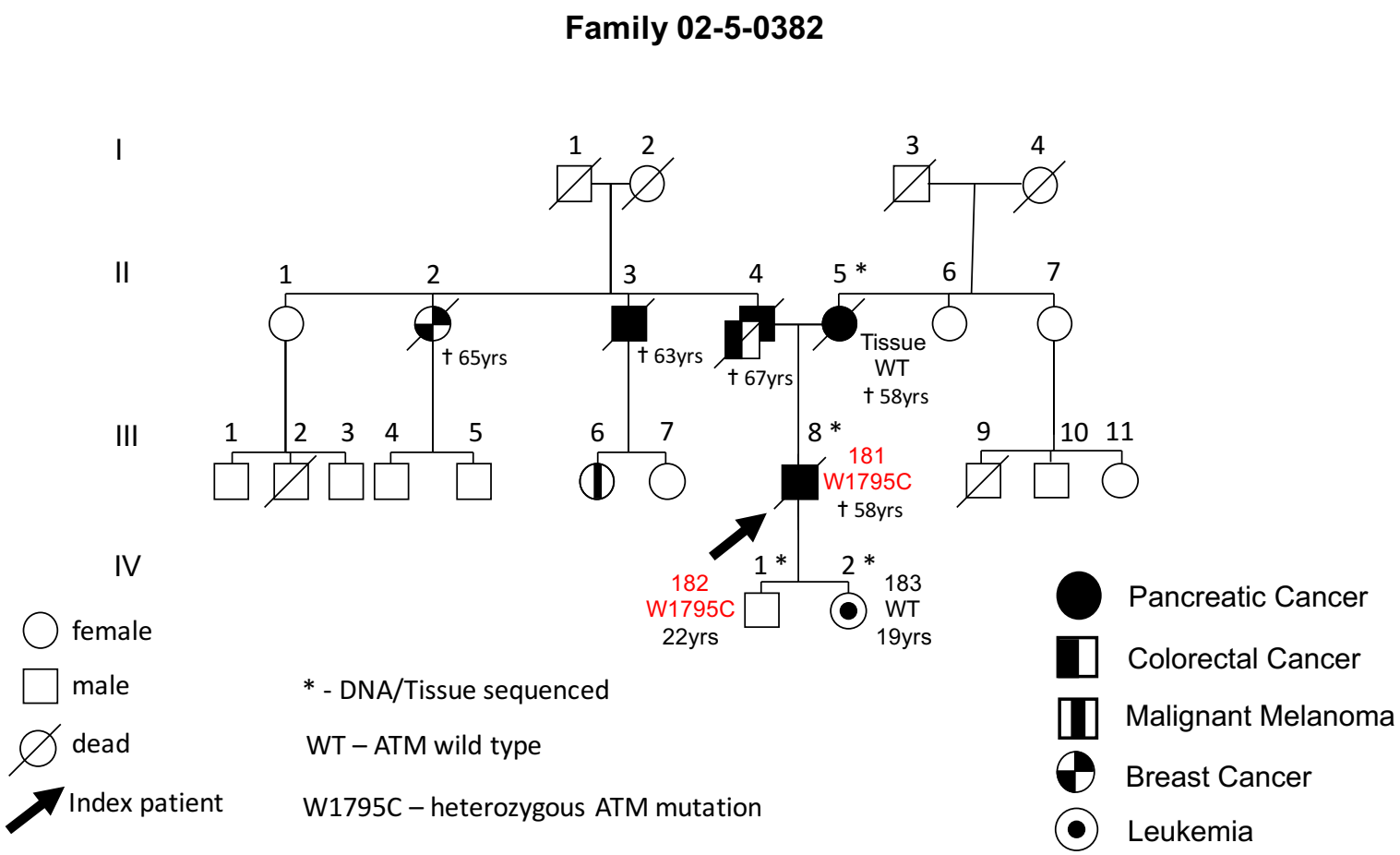
Interestingly, one FPC patient presented with a deleterious single base substitution (ATM:NM_000051, exon36, c.G5385T, p.W1795C), detected in exon 36 of the *ATM* gene which has been previously been described as deleterious (Roberts et al. 2012, Roberts et al. 2016).

## *3.3 Sanger Sequencing*

The ATM mutation was detected in FPC patient with lab number 181 from family 02-5-0382. EDTA-treated blood was available from patient 181 (III-8) and his children 182 (IV-1) and 183 (IV-2). The parents (II-4/5) of the index patient (181) both had PDAC and died from this malignant disease. There was tissue from the patient's mother which was tested negative for the mutation described below, no tissue from the father (II-4) was available for molecular genetic analyses. The mother (II-5) died at the age of 58 (PDAC diagnosis age: 57) and the father (II-4) died at the age of 67 (PDAC diagnosis age: 66). In addition to his PDAC diagnosis, he also had a rectum carcinoma, diagnosed at the age of 56.

The father (II-4) of the index patient (III-8) was one of four siblings. His brother (II-3) also succumbed to PDAC at the age of 63 and a daughter (III-6) of this brother had malignant melanoma. A sister (II-2) died of breast cancer at the age of 65 after diagnosis at the age of 60 (see Figure 9).

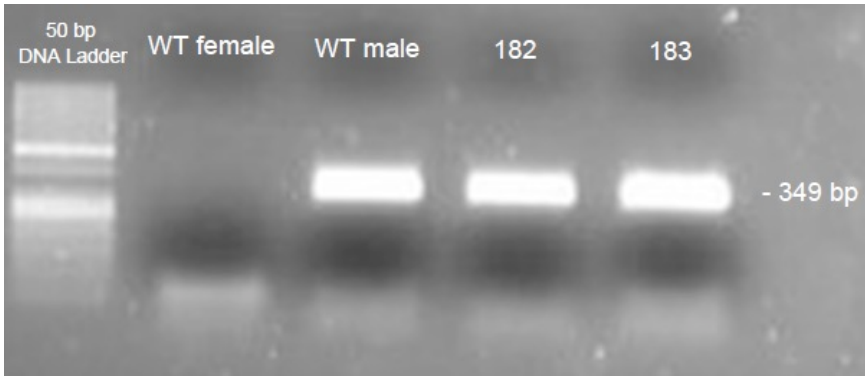
**Figure 9: Pedigree of family 02-5-0382 with deleterious W1795C mutation**



The index patient initially sequenced is indicated by the arrow. Current age (in years) or age at death is given below each figure. Family members who are heterozygous for the W1795C missense mutation are labelled in red.

EDTA-treated blood was also available from the children (IV-1/2) of the index patient (III-8) and was used to perform Sanger Sequencing of the *ATM* gene. The actually healthy son 182 (age: 22, at the time of last contact) was detected also to be a carrier of this heterozygous mutation. There are no known cancer diagnoses or precursor lesions, which is not surprising due to the young age of the patient. The daughter 183 was diagnosed with Acute Lymphoblastic Leukemia (ALL) in 1989, 1993 and 1997 and she was curatively treated by allogenic bone marrow transplantation (BMT) from a male donor. Her EDTA-treated-blood was taken in 2001 and Sanger Sequencing showed that both alleles from the daughter 183 carry the wildtype sequence, as shown in the chromatogram below (Figure 11). To clarify, whether the sequenced leukocyte DNA originated from the female person 183 or her male BMT donor, a PCR of an amplicon (349bp), located in the *SRY* gene on Y-chromosome was performed. Thereby it could be shown (Figure 10) that the analysed DNA contains a Y-chromosome and consequently originates from a man and cannot be used to rule out the mutation W1795C at the female person 183.

**Figure 10: SRY-Amplicon Agarose gel Picture**




Unfortunately, there was no other tissue available from 183 than the EDTA-blood, thus, no further DNA analysis could have been performed.

The heterozygous point mutation (ATM:NM_000051, exon36, c.G5385T, p.(W1795C)), identified in WGS, was then validated with Sanger Sequencing. Corresponding to reference sequence NM_000051, there is a substitution from guanine to thymine in the mutated allele of exon 35 at position 5385 from start-codon. The affected base triplet is thus changed from 'TGG' to 'TGT'. In the encoded protein, this substitution leads to the incorporation of the amino acid cysteine (C) at position 1795, instead of tryptophan (W). This variant is a missense mutation that tested to be deleterious. In this case, the mutation leads to a loss of function in modified ATM kinase. The mutation is not yet listed with a specific phenotype based on this polymorphism in the NCBI database ClinVar, which is a public archive of relationships among sequence variations and human phenotypes (Landrum et al. 2014).

Deleteriousness was proven to be pathogenic by results obtained with Mutation Taster, PolyPhen-2, SIFT and PROVEAN, from which all four tools predicted the variant to be deleterious:

**MutationTaster**: According to the Ensembl transcript ID: ENST00000278616 and single base exchange G5385T, MutationTaster predicts the variant to be disease causing, using the model: simple_aae, with a **probability value of 0.995**.

**PolyPhen-2**: The mutation W1792C in ATM kinase is predicted to be 'probably damaging' with a **score of 1.000** (sensitivity: 0.00; specificity: 1.00).

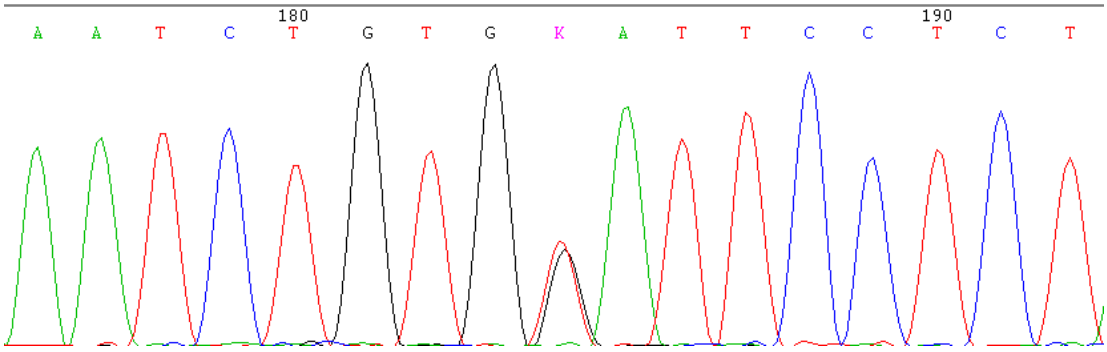
**SIFT**: According to sequence ENSP00000388058 with variant W1795C, SIFT predicts the mutation to be damaging with a **SIFT Score: 0**, the Median Information Content: 2.5, and 46 Sequences at position.

**PROVEAN**: Variant W1795C is predicted to be deleterious with **PROVEAN Score -8.942** (cutoff=-2.5).

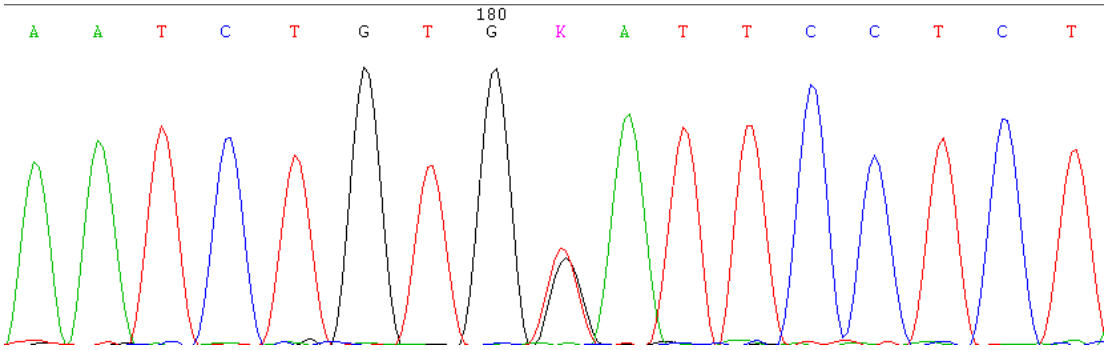
Even though using leucocyte DNA from EDTA-treated blood is very common, Roberts describes the possibility that somatic mutations in haematological malignancy driver genes can confound the findings of germline genomic sequencing studies in older populations (Roberts et al. 2016). However, the *ATM* mutation in patient 181 was also verified in his son's DNA (182), so we

can assume that it is a germline mutation and not a somatic mutation originating in haematopoiesis. In contrast to this, the leukocyte DNA from person 183 could not be used to identify germline mutations as she has leucocytes with a male chromosome set, based on the BMT nine years before EDTA-blood was taken. Rare cases like this are only to be identified by WGS and MLPA, not by Sanger Sequencing of single genes on autosomes.

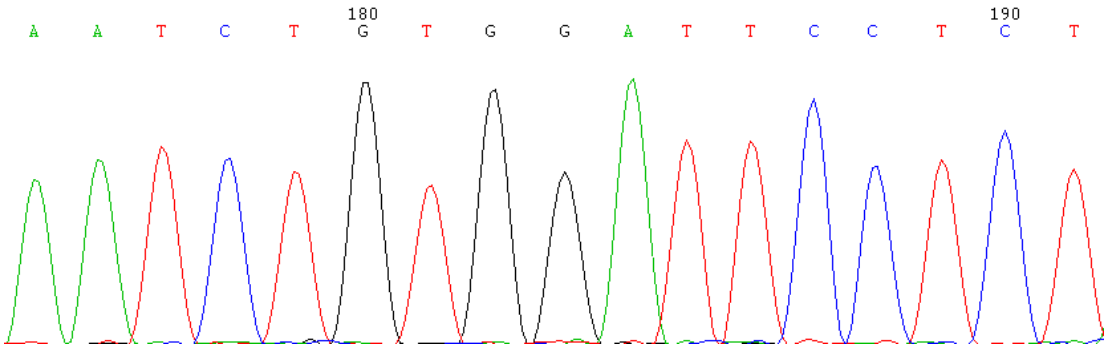
**Figure 11: Sanger sequencing results**




FPC patient 181: heterozygous single base substitution




Family member 182: heterozygous single base substitution




Family member 183: homozygous wild type

Besides this deleterious mutation, there have been detected 20 non-pathogenic variants in exons and exon-surrounding regions of the *ATM* gene. Some variants have been further described and published with a reference number, as they have been detected in other genetically studies focussing on *ATM* gene. All identified variants have been confirmed by using the forward and reverse primer for sequencing. All variants and the sequenced family members (index patient with PDAC) with homozygous (homo) or heterozygous (het) affection are listed in the table below (Table 11: ATM Variants). Only one of the 30 sequenced patients does not carry any variants in the analysed parts of the gene (patient lab number: 523, family ID: 25-7-000100).

In Table 10 is first named the affected exon (E) and the type of mutation with the affected amino acids in parentheses and its exact position. The leading sign (+/-) characterises the position downstream (-) or upstream (+) of the named exon, a number without mathematical sign defines the position in the exon starting from the beginning of the exon, the position from the start of the gene is put in parentheses. In case of amino acid change by the mutation, the single letter amino acid code is used to declare the change, the affected amino acid position is named in parentheses. Known reference numbers of ‘single nucleotide polymorphisms’ (refSNP), registered at NCBI, are listed at the end of the variant description.

**Table 11: ATM Variants**

| Patient number | E2, 2bp deletion (AA), position +37/+38, no AA Change, NCBI refSNP: rs2066734 | E3, 2bp deletion (AA), position -43/-42, no AA Change, NCBI refSNP: rs2066734 | E3, 1bp substitution (C->G), position 74 (146), AA Change: S->C (49), NCBI refSNP: rs1800054 | E6, 1bp substitution (T->C), position +16, no AA Change | E7, 1bp substitution (C-YT), position 73 (753), no AA change – V->V (245), NCBI refSNP: rs3218674 | E13, 1bp substitution (T->C), position 221 (2119), AA change: S->P (707), refSNP: COSM4159 | E17, 1bp substitution (T->C), position 106 (2572), AA Change: F->L (858), refSNP: COSM2182 | E22, 1bp substitution (C->G), position 8 (3161), AA Change: P->R (1054), refSNP: COSM2182 | E23, 1bp deletion (T), position -9, no AA Change | E24, 1bp substitution (T->A), position -15, NCBI refSNP: rs3218681 | E24, 1bp duplication (A), position -12, no AA Change | E29, 1bp substitution (C->T), position 22 (4258), AA Change I->F (1420), NCBI refSNP: rs1800058 | E30, 1bp subtitution (C->T), position 142 (4578), no AA Change - P->P (1526) , NCBI refSNP: rs1800889 | E37, 1bp substitution (T->C), position -8, no AA Change, NCBI refSNP: rs3092829 | E37, 1bp substitution (G->A), position 61 (5557), AA Change: D->N (1853), refSNP: COSM3736 | E37, 1bp substitution (A->T), position 62 (5558), AA change: D->V (1853), refSNP: COSM 3752 | E41, 1bp substitution (G->A), position 61 (6067), AA Change: G->R (2023), refSNP: CSOM2463 | E47, 3bp deletion (ATT), position -60...-58, no AA Change | E52, 1bp substitution (G->T), position +8, NCBI refSNP: rs11277590 | E60, 1bp substitution (A->C), position +8, NCBI refSNP: rs4986839 | number of PDAC in family | other tumour entities in family |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | | |
| **31** | homo | homo | | | | | | | | | homo | | | | | | | homo | | | 4 | Renal CA |
| **54** | het | het | | | | | | | | | het | | | | | | | het | | | 5 | Colon CA, Cervix CA |
| **153** | het | het | | | | | | | | | het | het | | | | | | het | | | 3 | Colon CA |
| **363** | het | het | | | | | | | | | het | het | | | | | | het | | | 5 | Breast CA |
| **432** | het | het | | homo | | | het | het | het | homo | | | | | het | | | homo | | | 2 | Colon CA, MM |
| **481** | het | het | | | | | | | | | het | | | | | | | het | | | 3 | Breast CA |
| **498** | homo | homo | | | | | | | | | homo | | | | | | | homo | | het | 2 | |
| **503** | | | | homo | | | | | het | | het | | het | het | het | | | het | | | 3 | Gastric CA |
| **523** | | | | | | | | | | | | | | | | | | | | | 4 | |
| **562** | | | het | | | | | | het | | het | | | | het | | | het | | | 4 | Breast CA, Colon CA, SCC, Renal CA |

| Patient number | E2, 2bp deletion (AA), position +37/+38, no AA Change, NCBI refSNP: rs2066734 | E3, 2bp deletion (AA), position -43/-42, no AA Change, NCBI refSNP: rs2066734 | E3, 1bp substitution (C->G), position 74 (146), AA Change: S->C (49), NCBI refSNP: rs1800054 | E6, 1bp substitution (T->C), position +16, no AA Change | E7, 1bp substitution (C-YT), position 73 (753), no AA change – V->V (245), NCBI refSNP: rs3218674 | E13, 1bp substitution (T->C), position 221 (2119), AA change: S->P (707), refSNP: COSM4159 | E17, 1bp substitution (T->C), position 106 (2572), AA Change: F->L (858), refSNP: COSM2182 | E22, 1bp substitution (C->G), position 8 (3161), AA Change: P->R (1054), refSNP: COSM2182 | E23, 1bp deletion (T), position -9, no AA Change | E24, 1bp substitution (T->A), position -15, NCBI refSNP: rs3218681 | E24, 1bp duplication (A), position -12, no AA Change | E29, 1bp substitution (C->T), position 22 (4258), AA Change I->F (1420), NCBI refSNP: rs1800058 | E30, 1bp subtitution (C->T), position 142 (4578), no AA Change - P->P (1526) , NCBI refSNP: rs1800889 | E37, 1bp substitution (T->C), position -8, no AA Change, NCBI refSNP: rs3092829 | E37, 1bp substitution (G->A), position 61 (5557), AA Change: D->N (1853), refSNP: COSM3736 | E37, 1bp substitution (A->T), position 62 (5558), AA change: D->V (1853), refSNP: COSM 3752 | E41, 1bp substitution (G->A), position 61 (6067), AA Change: G->R (2023), refSNP: CSOM2463 | E47, 3bp deletion (ATT), position -60...-58, no AA Change | E52, 1bp substitution (G->T), position +8, NCBI refSNP: rs11277590 | E60, 1bp substitution (A->C), position +8, NCBI refSNP: rs4986839 | number of PDAC in family | other tumour entities in family |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | | |
| 571 | | | | | | | | | het | | het | | | het | het | | | het | | | 3 | Colon CA, MM |
| 605 | het | het | | | | | | het | | | het | | | | | | het | het | | | 3 | Prostate CA |
| 685 | homo | homo | | | | | | | | | homo | | | | | | | homo | | | 6 | MM |
| 696 | homo | homo | | | | | | | | | homo | | | | | | | homo | | | 3 | |
| 701 | homo | homo | | | | het | | | | | homo | | | | | | | homo | | het | 3 | Colon CA, Breast CA |
| 704 | het | het | | | | | | | | | het | | | | | | | het | | | 3 | SCC |
| 709 | | | | | | | | | het | | het | | | | het | | | het | | | 2 | Colon CA, Prostate CA, Lung CA |
| 715 | het | het | | | | | | | | | het | | | | | | | het | | | 3 | Colon CA, Breast CA |
| 717 | het | het | | | | | | | | | het | | | | | | | het | | | 3 | |
| 718 | het | het | | | | | | | | | het | | | | | | | het | | | 4 | |
| 721 | het | het | | | | | | | | | het | | | | | | | het | het | | 3 | |

| Patient number | E2, 2bp deletion (AA), position +37/+38, no AA Change, NCBI refSNP: rs2066734 | E3, 2bp deletion (AA), position -43/-42, no AA Change, NCBI refSNP: rs2066734 | E3, 1bp substitution (C->G), position 74 (146), AA Change: S->C (49), NCBI refSNP: rs1800054 | E6, 1bp substitution (T->C), position +16, no AA Change | E7, 1bp substitution (C-YT), position 73 (753), no AA change – V->V (245), NCBI refSNP: rs3218674 | E13, 1bp substitution (T->C), position 221 (2119), AA change: S->P (707), refSNP: COSM4159 | E17, 1bp substitution (T->C), position 106 (2572), AA Change: F->L (858), refSNP: COSM2182 | E22, 1bp substitution (C->G), position 8 (3161), AA Change: P->R (1054), refSNP: COSM2182 | E23, 1bp deletion (T), position -9, no AA Change | E24, 1bp substitution (T->A), position -15, NCBI refSNP: rs3218681 | E24, 1bp duplication (A), position -12, no AA Change | E29, 1bp substitution (C->T), position 22 (4258), AA Change I->F (1420), NCBI refSNP: rs1800058 | E30, 1bp subtitution (C->T), position 142 (4578), no AA Change - P->P (1526) , NCBI refSNP: rs1800889 | E37, 1bp substitution (T->C), position -8, no AA Change, NCBI refSNP: rs3092829 | E37, 1bp substitution (G->A), position 61 (5557), AA Change: D->N (1853), refSNP: COSM3736 | E37, 1bp substitution (A->T), position 62 (5558), AA change: D->V (1853), refSNP: COSM 3752 | E41, 1bp substitution (G->A), position 61 (6067), AA Change: G->R (2023), refSNP: CSOM2463 | E47, 3bp deletion (ATT), position -60...-58, no AA Change | E52, 1bp substitution (G->T), position +8, NCBI refSNP: rs11277590 | E60, 1bp substitution (A->C), position +8, NCBI refSNP: rs4986839 | number of PDAC in family | other tumour entities in family |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | | |
| **723** | het | het | | | | | het | het | | | het | | het | | | | | het | | | 4 | |
| **735** | het | het | | | | | | het | het | | homo | | | het | het | | | homo | | | 3 | Breast CA, MM |
| **748** | het | het | | | | | het | het | | | | | het | | | | | het | | | 2 | Breast CA, Oesophagus CA |
| **756** | | | | | het | | | | | | | | | | | | | | | | 3 | Breast CA, Prostate CA |
| **767** | het | het | | | | | | | | | het | | het | | | | | het | | | 3 | Colon CA |
| **768** | homo | homo | | | | | | | | | homo | | | | | het | | homo | | | 3 | |
| **774** | het | het | | | | | | | | | het | | | | | | | het | | | 3 | |
| **788** | homo | homo | | | | | | | | | homo | | | | | | | homo | | | 3 | |
| **792** | het | het | | | | | het | het | het | | homo | | | | het | | | homo | | | 2 | Colon CA |

## *3.4 Multiplex ligation-dependent probe amplification (MLPA)*

The same 30 FPC patients sequenced in Sanger Sequencing have been analysed with MLPA to detect INDELs. No variants were found in comparison the reference sequences.

Figure 12 is the electropherogram of the index patient of family 47 as a typical regular example without abnormalities, created by GeneMapper 4.0 which was used to analyse the measured data. The relative height of each individual probe peak (blue) in the electropherogram, as compared to the size marker probe peak (orange) height in various reference DNA samples, illustrates the relative copy number of the target sequence in the sample. A deletion of one or more target sequences thus becomes apparent as decrease in peak height; an increased relative peak height stands for an amplification of the target sequence.

The probe mixes (P041-B1 and P042-B2) each contain 45 MLPA probes with amplification products between 130 and 485nt in length including 34 probes for the ATM gene and 11 reference probes. Thus, we had a probe for each ATM exon, including one probe for intron 1, two probes for exon 1 and 61 and two probes for intron 61. The reference sequences contain nine quality control fragments generating amplification products between 64 and 105nt, four DNA Quantity Fragments (Q-fragments), three DNA Denaturation Fragments (D-fragments), and one chromosome X and one chromosome Y-specific fragment. A ‘no DNA control’ was added included in each run.

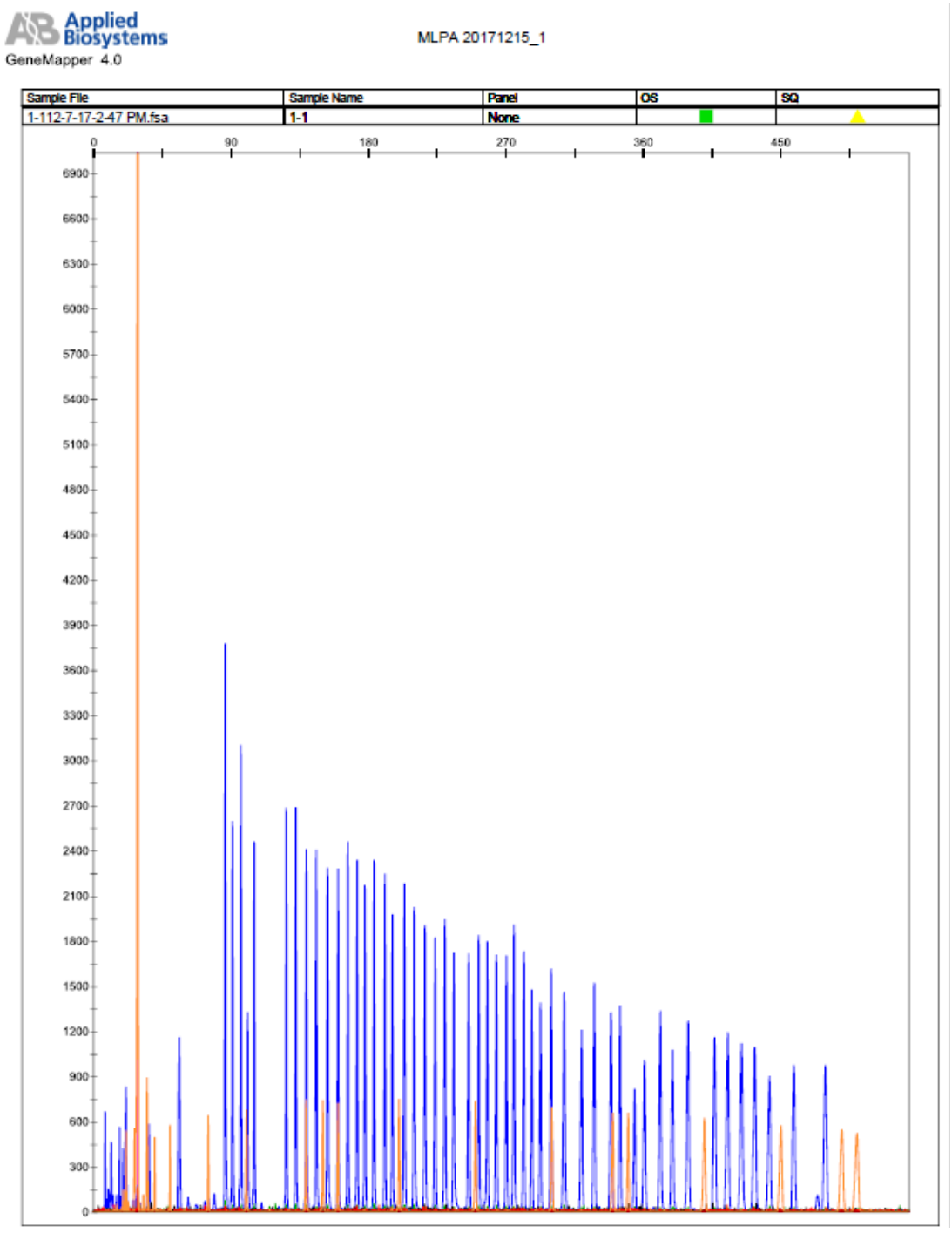
The four Quantity Fragments (Q-fragments, at 64-70-76-82 nt) are complete fragments that do not need to hybridise to DNA or to be ligated to be amplified during PCR. The more sample DNA is added, the lower these become. That is why in the ‘no DNA control’ sample, only the four Q-fragments are visible.

The two D-fragments (88nt and 96nt) detect sequences in exceptionally strong CG-regions, which are difficult to denature. When the 88nt and 96nt fragments are low, it indicates denaturation problems of the sample DNA, possibly due to the presence of an excess of salt in the DNA sample that can lead to false positive deletions.

The MLPA peak of a DNA sample without genomic abnormalities will be identical to that of the reference samples, which means Dosage Quotient (DQ) of ~1. For heterozygous deletions, DQ will be ~0.5, homozygous deletion without allele copies have a DQ=~0. At duplications of the allele of interest, DQ will increase to ~1.5 (3 copies, heterozygous duplication) or ~2 (4 copies, homozygous duplication).

The GeneScan™ 500 LIZ® size standard has been used to analyse the size of amplified oligos after capillary electrophoresis in the range of 20-500nt (size standards: 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 und 500nt).

**Figure 12: MLPA**



## *3.5 Summary*

The deleterious ATM mutation, detected in the WGS-project, was verified by Sanger-Sequencing as heterozygous point mutation. The substitution from guanine to thymine in the mutated allele leads to the incorporation of the amino acid cysteine instead of tryptophan. This variant is a missense mutation that tested to be deleterious with a loss of function in modified ATM kinase.

Furthermore, Sanger sequencing of the germline DNA from a group of 30 unrelated FPC patients detected no further pathogenic variants in the ATM-gene. Besides this deleterious mutation, there have been detected 20 more variants in exons and exon-surrounding regions of the *ATM* gene, all of which were tested to be non-pathogenic in the deleterious tests. Some variants have been further described and published with a reference number, as they have been detected in other genetically studies focussing on *ATM* gene.

The same 30 unrelated FPC patients, of whom coding DNA from the ATM-gene had been sequenced by Sanger-Sequencing, were analysed by MLPA. Again no further pathogenic variant was detected.

In total, we identified a deleterious mutation in the ATM-gene in 1 of 35 (2.9%) FPC families. Considering only the more severely affected FPC families with three or more PDAC cases, the prevalence of deleterious ATM mutations was 3.4% (1 of 29 FPC families).

# 4 Discussion

Cancer related death is the second most common cause for disease related death in Germany and the western world. PDAC even stands out with the worst prognosis of all malignant cancers and in comparison to other entities, the PDAC prognosis has not improved significantly in the last years (Statistisches Bundesamt 2017). Approximately 10% of PDAC patients have an increased risk, because of a distinctive familial aggregation of malignancies, probably due to pathogenic variants in germline DNA (Fendrich, Langer, and Bartsch 2014). In hereditary tumour syndromes the underlying deleterious germline mutations segregate with the disease. However, for FPC, there are only a few known susceptible genes, like *BRCA1* (Al-Sukhni et al. 2008), *BRCA2* (Murphy et al. 2002), *CDKN2A* (Slater, Langer, Fendrich, et al. 2010), *PALB2* (Slater, Langer, Niemczyk, et al. 2010) and *CHEK2* (Bartsch et al. 2006), where pathogenic variants cause the increased PDAC risk in the affected families. Even in families with known mutations in the FPC susceptibility genes, there is still an incomplete penetrance (Roberts et al. 2016). The hereditary background of tumour syndromes with increased PDAC risk is very heterogeneous. Several other tumour entities or organ dysfunctionalities combined with PC in one patient or in one family have been described. The genetic basis underlying disease susceptibility in the remaining 80 – 90% of FPC patients is unknown (Roberts et al. 2016). Defining further FPC susceptibility genes might be an approach to improve the poor prognosis of PDAC, as there might be possibilities for a specific cancer screening for earlier diagnosis (Cohen et al. 2018) and targeted therapies (Choi, Kipps, and Kurzrock 2016) that would complement surgical resection, now the only curative option in PDAC therapy.

The search for new candidate genes conferring susceptibility to FPC or other hereditary tumour syndromes is performed with WGS. This method allows the analysis of the majority of human DNA, much more than just protein coding sequences, as these make up only 1% of human DNA (Liang et al. 2018). However, WGS projects that have been performed in recent years, were only able to confirm the low rate of the above named FPC susceptibility genes

(Roberts et al. 2016). *ATM* was described as a new FPC susceptibility gene, with mutations identified in the North American population with a frequency of 2.4% (Roberts et al. 2012), as low as the other identified FPC susceptibility genes. Apart from that, WGS studies could not identify further candidate genes with a frequency comparable to the named FPC susceptibility genes.

In our WGS-project, we identified the mutation c.G5385T/p.W1795C in the *ATM* gene in one family that was tested to be deleterious. We verified the mutation by Sanger Sequencing.

Sanger Sequencing and MLPA of all 63 coding *ATM* exons were then used to analyse DNA from additional FPC families, as there is no one area of the *ATM* gene especially susceptible to mutations (Telatar et al. 1998, Rothblum-Oviatt et al. 2016). DNA from peripheral white blood cells has been used, knowing that detected mutations can originate from somatic mutations in haematopoiesis and therefore need to be confirmed in DNA from a second tissue or in other family members, before expecting it to be germline DNA. Within the further analysed families, we could show, that the frequency of ATM-mutations in the analysed German population is at 2.9%. Knowing that the genes responsible for the majority of cases with increased PDAC risk in FPC families are still to be discovered, it is a small but important fact that ATM joins the low number of FPC susceptibility genes.

The MLPA did not uncover variants in German FPC patients. It was well known, that the frequency of INDELs in other tumour predisposing syndromes is quite low but variable. The frequency of ATM deletions or duplications e.g. in AT patients is around 2-5% (Podralska et al. 2014, Cavalieri et al. 2008), whereas in breast cancer this is less than 0.1% (Susswein et al. 2016). Even if the used population is small, it does not seem to be necessary to regularly screen FPC patients by MLPA of the ATM gene.

It needs to be noted, that the ATM-mutations, detected in ‘North American population’, as well as in ‘German population’, have been identified in families with more cancer entities than just PC. The North American families described also included patients with breast cancer, colon cancer, lung cancer, prostate

cancer, uterine cancer, ovarian cancer, malignant melanoma and lymphoma (Roberts et al. 2012). None of these patients' DNA was sequenced in this study, thus, whether these cancer patients also carry the ATM-mutation remains unknown. The German ATM-mutation family includes members with breast cancer, malignant melanoma and rectal cancer, as shown in the pedigree above (Figure 9). Unfortunately, there was no DNA available for mutation analysis from family members other than the index patient and his children. The parents of the index patient both died of pancreatic cancer, but it was not possible to identify, whether the germline mutation was inherited from the father's or the mother's side. Regarding the North American families, the ATM mutation might be the reason for the diversity of malignancies in the family on the father's side of the index patient. It is important to detect and analyse further families with deleterious ATM mutations in germline DNA to identify a segregation of the mutation with the diagnosed cancers. This is the only way to define cancer entities that develop based on ATM mutations and to identify or exclude additional, mutated genes.

The *ATM* gene is not the only gene with pathogenic variants that result in different tumour entities. The most common example is the *BRCA2* gene that has been proven to be an FPC susceptibility gene (Murphy et al. 2002, Slater, Langer, Fendrich, et al. 2010), in addition to playing a role in the HBOC-syndrome (Ford et al. 1998). The reason that some mutations in the *BRCA2* gene lead only to breast cancer and some to pancreatic and breast cancer is not yet clarified.

Further studies in larger cohorts are needed to verify the frequency of *ATM* mutations in FPC families and to define the fact whether a single inherited heterozygous *ATM* mutation in germline DNA is able to cause this diversity of malignancies. Furthermore, it has to be determined, if mutations in certain regions of the *ATM* gene lead to an aggregation of only pancreatic cancer while families with different mutations have an aggregation of several different cancer entities.

As a consequence of including *ATM* to the FPC-susceptibility genes, further studies need first to identify, which influence routinely screening of FPC patients on pathogenic variants in *ATM* gene can achieve in clinical daily routine. Supposing, that single pathogenic variants in the named FPC-susceptibility genes can cause the high PDAC risk in FPC-families, there should be an intensified screening of individuals carrying the mutation. There further should no screening necessary of wild type tested individuals, as they then seem to have the same risk as the ‘German population’, which is not regularly screened on PDAC because of the low prevalence of the disease and the rapidly growing tumour. The second point to be tested in future studies is the influence of identified pathogenic variants in *ATM* gene on therapeutic treatment. As explained above (4.4.6), there are several approaches in mostly cell cultural studies, which show the possibility of using the damaged ATM-kinase in the tumour cells with chemotherapy to supplement the curative surgical treatment and the common chemotherapeutics with individualized drugs to improve the poor prognosis of PDAC.

# 5 Abstract

Pancreatic ductal adenocarcinoma (PDAC) is predicted to become the second leading cause of cancer related death in Germany by 2030. Despite extensive research in recent years, PDAC still has a dismal prognosis. About 5-10% of PDAC cases accumulate in families, due to the familial pancreatic cancer syndrome, other hereditary cancer syndromes or hereditary pancreatic dysfunctionality syndromes. Families with at least two first-degree relatives affected with PDAC without fulfilling the criteria of other cancer syndromes are defined as familial pancreatic cancer (FPC). So far *BRCA1, BRCA2, CDKN2A, PALB2, CHEK2* have been identified as susceptibility genes for FPC, but predisposing germline mutations in these genes have been identified in only about 10% of FPC families. Previously, it was hypothesized that the Ataxia telangiectasia mutated (*ATM)* gene might also be a low penetrance FPC susceptibility gene.

Therefore, we analysed 35 FPC families of the National Case Collection for familial pancreatic cancer (FaPaCa) registry by Whole Genome Sequencing, Sanger Sequencing and Multiplex ligation-dependent probe amplification (MLPA) to determine the role of ATM in FPC. A deleterious ATM germline mutation (X175Y) was detected 1 of these 35 FPC families according to a prevalence of 2.9%. Deleteriousness of the mutation tested positively using MutationTaster, PolyPhen2, SIFT and PROVEAN.

*ATM* can be considered as a low penetrance FPC susceptibility gene, which might also predispose to other cancers despite PDAC. Further studies are needed to clarify whether there might be a genotype/phenotype correlation.

# 6 German Abstract

Das duktale Adenokarzinom des Pankreas (PDAC) gewinnt in Deutschland und der westlichen Welt zunehmend an Bedeutung. Das ‘Deutsche Statistische Bundesamt’ prognostiziert bis 2030 eine Zunahme zur zweithäufigsten krebsbedingen Todesursache, denn trotz intensiver Forschung der letzten Jahre hat das Pankreaskarzinom weiterhin eine schlechte Prognose. Bei 5-10% der Patienten mit malignen Neoplasien des Pankreas, deckt die Anamnese weitere PDAC Fälle unter erstgradig Verwandten auf, weshalb bei der insgesamt sehr niedrigen Prävalenz dieser Erkrankung von einem erhöhten familiären Risiko mit weitgehend ungeklärtem molekularen Hintergrund gesprochen werden kann. Diese familiäre Häufung kann auf das ‘familiäre Pankreaskarzinom’ (FPC), andere hereditäre Karzinom-Syndrome oder Syndrome mit Dysfunktionalität des Pankreas mit daraus resultierender erhöhter Karzinomneigung zurückgeführt werden.

Per Definition müssen beim FPC mindestens zwei erstgradig Verwandte am Pankreaskarzinom erkranken, ohne dass sie gleichzeitig die Kriterien für ein anderes Tumorsyndrom erfüllen. Mutationen in den Genen *BRCA1, BRCA2, CDKN2A, PALB2* und *CHEK2* konnten in anderen Studien für die erhöhte Pankreaskarzinom Inzidenz in wenigen betroffenen Familien verantwortlich gemacht werden. In 80 - 90% der Familien sind in den genannten Genen keine Mutationen nachweisbar, deshalb bleibt hier die molekulare Ursache für das erhöhte Risiko weiterhin unklar.

Die Suche nach Mutationen im *ATM* Gen in 35 FPC Familien sollte klären, ob das, bereits als potentiell relevantes FPC-Gen beschriebene, *ATM-Gen* in die Liste der FPC-Gene aufgenommen werden kann. Hierfür wurde Leukozyten-DNA aus EDTA-Blut der FPC-Patienten mit ‘Whole Genome Sequencing’, ‘Sanger Sequencing’ und ‘MLPA’ auf Mutationen im *ATM* Gen untersucht. In einer von 35 Familien (2.9%) wurde eine pathogene Mutation identifiziert, deren Funktionsverlust für die ATM-Kinase mittels MutationTaster, PolyPhen2, SIFT und PROVEAN bestätigt werden konnte. Mit einer Mutations-Prävalenz von 2,9% in FPC-Familien der untersuchten Population

reiht sich ATM bei den bereits anerkannten FPC-Genen mit ihrer ebenfalls niedrigen Prävalenz ein. Zukünftige Studien müssen die Korrelation zwischen Geno- und Phänotyp bestätigen.

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# 8 Appendix

## *8.1 Ethikvotum*

## *8.2 Tabellarischer Lebenslauf*

## *8.3 Verzeichnis der akademischen Lehrer*

Meine akademischen Lehrenden in Marburg waren:

Bartsch, Becker, Becker, Cetin, Czubayko, Daut, Dettmeyer, Feuser, Fritz, Geraedts, Gress, Hertl, Hofmann, Hoyer, Kinscherf, Kircher, Kruse, Lill, Lohoff, Mahnken, Maier, Moll, Neubauer, Neumüller, Nimsky, Oberwinkler, Oliver, Opitz, Pagenstecher, Peterlein, Rastan, Renz, Richter, Ruchholz, Sahmland, Schieffer, Schneider, Schratt, Seitz, Sekundo, Stuck, Thieme, Timmermann, Timmesfeld, Vogelmeier, Wagner, Weber, Weihe, Worzfeld, Wrocklage, Wulf.

## *8.4 Danksagung*

## *8.5 Ehrenwörtliche Erklärung*