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DISRUPTION OF DNA DAMAGE RESPONSE PATHWAYS IN TUMORIGENESIS: INVESTIGATING THE ROLE OF ATM AND BAP1 IN HEREDITARY CANCERS.

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DECLARATION

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of "Good Scientific Practice".

Individuals and organizations I collaborated with during my PhD that have contributed to the research for this dissertation are:

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HEREDITARY CANCERS

Cancer is a multifactorial disorder, in which genetic aberrations play a major role. Indeed, cancer arise and progress due genomic aberration involving cell cycle control and DNA damage repair, which result in uncontrolled cell proliferation[1,2].

The majority of cancers are sporadic, and are due to acquired genomic aberration due to a combination of stochastic reasons, environmental exposures, there is a subset of cancers arise in familial contexts. Familial cancers are cancers that occur in multiple members of a family with a frequency higher than that expected by the incidence in the general population, and these families are frequently characterized by multiple cancers in one individual and/or early age of onset. Familial cancers may occur due to an underlying oncogenic germline DNA alteration, shared exposure to environmental risk factors, or both. Inherited pathogenic variants in a single gene characterize most hereditary cancer syndromes, which are often due to high-penetrance genes and in which cancer risk is transmitted with an autosomic dominant pattern of inheritance.

DNA DAMAGE RESPONSE AND CANCER PREDISPOSITION

DNA damage response (DDR) is composed by multiple pathways which lead to DNA repair or, if not possible, to apoptosis or cellular senescence upon single or double strand DNA breaks caused by oncogene induced replication stress or carcinogenic agents such as ultraviolet and ionizing radiations, reactive oxygen species (ROS), alkylating agents. Acquired dysregulation of one or more DDR pathways can lead to genomic instability, one of the hallmarks of cancer[2,3].

Moreover, several cancer predisposition syndromes have been linked to germline anomalies in the DDR pathways[4]. For instance, germline loss-of-function in the homologous recombination repair

(HRR) genes *BRCA1* and *BRCA2* underlie the hereditary breast-ovarian cancer predisposition syndrome (HBOC)[5]. Moreover, pathogenic variants in *BAP1,* a gene encoding for a deubiquitinase that interacts with *BRCA1*, are responsible of a rare multi-cancer predisposition syndrome. Pathogenic variants in the mismatch repair genes *MSH2* and *MLH1* cause Lynch syndrome, a hereditary syndrome characterized by increased susceptibility to colorectal and other gastrointestinal cancers, as well as malignancies of the ovary, urinary tract, brain and skin[6]. Among genes implicated in non-homologous end joinining (NHEJ) are *TP53*, whose germline variants cause Li-Fraumeni syndrome and *ATM*, which causes Ataxia-Telangiectasia[7] and is an established breast cancer predisposition gene[8].

PART I: *ATM* AND MELANOMA

GERMLINE MULTI-GENE PANEL SEQUENCING IN MELANOMA AND RELATED CANCERS

Our Cancer Genetics group's research is focused mainly on susceptibility to melanoma, as well as cancers included in the spectrum of hereditary multi-tumor syndromes that include melanoma in their spectrum.

Melanoma is the most frequently lethal form of skin cancer, and its incidence is increasing in countries with a high prevalence of light-skinned individuals. Most melanoma cases are sporadic, and the main predisposing causes are phototype and environmental risk factors, in particular light complexion combined with exposure to ultraviolet radiation.

However, up to 5-15% of melanomas arise in individuals belonging to melanoma-prone families or melanoma-related multi-cancer syndromes. The gene most frequently responsible for melanoma predisposition is *CDKN2A*, found in 20 to 40% of familial melanoma patients, whereas a small (<10%, but still to be clearly defined) proportion of familial patients carry a variant in other melanoma predisposition genes, namely *BAP1*, *POT1, MITF, ACD*. However, in more than half of high-risk melanoma patients, an underlying genetic cause cannot be determined.

In the last three decades, researchers' efforts coupled with a progressive improvement of DNA sequencing methods led to the discovery and validation of several melanoma predisposition genes, and additional candidate predisposition genes are currently being studied (**Figure 1**). However, despite these scientific advances, only part of melanoma heritability can currently be explained by an inherited pathogenic variant in a known melanoma predisposition gene. It is possible that additional high penetrance genes yet to be discovered contribute to the risk of melanoma. It is more likely however that, at least in part, the unexplained heritability could be due to inheritance of variants in low to intermediate penetrance genes, which are more difficult to identify due to the lack of complete

co-segregation with the disease. Moreover, a variable combination of multiple low-risk alleles and/or their interaction with the environment could play a role in melanoma development (**Figure 2**).

In an effort to to determine whether multi-gene panel testing could lead to an increased mutational yeld of known melanoma-predisposition genes, and identify novel melanoma-predisposition genes to tackle the issue of missing heritability, our research group performed germline genetic testing on cohort of 273 *CDKN2A*/CDK4 negative malignant melanoma index patients enrolled at multiple Italian centers and considered high-risk based on current criteria[9], using an in-house custom panel which included *POT1, BAP1, MITF* (for diagnostic purposes) and the novel candidate melanoma susceptibility genes *TERT, ACD, TERF2IP, MC1R*. In addition, *ATM* and *PALB2*, candidate pancreatic cancer susceptibility genes, were included in our panel due to the presence of *CDKN2A*/*CDK4* negative families prone to both melanoma and pancreatic cancer.

This study showed that panel testing proves to be advantageous in the clinical practice, as it provided an increase of 6% of the mutational yeld in the diagnostic setting compared to genotyping of *CDKN2A* and *CDK4* alone. However, an even more interesting result derived from the assessment on novel candidate predisposition genes, as *ATM* pathogenic variants were found in 6 out of the 273 patients enrolled in the study (2,6%). This result prompted us to carry out the following study.

GERMLINE *ATM* VARIANTS PREDISPOSE TO CUTANEOUS MELANOMA

BACKGROUND AND RATIONALE

The Ataxia-Telangiectasia mutated (*ATM*) gene was firstly identified by linkage analysis and positional cloning[10]. *ATM*[1q22.3] is a 146619 nucleotides gene, which encodes for a 370-kDa serine-threonine kinase protein composed of 3055 amino acids[11].

ATM is a serine/threonine kinase belonging to the PI3/PI4-kinase family protein that plays a key role in genome stability, being part of several cell cycle pathways involved in DNA damage response. In particular, *ATM* is activated upon DNA double strand breaks caused by ionizing radiations, ultraviolet radiation and oxidative stress (ROS), and is involved in non-homologous end join, among other functions (**Figure 3**). Indeed, null *ATM* cells have a high rate of chromosomal aberrations, and show lack of DNA damage repair following exposure to ionizing radiations.

Homozygous loss-of-function (LOF) *ATM* variants cause Ataxia-Telangiectasia (AT), also known as Louis-Bar Syndrome, an autosomal recessive syndrome characterized by progressive cerebellar degeneration, ocular telangiectasias, immunodeficiency and radiosensitivity[7]. Moreover, hematologic malignancies and solid cancers have been found in association with AT, such as breast cancer, gastric cancer and pancreatic cancer[12].

Following the detection of an increased incidence of cancer, especially breast cancer in heterozygous parents of AT patients, epidemiological studies showed that truncating *ATM* variants, as well as specific missense variants moderately increases the risk of breast cancer. However, the heterogeneity of risk magnitude across different cohort studies, as well as the variability of familial co-segregation

for different types of variant in the same study cohort[13]. suggests that different *ATM* variants may confer cancer risk with different penetrance.

In addition to breast cancer, there have been several reports of *ATM* association with other cancers. For instance, *ATM* pathogenic and likely pathogenic variants are enriched in familial pancreatic cancer, suggesting that *ATM* is a pancreatic cancer predisposition gene[14]. Moreover, *ATM* has been implicated in gastric cancer and prostate cancer [15,16], suggesting that the real extent of *ATM* tumor spectrum is likely broader.

Even in cancers for which *ATM* link has been established, such as breast cancer, effects on clinical practice are heterogeneous in different countries, ranging from annual mammographic screening from the age of 40 in the USA/Canada/GB, to the choice of not reporting *ATM* results from breast-ovarian cancer syndrome diagnostic multi-gene panels in France[17–19].

In 2008, a population study found that a specific *ATM* common variant, S49C, was more frequent in melanoma patients compared to the general population[20]. Moreover, in a recent Genome-wide association study (GWAS) carried out by the GenoMel consortium, two variants in the *ATM* gene were associated with melanoma[21,22]. Conversely, a study conducted on melanoma-prone families found specific *ATM* variants in a subset of families, but the global burden test on rare variants showed no relationship between *ATM* and melanoma[23].

AIM OF THE STUDY

Based on our preliminary findings on *ATM* pathogenic variants in cutaneous melanoma mentioned in the previous chapter, we started and coordinated a multi-centric study with the collaboration of the GenoMEL and MelaNostrum research consortia to assess whether *ATM* is a melanoma predisposition gene.

PATIENTS AND METHODS

Study datasets

From 10 countries of the GenoMel and MelaNostrum consortia across Europe, the United States and Australia, we retrospectively collected information on germline *ATM* status of 1936 cutaneous melanoma cases, either probands of melanoma-prone families, apparently sporadic multiple primary melanoma cases, or sporadic melanoma cases belonging to case-control study cohorts. *ATM* genotyping was performed via panel, exome or whole genome sequencing at each recruiting centers.

The Genome Aggregation Database (GnomAD), version 2.1, consists of 125748 exomes and 15708 genomes from 141456 germline DNA samples belonging to individuals enrolled in disease-oriented and population case-control studies. Considering that, unlike the GnomAD database, our study cohort includes cases enrolled in countries with predominantly non-Finnish European ancestry, we only analyzed the GnomAD Non-Finnish European (NFE) subset, which consists of 64603 individuals (56885 exomes and 7718 genomes).

Variants selection

Out of all *ATM* variants found in our study cohort, the GnomAD cohort or both, nonsense, frameshift, splice acceptor and splice donor variants were considered LOF and included in this study.

Filtering of missense variants was done according to a frequency criterion. Considering that AT prevalence worldwide ranges from 1:40000 to 1:100000[24], the estimated allele frequency of AT heterozygotes is 0.002-0.005. Therefore, all missense variants with an AF above 0.005 were considered likely benign and excluded from this study. Moreover, missense variants found homozygous in more than two GnomAD subjects or whose annotation was considered unreliable in GnomAD were also excluded. All remaining missense variants were considered VUS and included.

Statistical analysis

Prior to assess whether the frequency of *ATM* variants differed between the GenoMEL cohort and the GnomAD cohort, we grouped all LOF variants together and considered them as a single variable, and we performed the same grouping on VUS.

We then compared the (AF) of selected *ATM* variants in our cohort and in GnomAD non-Finnish Europeans (NFE) using Fisher's exact test.

All analyses were two-sided and a Bonferroni-corrected p-value of 0.05 was used as a cut-off for significance.

RESULTS

Our study cohort consisted of 1936 cases (941 apparently sporadic from case-control cohorts, 261 apparently sporadic MPM, and 734 probands of melanoma-prone families). After filtering, we included 1006 unique *ATM* variants, 158 LOF (15 found in the Genomel db, 140 in GnomAD db, 3 in both) and 848 unique VUS (42 found in the Genomel db, 731 in GnomAD db, 75 in both).

In our study cohort we found 19 LOF alleles in 10 familial, three MPM cases, and six sporadic SPM cases. Moreover, 193 VUS alleles were found in 136 familial, 22 sporadic MPM and 35 sporadic SPM cases. The NFE subset of the GnomAD cohort consists of 64603 individuals, for a total allele count of 129206. In this dataset, 4468 alleles had an *ATM* variant, 223 LOF and 4245 VUS.

Overall, *ATM* variants were more represented in our study cohort then in the GnomAD NFE cohort (AF 0.05475207 and 0.03458044, OR= 1.6, CI=1.39 - 1.85, p= 7.358e-10). The highest difference was observed for LOF variants (AF 0.005 and 0.002, OR=2.68, CI=1.59-4.29, p=0.0002), especially when we restricted the analysis to familial and MPM cases only (AF 0.006 and 0.002, OR=3.66, CI=1.66-5.54, p=0.0004).

VUS showed a similar, albeit smaller, association, both when considering all cases (AF 0.05 and 0.033, OR=1.53, CI=1.32-1.78, p=7.947e-08) and the familial/MPM subset (AF 0.07 and 0.033, OR=2.22, CI=1.87-2.61, p< 2.2e-16).

ATM genotyping on all affected family members was only available for four probands. Of these, two showed complete co-segregation of the variant with melanoma. One family showed partial co-segregation, whereas in another family only one out of six affected members had the variant.
Family 11 showed no co-segregation, as only one of three analyzed affected family members had the variant; however, no information is available of four additional family members, who had melanoma but were not genotyped for *ATM* (**Table 3**).

Of all probands with LOF variants, three individuals were also diagnosed with a non-melanoma cancer: one had CLL, another had endometrial cancer, whereas a third individual had a diagnosis of pancreatic cancer after being diagnosed with melanoma. Non-melanoma ATM-related tumors were found in a subset of families: patient 18 had a sister with breast cancer, whereas the mother of patient 17 was diagnosed with pancreatic cancer. Two more probands, patients 14 and 19, had a second-degree relative diagnosed with pancreatic cancer; in one case, however, the relative was more than 80 years old at the time of diagnosis

DISCUSSION AND CONCLUSION

Despite the technological advances that have occurred during the last decades that led to multiple discoveries in the study of the human genome and cancer, several questions are still partially unanswered. For instance, the majority of heritability of melanoma cannot be explained by germline pathogenic variants in a single established predisposition gene and, on the other hand, an increased

prevalence of melanoma cases is being found in known familial cancer syndromes for which an association with melanoma was not previously established.

An example is *ATM*, an intermediate breast cancer susceptibility gene that is also a melanoma GWAS hit and may be implicated in melanoma susceptibility. However, *ATM* is a long gene, with a high probability of genomic variation due to stochastic reason. Moreover, epidemiological studies on breast cancer cohorts show that *ATM* it confers cancer risk with an intermediate penetrance with incomplete co-segregation in families. Considering that the penetrance for melanoma may be analogous or even lower than that of breast cancer, collecting information on pedigrees large enough to investigate cancer predisposition is a challenge. An additional level of uncertainty is presented by the possibility that different types of variants may confer variable cancer risk, which can be hypothesized based on prior knowledge deriving from studies on AT patients and high-risk breast cancer patients[13].

However, to our knowledge, this is the first hypothesis-driven study to investigate the presence of *ATM* rare variants in a large international multicentric melanoma cohort. Here, we demonstrate that *ATM* pathogenic variants are more frequent in melanoma patients compared to that in the GnomAD database, suggesting that *ATM* may be a melanoma predisposition gene. Indeed, the association with melanoma reached its maximum in familial cases, and the analysis of available pedigrees showed co-segregation with melanoma in a subset of these families. VUS association with melanoma was weaker, albeit present, than that of LOF variants. A possible explanation is that, as for AT, missense variants confer a lower risk compared to truncating variants. However, at least in part, an intragroup heterogeneity in terms of pathogenicity might have affected our results. Indeed, although we only included among VUS rare missense variants that were not found in homozygous healthy individuals, *ATM* is a long gene with 63 exons and, therefore, here is a high probability that innocuous single nucleotide substitutions occur for stochastic reasons. Therefore, a variable amount of non-pathogenic

variants in this group might have diluted an association of missense pathogenic variants with melanoma.

Our study design presents some limitations. The unavailability of healthy individuals from the majority of the study groups made it impossible to perform a case-control study with in-house controls and, therefore, we reverted to comparing our study cases against population controls. Aware of the risk of population stratification bias due non-overlapping distribution of ethnic groups, we chose as our control group only the GnomAD NFE cohort, as the affected individuals in our study cohort either come from European countries excluding Finland, and melanoma patients from USA and Australian centers are likely to be composed mainly of individuals of European descent. However, even with this adjustment, the possibility of population stratification bias cannot be completely excluded.

In addition to that, although cleared of individuals with severe pediatric conditions and their affected relatives, the GnomAD dataset is composed of several studies datasets, it includes cancer cohorts, such as the TCGA, and, therefore, the detection of pathogenic *ATM* variants in this dataset could be due, at least in part, to the presence of affected individuals with *ATM*-induced germline cancer predisposition. Therefore, the real association of *ATM* pathogenic variants with melanoma could be actually higher than the one we observed. To avoid the inclusion of TCGA samples, the analysis could have been performed on the whole-genome sequencing (WGS) GnomAD subset but, as only a minority of our study samples were sequenced by WGS, this option would have increased sequencing platforms differences between cases and controls. We also chose not to limit our assessment to the GnomAD controls (control groups of case-control studies for common diseases) and non-cancer (individuals not ascertained for having a neoplasm) subsets because they are not a guarantee of being a cancer-free control group, as controls for diseases other than cancers might have had a cancer diagnosis prior or even at the time of enrollment, depending on the study design and the condition(s) being studied, such as for instance psychiatric disorders.

If confirmed by further studies, our results could provide benefits in the clinical setting. In the era of personalized medicine, DNA damage repair genes are promising targets for novel cancer therapies. PARP inhibitors, for example, are used for *BRCA1*/2-positive breast, ovarian and pancreatic cancer[25,26] and have been FDA approved in the USA for castration-resistant *ATM*-deficient prostate cancer following a recent clinical trial[27], but recent studies have shown their potential role in the treatment of other *ATM*-deficient cancers[28–30].

This study is the preliminary part of a broader project aimed at exploring the link between *ATM* and melanoma. In addition to LOF variants, rare missense variants were enriched in our melanoma cohort. To gain a clearer picture on *ATM* impact in melanoma, it will be necessary to carry out pathogenicity assessment of rare missense variants through functional testing. Moreover, an assessment of *ATM* penetrance in melanoma will be crucial to determine the potential clinical utility of germline *ATM* assessment in terms of surveillance.

Figure 1. Timeline showing the discovery of melanoma risk genes/loci over time. Genes in italics are high risk candidate genes of recent identification

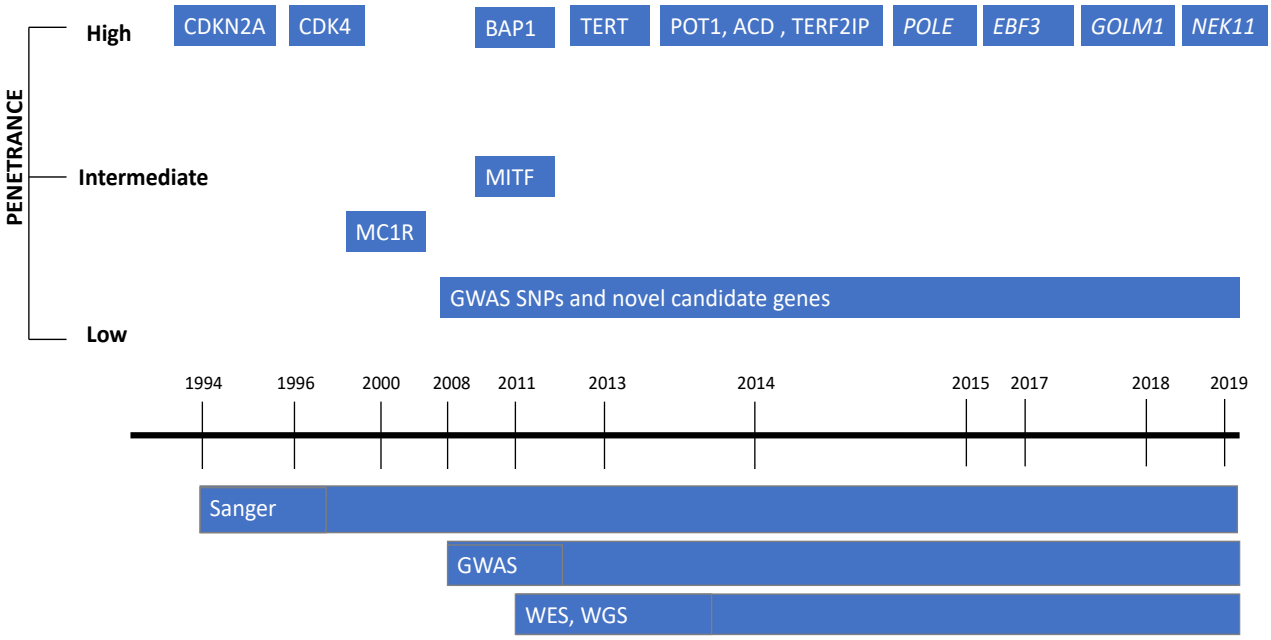


Figure 2. Overview of melanoma susceptibility: interplay between genetic, host and environmental factors

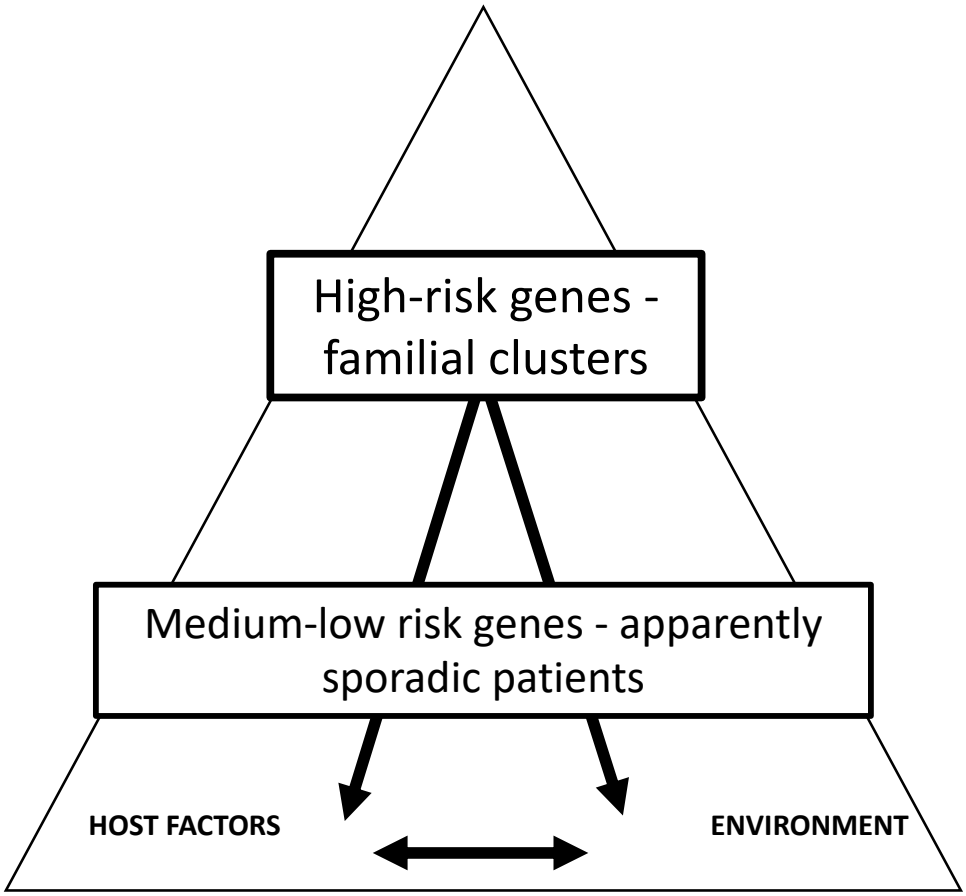
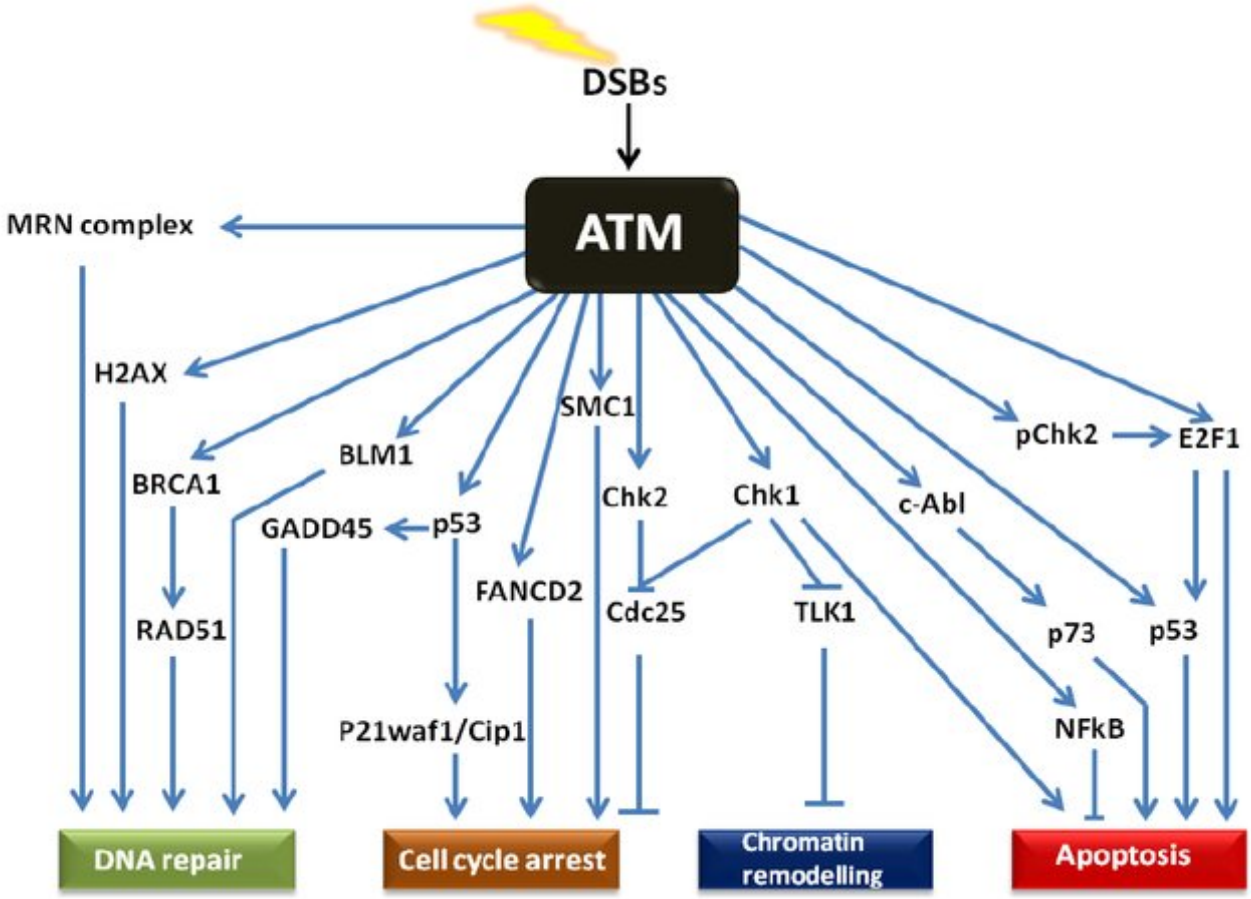


Figure 3.



From Khalil, Hilal S., et al. "Targeting ATM pathway for therapeutic intervention in cancer." Biodiscovery 1 (2012): e8920.

Figure 4. ***ATM*** **variants selection criteria**

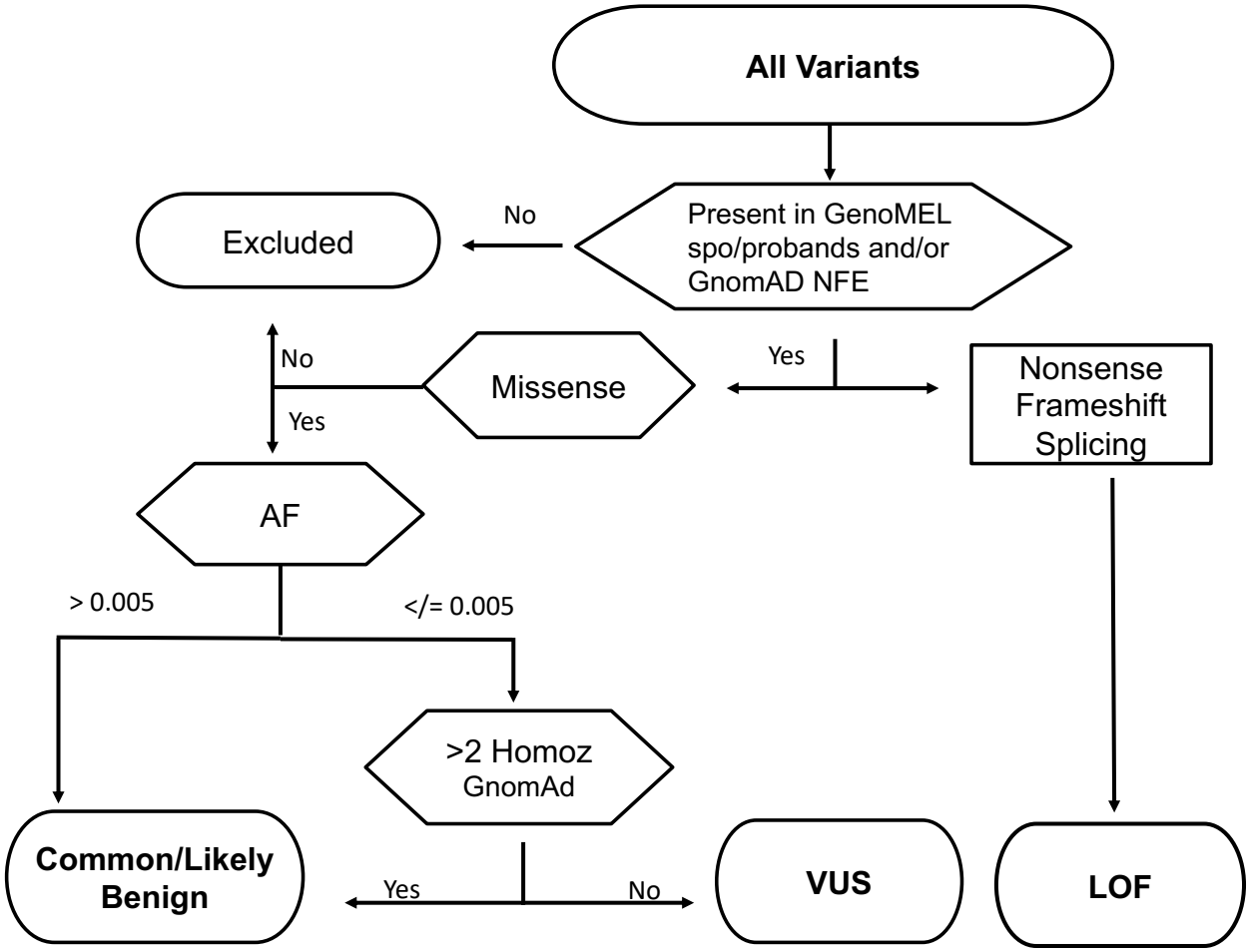


Table 1. Frequency of *ATM* LOF variants in the GenoMEL cohort and in the GnomAD database

	GenoMel (All)	GenoMEL (Fam + MPM)	GnomAD NFE
LOF alleles	19	13	237
Total alleles	3872	2246	129206
AF	0.005	0.006	0.002
OR* (95%CI)	2.68 (1.59-4.29)	3.66 (1.66-5.54)	
p-value	0.0002	0.0004	

OR= odds ratio, CI= confidence interval. *Odds of finding in the GenoMEL cohort compared to the odds of finding the variants in the GnomAD NFE cohort

Table 2. Frequency of *ATM* VUS in the GenoMEL cohort and in the GnomAD database

	GenoMel (All)	GenoMEL (Fam + MPM)	GnomAD NFE
VUS alleles	193	158	4268
Total alleles	3872	2246	129206
AF	0.05	0.07	0.033
OR* (95%CI)	1.53 (1.32-1.78)	2.22 (1.87-2.61)	
p-value	7.947e-08	2.2e-16	

OR= odds ratio, CI= confidence interval. *Odds of finding in the GenoMEL cohort compared to the odds of finding the variants in the GnomAD NFE cohort

Table 3. Overview of probands harbouring *ATM* LOF variants

Country	Selection criteria	ID (anonimized)	N affected members in family	N affected members sequenced in family	N affected members with the variant/total affected members	Co-segregation	Nucleotide base change	Amino Acid Change	Effect of variant	Total number of melanomas
Norway	MPM	1	1	1	1/1	-	c.3244_3245insTG	p.H1082fs	FS	3
Norway	MPM	2	1	1	1/1	-	c.3284+1G>A		Splice	3
USA	S	3	1	1	1/1	-	c.717_720del		FS	1
USA	S	4	1	1	1/1	-	c.902/1G>T		Splice	1
USA	S	5	1	1	1/1	-	c.6227delT		FS	1
USA	S	6	1	1	1/1	-	c.7629+2T>C		Splice	1
USA	S	7	1	1	1/1	-	c.7928/2A>T		Splice	1
France	F	8	3	1	1/1	-	c.8850+2insA(IVS61)		Splice	3
Australia	F	9	3	3	3/3	Yes	c.1236/2_1236/1insG	p.W412fs	FS	NA
Australia	F	10	6	6	1/6	No	c.7878_7882del	p.I2629Sfs*24	FS	1
Australia	F	11	3	3	2/3	Partial	c.7828_7829del	p.R2610Kfs*1	FS	12
Denmark	F	12	7	3	2/3	Partial	c.1236/3_1237delinsATTT		FS	
Australia	F	13	3		3/3	Yes	c.1561/1562	p.R521X	NS	
Italy	S	14	1	1	1/1	-	c.3275C>A	p.Ser1092Ter	NS	1
Italy	F	15	2	1	1/2	-	c.3576G>A	p.Lys1192=	Splice	2
Italy	F	16	2	1	1/2	-	c.3576G>A	p.Lys1192=	Splice	1
Italy	F	17	2	1	1/2	-	c.4451delT	p.Met1484ArgfsTer15	FS	2
Italy	MPM	18	1	1	1/1	-	c.5979_5983delTAAAG	p.Ser1993ArgfsTer23	FS	3 (1CMM, 2UM)
Italy	F	19	2	1	1/2	-	c.8319_8323dupTGTCC	p.Pro2775LeufsTer33	FS	3

PART II: *BAP1*-TUMOR PREDISPOSITION SYNDROME AND BREAST CANCER

BAP1 -TUMOR PREDISPOSITION SYNDROME

The *BRCA1* associated protein-1 (*BAP1*) [3p21.1] is a 9343 bases long gene which encodes for a deubiquitinase with tumor suppressor activity. Its name derives from its ability to bind the *BRCA1* RING finger domain[31], and a recent study suggested that *BAP1* can regulate *BRCA1*/ BARD1 heterodimer activity[32]. Moreover, *BAP1* has been shown to interact with a wide array of other proteins, and, although the mechanisms by which it exerts its tumor suppressor functions are largely unknown, *BAP1* is involved in multiple processes, such as DNA damage response and chromatin repair, endoplasmic reticulum metabolic stress response, cell cycle regulation, cell growth and inflammatory response[33–42].

BAP1 somatic loss-of-function variants are a common feature of several solid tumors[43–48]. Moreover, germline loss-of-function variants in this gene are responsible for the *BAP1* Tumor Predisposition Syndrome (*BAP1*-TPDS), a hereditary condition characterized by susceptibility to specific types of cancers[49]. Uveal melanoma, malignant mesothelioma, clear cell renal cancer and cutaneous melanoma are the most frequent tumors in *BAP1*-TPDS families, and are therefore considered the core tumors of the syndrome[50–54] and, especially in the case of mesothelioma, BAP penetrance can be enhanced by the concurrent exposure to environmental carcinogens, such as asbestos[55]. Other types of cancer have been reported in families with germline *BAP1* pathogenic variants[56–66]. However, the *BAP1* syndrome is rare as, although its prevalence is not known, it is estimated to be 1/53015 individuals[67], and it is difficult to obtain study samples adequate to perform association studies.

COLLABORATIVE META-ANALYSIS ON *BAP1* AND CANCER PREDISPOSITION

In the last two years, we participated in a meta-analysis coordinated by Prof. Nicholas Hayward of QIMR Berghofer Medical Research Institute (Brisbane, QLD, Australia) that included both 106 published and 75 unpublished families with *BAP1* LOF variants, the largest *BAP1* study cohort to date, and was aimed at identifying the actual tumor spectrum of the *BAP1*-TPDS, as well as the gene's penetrance for each tumor[68].

Based on the results of this meta-analysis, the updated numbers on *BAP1*-TPDS are as follows:

- The total number of known *BAP1* unique germline variants has increased to 140 (104 LOF and 36 missense);
- Individuals with *BAP1*-TPDS-related cancers had a lower age of onset compared to US individuals with the same cancer from the Surveillance, Epidemiology and End Result Program (SEER) database[69], and the difference was even higher when considering LOF variants only;
- Uveal and cutaneous melanoma, mesothelioma and renal cell carcinoma are confirmed to be the most frequent tumors in the *BAP1*-TPDS spectrum. However, the spectrum should also include nonmelanoma tumors of the skin (especially basal cell carcinoma), meningioma and cholangiocarcinoma.

After the meta-analysis, we included additional published and unpublished families in the *BAP1* database of the Oncogenomics Laboratory (QIMR Berghofer, AU) and the BAP1 Interest Group (BIG), reaching a total of 373 families. Interestingly, in 181 families with LOF BAP1 variants, 27 (15%) included members with breast cancer.

GERMLINE *BAP1* VARIANTS IN BREAST CANCER PATIENTS

BACKGROUND AND RATIONALE

Breast cancer is the second most common cancer in the world, as well as the most commonly diagnosed cancer and the leading cause of cancer death among women[70]. Although the majority of breast cancers are sporadic, a small proportion of breast cancer arise in cancer-prone families[71]. Pathogenic variants in the high-penetrance *BRCA1, BRCA2*, *CDH1*, *PTEN*, *TP53* and *STK11* genes account for no more than 25% of high-risk individuals, whereas less than 3% of breast cancer risk can be attributed to intermediate-penetrance genes, namely *ATM*, *PALB2*, and *CHEK2*[72–78]. However, in the majority of familial breast cancers, the predisposing cause cannot be identified, and although pathogenic variants in other genes such as *BRIP1*, *RAD51C* and *RAD51D* have been found in high-risk individuals and families, reports are equivocal, and their role in breast cancer is still debated[79–82]. Therefore, researchers are exploring the possibility that novel yet unknown high-risk predisposition genes, or different combinations of multiple low-penetrance alleles and environmental risk factors, might be responsible of breast cancer predisposition in some families.

Recently, somatic biallelic inactivation of the *BAP1* gene in breast cancer samples has been reported in the COSMIC database[83]. Moreover, it has been suggested that *BAP1* loss leads to chromosomal instability in breast cancer cells[33].

The nature of the relationship between breast cancer and the *BAP1*-TPDS is, however, difficult to assess, as studies published so far have provided conflicting results[68,84,85]. Besides, the occasional presence of breast cancer in families with *BAP1*-TPDS could merely reflect random chance, due to the high incidence of this tumor type in the general population. Based on these considerations, we

performed this study to assess, through the analysis of both published and novel families, whether *BAP1* plays a role in breast cancer susceptibility.

AIM OF THE STUDY

To assess whether the *BAP1* gene is implicated in breast cancer susceptibility.

PATIENTS AND METHODS

6088 high risk Breast cancer (familial and/or young) patients, were recruited during Genetic counselling at the Parkville Familial Cancer Centre (Melbourne, AU). In addition, a series of 5847 apparently healthy controls, belonging to the Lifepool cohort[86] was included in the study. Both cases and controls, were tested through comprehensive breast cancer panels using Agilent HaloPlex technology were negative for *BRCA1* and *BRCA2* germline pathogenic variants.

Loss of heterozygosity assessment was performed on available tumor samples using a mouse monoclonal antibody raised against amino acids 430–729 of human *BAP1* (C-4, Cat. no. sc-28383; Santa Cruz, USA)

RESULTS

Overall, we found 4 LOF variants, in 7 cases and 1 control (Table 3). Moreover, we found 58 missense variants in 59 cases (0.9%) and 38 controls (0.6%). One of the missense variant, the c.1735G>A was present in 11 cases, all of East Asian origin.

Tumor samples, which were available for three of the seven cases with LOF variants and for six of the 59 cases with missense variants, were evaluated for loss-of heterozygosity through Immunohistochemistry. All three samples with the c.783+2T>C splice donor variant showed no nuclear expression of the *BAP1* protein.

As for the missense variants, one case with the c.1946G>A (p.Cys649Tyr) showed LOH, whereas a second case retained nuclear *BAP1* expression. Similarly, the c.944A>C (p.Glu315Ala) was found in

two cases, one with and one without LOH. A fifth breast cancer case showed LOH for the c.176G>A (p.Arg59Gln) variant, whereas for the sixth case, who had the c.1547C>T (p.Pro516Leu) variant, the analysis failed.

Although the c.783+2T>C is a canonical splice variant, a functional analysis with a minigene assay, to confirm that this variant affects splicing is ongoing (no RNA sample from the patient was available). Moreover, we plan to perform functional study to assess the potential pathogenicity of the two missense variants that showed LOH.

DISCUSSION

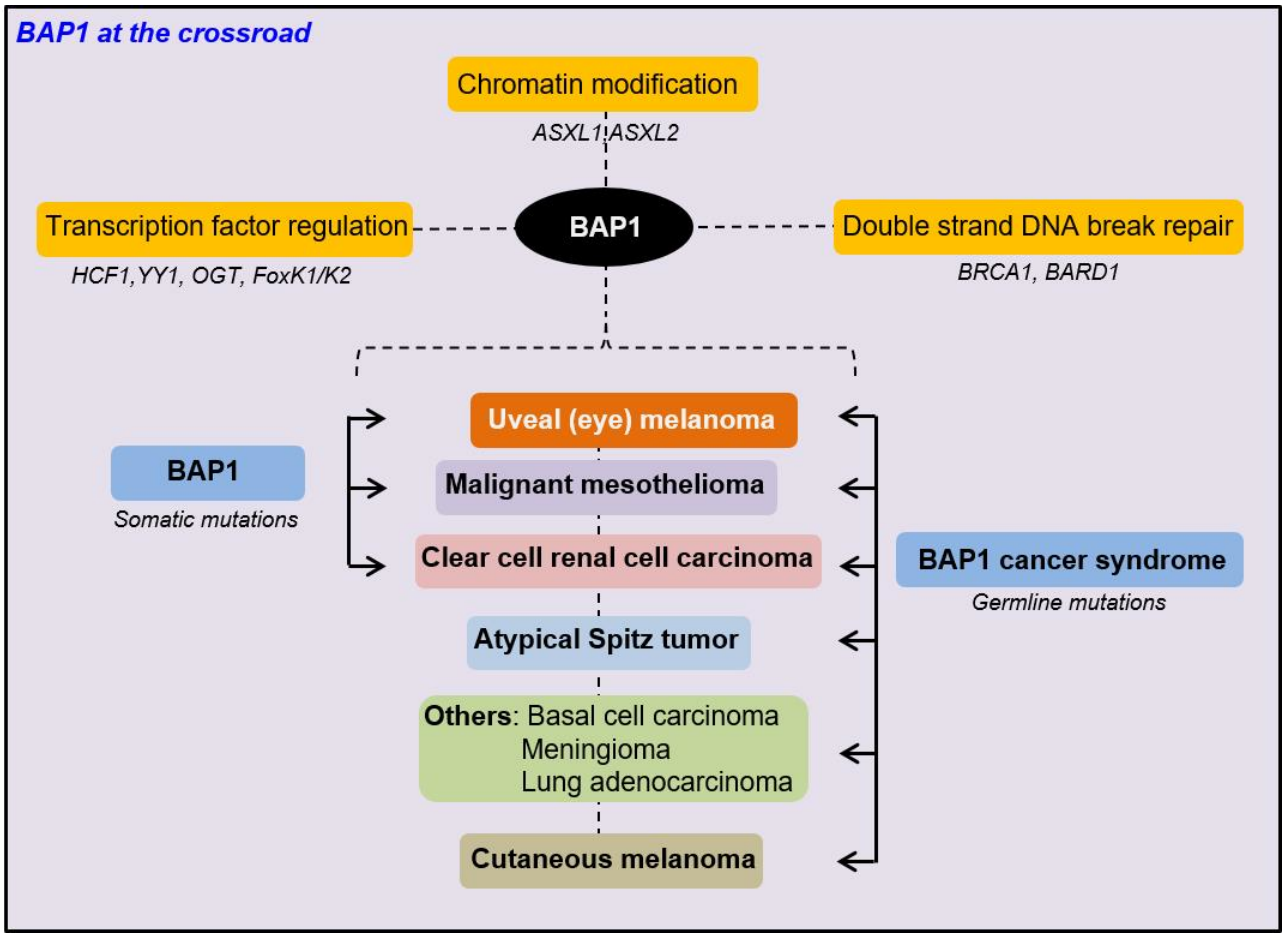
Molecular links between *BAP1* and *BRCA1* and the occasional finding of breast cancer individuals among relatives of individuals with *BAP1*-TPDS have led researcher to hypothesize that *BAP1* may be a breast cancer predisposition gene[60]. However, breast cancer is a high incidence cancer whose occurrence may be unrelated to the *BAP1*-TPDS, in which it hasn't been found at frequencies higher than expected, and LOH assessment of tumor samples from breast cancer relatives of *BAP1*-TPDS individuals is seldom performed, although in one study IHC showed LOH in one individual with breast cancer[83].

A reason for the fact that the majority of individuals and families with *BAP1* do not develop breast cancer may be due either the occurrence of different phenotypes depending on different variants, or to the influence of genes modifiers of penetrance and/or phenotype. The first of the two hypothesis stems from the knowledge that for other genes, such as *ATM*, there are reports of milder or different phenotypes for rare LOF variants and missense variants. However, c.783+2T>C, the only LOF variant for which we could assess and demonstrated LOH, has already been found in a family satisfying the criteria for *BAP1*-TPDS (three first degree relatives diagnosed with mesothelioma, unpublished data). On the other hand, the hypothesis that unknown modifiers could influence the prevalent tumor phenotype in families is intriguing. We already know that, although several cancers

have been included in the *BAP1*-TPDS spectrum, there are *BAP1*-deficient individuals with only one or two prevalent BAP-1 related tumors in the family, which may be due to variants in additional genes and/or environmental modifying factors. Interestingly, none of the cases with LOF variants, nor those with missense variants showing LOH had a single criteria for the BAP-TPDS. Namely, the only other cancer found in a subset of these families, besides breast cancer, was colorectal cancer.

Apart from high-risk and familial cases, breast cancer is a common cancer in the general population and, regardless of its possible association with *BAP1* in terms of susceptibility, it can occur also by chance. Therefore, the introduction of breast cancer in the spectrum of the *BAP1*-TPDS could represent a challenge in terms of assigning a weighted value to breast cancer within the diagnostic criteria of the syndrome. Given this major issue, we believe that the relationship between *BAP1* and breast cancer needs further investigation, in terms of pathogenicity of variants found in relationship to the specific cancer, also with LOH assessment and functional validation of variants found at the germline level.

Figure 5



From Sharma, Amit, et al. "Mutational Landscape of the BAP1 Locus Reveals an Intrinsic Control to Regulate the miRNA Network and the Binding of Protein Complexes in Uveal Melanoma." Cancers 11.10 (2019): 1600.

TABLE 3. Germline *BAP1* variants found in breast cancer cases and healthy controls

cDNA change	Protein change	Effect of the variant	N cases	N controls
c.37+1G>T	-	splicing	1	0
c.783+2T>C	-	splicing	5	0
c.876_880delCCCGC	p.Pro293GlyfsTer12	missense	1	0
c.1203T>G	p.Tyr401Ter	missense	0	1
			7	1

PART III: OTHER PROJECTS

CDKN2A AND SURVIVAL IN CUTANEOUS MELANOMA PATIENTS

CDKN2A is a three-exon gene located on chromosome 9p21.3 encodes for two protein, p16INK4 and p14ARF, and is a regulator of cell cycle progression. Although not being a DNA damage repair gene itself, *CDKN2A* expression is essential for the correct functioning of DNA damage response mechanisms. Indeed, p14ARF presence is crucial in p53 stability, as it prevents p53 degradation by MDM2[87].

BACKGROUND AND RATIONALE

The incidence of malignant melanoma in Italy is rapidly increasing [88,89]. Although most melanoma cases are sporadic and linked to interactions between phenotype and environmental risk factors, 6-12% of all melanoma patients are clustered within melanoma-prone families, up to 40% of which harbour inherited germline variants in the Cyclin Dependent Kinase Inhibitor 2A (*CDKN2A*)/p16Ink4 tumor suppressor gene [9,90,91]. Individuals harbouring *CDKN2A* pathogenic variants have a lifelong increased risk of developing MPM, as well as other cancers, and at a younger age compared to their sporadic counterpart[92–94]. In fact, the occurrence of pancreatic cancer in those individuals is estimated to be up to 17% [95–100].

CDKN2A pathogenic variants are found in up to 2% of individuals diagnosed with melanoma, and the prevalence of these variants is 20- 40% in melanoma-prone families depending on the country, being inversely associated with local incidence of melanoma [92,101,102]. Moreover, *CDKN2A* penetrance can be modified by several environmental factors, such as tobacco[103,104], and molecular factors. Among the latter, Melanocortin 1 Receptor gene (*MC1R*) variants seem to increase

the risk of melanoma, although studies performed on our population have not confirmed, as yet, this finding [93,105,106].

Melanoma prognosis is influenced by several factors, such as tumor thickness at diagnosis, nodal status, ulceration and mitotic rate [107]. Moreover, several studies are investigating gene expression signatures and other molecular tools to help estimate melanoma prognosis[108].

Recently, a study conducted on a Swedish cohort showed that germline mutation positive (MUT+) familial melanoma patients had worse prognosis, in terms of survival, compared to germline mutation negative (MUT-) and to untested sporadic melanoma patients [109]. In this Swedish study, MUT+ and MUT- patients from melanoma-prone families underwent similar follow-up programmes. Similarly, *CDKN2A* pathogenic variants have been associated with worse survival in another Swedish study involving individuals affected by multiple primary melanomas (MPM) [110].

Our population is among those with the highest incidence of *CDKN2A* germline pathogenic variants, mainly due to the founder effect of the p.G101W variant [91,111–113]. The multidisciplinary melanoma group in our hospital provides an intensive follow-up regimen for all affected individuals harbouring a *CDKN2A* pathogenic variant, regardless of family history. Despite the inherited risks of multiple melanomas and of other malignancies, no information on the *CDKN2A* effect on our melanoma patients' prognosis in terms of survival has been available thus far. Based on the above-mentioned findings, and considering that variants in the same genes can lead to non-identical effects in different populations (due to other interfering genetic/environmental factors), we decided to investigate the relationship between *CDKN2A* and survival in our melanoma cohort.

AIM OF THE STUDY

To assess the impact of *CDKN2A* gemline variants in an Italian cohort of melanoma patients undergoing a mutation-specific follow-up.

PATIENTS AND METHODS

Patients characteristics

Between 2000 and 2015 we enrolled, among all patients diagnosed with melanoma in our hospital, a consecutive series of 1239 patients on whom we performed genetic testing for *CDKN2A* and collected and stored clinical and pathological information.

Part of this cohort consisted of probands of melanoma-prone families and apparently sporadic patients with MPM, tested either for diagnostic or research purposes. Additionally, a series of apparently sporadic single melanoma patients, on whom genetic testing was performed for research purposes, was included. For 448 patients, molecular and clinical information has previously been described [106].

Follow-up was carried out according to standard practice of our hospital. Briefly, follow-up of *CDKN2A* mutation positive (MUT+) patients is carried out by our multidisciplinary team every four months starting from diagnosis, regardless of familial status, or more frequently if justified by tumor stage according to the guidelines of the Italian Association of Medical Oncology (AIOM)[88]. Skin examination by a dermatologist is performed every four months. Familial *CDKN2A* mutation negative (MUT-) patients are followed-up at least every 6 months, or more frequently if justified by phenotype or tumor stage. Follow up of sporadic MUT- patients is carried out at least every six months for five years, and then once a year, according to phenotype and tumor stage. Skin examination by a dermatologist is performed every six months for five years, then once a year (or every six months even after five years for patient with high-risk phenotype).

Collection of clinical and pathological data

Clinical information was obtained through a questionnaire, administered by a trained interviewer, and included: personal information, phenotype, personal/family history of other melanomas and other

tumors as previously described[91,106]. Clinical records and/or local cancer registry data were used to collect follow-up information and confirm causes of death. Pathological information included: tumor histology and staging according to AJCC's TNM staging system [114].

Molecular Analysis

All patients provided a blood sample from which we extracted genomic DNA. Sanger sequencing to assess the mutational status of *CDKN2A*, *CDK4* and *MC1R* were performed as previously described [106,115].

Selection of patients for Survival Analysis

From our cohort of 1239 patients, we filtered out all patients with incomplete data concerning follow-up, mutational status, stage, and those with non-cutaneous melanoma. Subsequently, we selected all MUT+ patients and grouped them together, regardless of familial status. Moreover, we selected a second group of MUT- patients, matched by age and sex, and with similar tumor stage distribution. Since our follow-up scheme is the same for both familial and sporadic MUT+ patients, whereas MUT- patients' follow up differs according to familial status, we decided to exclude familial MUT- patients from the survival analysis, so to have a higher homogeneity of follow-up within groups. The resulting dataset consisted of 305 patients, 106 MUT+ and 199 MUT.

Patient selection workflow is outlined in **Figure 6**.

In order to make sure that our results were not affected by events occurred in the time elapsed between melanoma diagnosis and the start of the mutation-based follow-up, we also verified whether survival analysis results remained consistent when only including incident patients, using the 24 months cut-off previously used to assess survival in melanoma patients[116]. To obtain this "incident patients-only" subset, the same selection process was again applied to patients from our melanoma cohort. Only patients enrolled within 24 months from melanoma diagnosis were selected, resulting in a total

of 199 incident patients matched for age and sex, and with similar of tumor stage distribution, 62 and 137 of whom were MUT+ and MUT-, respectively.

Informed consent was signed by all patients prior to enrollment, according to local ethics committee approved protocol.

Endpoints and Statistical analyses

Our endpoints were to investigate whether *CDKN2A* mutational status is linked to patients' prognosis in terms of overall survival (OS) and melanoma-specific survival (MSS) in our melanoma cohort undergoing a mutation-based follow-up.

When evaluating the difference of a numerical variable between two groups, we performed the Mann-Whitney U test. To assess the association between two categorical variables, we used the Fisher's exact test. Association between one ordinal and one categorical variable was performed using the Kruskal-Wallis test. OS and MSS were calculated with Cox-proportional hazard regression models and Kaplan-Meier curves.

To analyze OS, events were defined as death by any cause. For MSS, only deaths by melanoma were considered events, whereas deaths by other causes were censored. Considering that mutation-based follow-up started after patients' enrollment and *CDKN2A* genetic testing, we calculated follow-up as months from inclusion to censoring or death.

All analyses were two sided, and threshold for statistical significance was set at p=0.05. Statistical analyses were performed within the R computational environment [117], using the packages stats, survival [118], and the R/Bioconductor package survcomp [119].

RESULTS

Patients Characteristics

Whole cohort

Our cohort, after filtering-out 30 patients with either missing information on *CDKN2A* mutational status or variants of uncertain significance (VUS), consisted of 1187 patients, 129 MUT+ and 1058 MUT-. Patients and tumor characteristics, and corresponding statistics are shown in **Table 4**.

Overall, 59/977 apparently sporadic patients (6%) and 70/210 (33%) of familial patients were MUT+. Median age was lower in MUT+ patients compared to MUT- (42 and 50 years, respectively, $p<0.01$). Conversely, sex, tumor stage and median Breslow thickness did not differ depending on *CDKN2A* mutational status. As expected, MPM were more frequent among MUT+ patients ($p<0.01$). Moreover, 31/130 (24%) of apparently sporadic patients diagnosed with MPM were MUT+, compared to only 28/847 (3%) of apparently sporadic patients with a single melanoma, as shown in **Table 5**.

Among MUT+ patients there was a higher rate of individuals who had one or more dysplastic nevi, and other skin lesion, surgically removed.

19% of MUT+ patients had been diagnosed with at least one non-melanoma cancer, compared with 12% of MUT- patients (OR=1.7%, $p=0.042$).

The most frequent *CDKN2A* variant was G101W (97 patients), followed by E27X (13 patients), A127P, P48T, R24P (3 patients each), and A36T, A68L, D74Y, F90S (1 patient each).

The distribution of *MC1R* variants was similar in MUT+ and MUT- patients, either using the 5-point score described by Davies et al.[116] or a dichotomous approach ($p>0.7$ in both cases, **Table 4**).

Study dataset

After patients filtering and matching for age and sex, median age was 43 in MUT+ and 44 in MUT- patients ($p=0.719$). Moreover, 48 (45%) and 95 (48%) patients were male, while 58 (55%) and 104

(52%) were female in MUT+ and MUT- groups, respectively (p=0.81). Tumor stage distribution and median Breslow thickness remained similar in MUT+ and MUT- patients (both p>0.8), with the majority of patients having stage Ia to IIc tumors. Similarly, the distribution of *MC1R* variants did not differ significantly between groups (p>0.7). Median follow-up length was 123 months (CI=43-182) in MUT+ patients and 105 months (CI=42-154) in MUT- patients.

As in the main cohort, patients diagnosed with MPM were more frequently MUT+ (42% compared to 11% of MUT- patients, OR=5.97, p<0.01). *CDKN2A* pathogenic variants were also associated with removal of one or more dysplastic nevi (OR=2.25, p=0.026), especially when only considering dysplastic nevi removed after genetic testing for *CDKN2A* (OR=3.06, p=0.023).

In line with previous reports, the MUT+ had a higher rate of individuals diagnosed with non-melanoma cancers compared to MUT- (17% vs 9%), albeit this difference was not significant (OR=2.08, p=0.054).

Descriptive statistics performed on the study dataset is shown in **Table 6**.

Survival Analysis

Overall event rate was 17% in both MUT+ and matched MUT- patients, whereas deaths by melanoma were 10.8% and 7.8% among MUT+ and MUT- patients, respectively.

As shown in **Figure 7a**, we did not detect differences in OS between MUT+ and MUT- patients, as confirmed by a Cox-proportional Hazard Regression Model: Hazard Ratio (HR)=0.85, p=0.592, CI=0.48-1.52. Similarly, MSS did not differ depending on *CDKN2A* mutational status, as shown in **Figure 7b** (HR=0.86CI= 0.38-1.95, p=0.718,).

To verify survival in an even more "follow-up-homogeneous" setting, we then performed the analyses in the "incident patients-only" subset, obtaining overlapping results both for OS (HR=0.64 CI= 0.28-1.45, p=0.282,) and MSS (HR=1.87, CI=0.39- 2.95, p=0.9). Kaplan-Meier curves along with Log-rank computed p-values of the "incident patients-only" subset are shown in **Figure 8.**

DISCUSSION

Our study shows that *CDKN2A* mutational status does not negatively affect survival in our melanoma cohort. Both OS and MSS did not vary according to *CDKN2A* mutational status, when known confounding variables such age, sex, and tumor stage were similar in MUT+ and MUT- patients.

Interestingly, our results are not in line with the previously described Swedish study, in which familial *CDKN2A* MUT+ patients had worse survival compared to familial MUT- and sporadic patients [109]. It is possible that intrinsic population differences may modulate *CDKN2A* impact. Indeed, although MUT+ and MUT- had a comparable distribution of *MC1R* variants which have been suggested to affect survival[116], we cannot rule out the possibility that somatic/germline variants in other genes may have had a protective effect on survival in our study cohort.

Moreover, survival according to *CDKN2A* variants in different regions might be modulated by an asymmetric distribution of non-genetic risk factors. For instance, it is not known yet whether different environmental exposures can influence prognosis in MUT+ individuals.

A third hypothesis is that intensive clinical surveillance may modulate survival in this high-risk population, possibly by counteracting negative effects on survival due to the *CDKN2A* variant itself. In fact, the impact of follow-up intensity on survival according to *CDKN2A* pathogenic variants is currently unknown, and it must be kept in mind that the Swedish study was performed on patients who underwent a familial melanoma-specific follow-up whereas our hospital adopts a different strategy. Considering the high *CDKN2A* mutation rate of our population, we have a considerable number of apparently sporadic MUT+ patients whose risk deriving from their first melanoma, as well as the risk of developing MPM, may not be inferior to that of their familial counterpart [91,111–113]. Indeed, we observed a 24% *CDKN2A* mutation rate in apparently sporadic patients who had MPM, compared to 3% in those who only had one melanoma diagnosis. Considering that close surveillance

in high-risk groups such as MUT+ is encouraged by multiple evidence in order to improve early diagnosis[9,120,121], our hospital offers an intensive follow-up to MUT+ individuals.

The proportion of individuals who had one or more dysplastic nevi removed was higher among MUT+ patients, especially when only considering dysplastic nevi removed after DNA testing. This difference was even higher when we restricted the analysis to incident patients. Hence, it is possible that intensive follow-up resulting in detection and removal of dysplastic lesions reduced the occurrence of metachronous melanomas in MUT+ patients.

Unfortunately, due to the retrospective nature of our cohort, our data on individual patients' follow-up is incomplete and thus we cannot compare median follow-up intervals between MUT+ and MUT, even though the higher rate of dysplastic nevi removal after DNA testing may be an indirect indicator of a more intensive follow-up in the MUT+ group. Therefore, although follow-up may be involved in the non-inferior survival of MUT+, this hypothesis needs to be verified prospectively.

Another limitation is that only probands of familial melanoma patients were included in our cohort: although providing the advantage of having independent observations, this lowered the size of our familial subset. Likely for this reason, we did not have enough data to analyze both the association of *CDKN2A* with the occurrence of specific cancers (such as pancreatic) and survival by non-melanoma cancers. The downsizing of our cohort needed to obtain an accurate dataset for survival analysis also prevented us from carrying out further analyses, such as direct comparison between MUT+ and MUT- among familial-only patients. In addition, the majority of patients in both groups were still alive at censoring time. Due to the above-mentioned exclusion of affected family members of the probands, along with the fact that the majority of our cohort's patients had Stage I and II melanomas, it is possible that the number of deaths we observed was insufficient to detect an effect of *CDKN2A* on survival.

In conclusion, despite recent findings, *CDKN2A* mutational status is not associated with survival in our cohort. Whereas several potential modifiers could be implicated in region-specific differences concerning *CDKN2A*-related melanoma survival, further studies are needed to verify these hypotheses.

This study, now published in the Journal of the American Academy of Dermatology (JAAD)[122], is part of an ongoing project aimed at unraveling the relationship between *CDKN2A*, disease characteristics and survival. Upon the enrollment of a higher number of patients, we plan to compare MUT+ and MUT- familial melanoma patients, and to analyze mortality by melanoma and other cancers in different subgroups, also extending the analysis to affected family members of the already included probands.

Figure 6. **Cutaneous melanoma. Patients selection workflow**

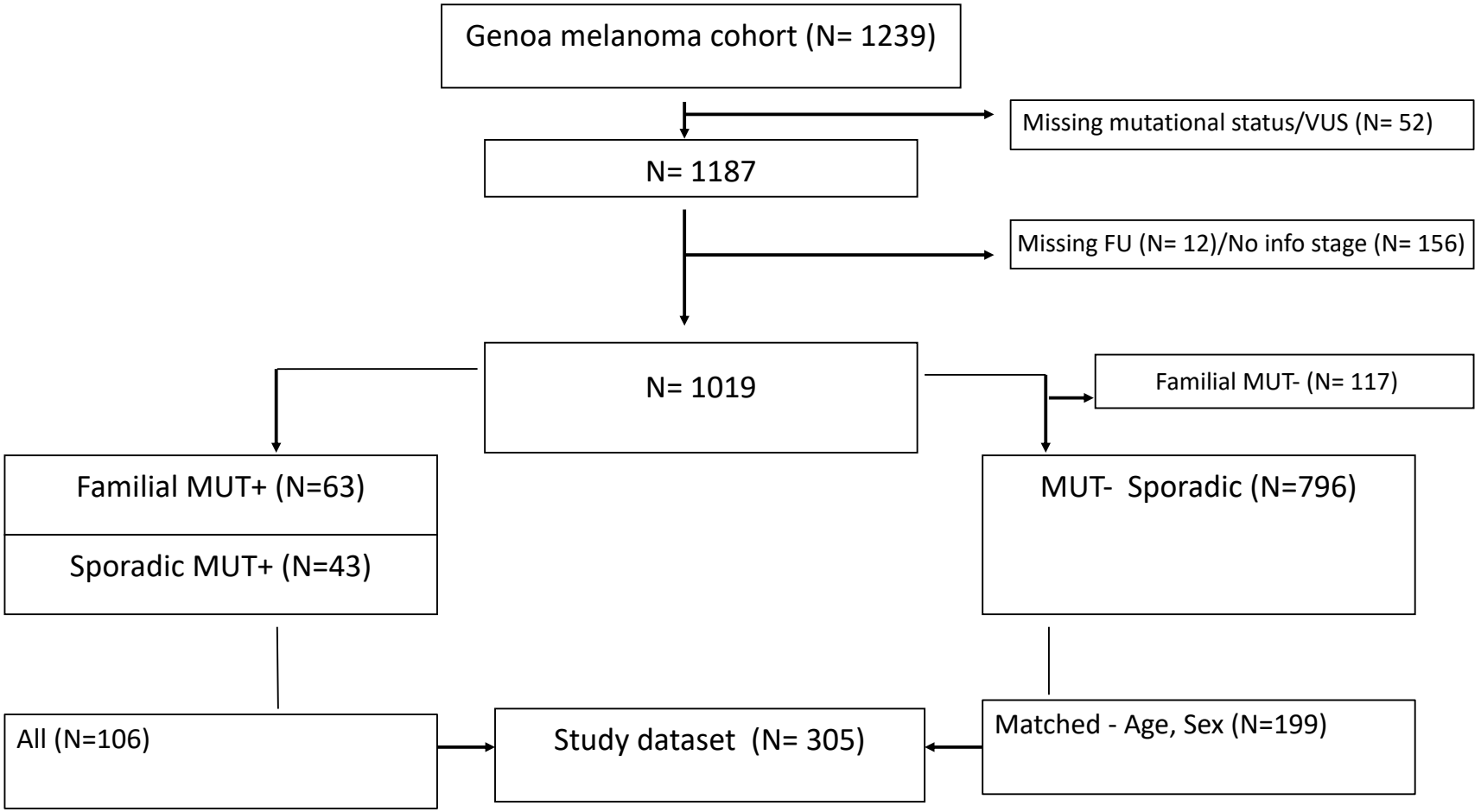


Table 4

Whole cohort. Clinical, pathological and molecular characteristics

		N	MUT	WT	OR	Lower CI	Upper CI	T	H	p
Sex	M	1187	56 (0.43)	485 (0.46)	1.1	0.75	1.63			0.64
	F		73 (0.57)	573 (0.54)						
Age	median (IQR)	1187	42 (32-53)	50 (39-63)				87372		**1.938E-07**
Stage	IS	1027	5 (0.02)	121 (0.06)					2.06	0.151
	I		71 (0.27)	537 (0.26)						
	II		20 (0.08)	171 (0.08)						
	III		7 (0.03)	56 (0.03)						
	IV		5 (0.02)	34 (0.02)						
Familial	fam	1187	70 (0.54)	140 (0.13)						
	spo		59 (0.46)	918 (7.12)						
N. of Melanomas	cum	1185						93989		**2.548E-30**
N. of Melanomas	1	1185	68 (0.53)	942 (0.89)	7.39	4.88	11.21			**1.687E-21**
	2+		61 (0.47)	114 (0.11)						
Breslow	median (IQR)	1133	0.9 (0.4-1.9)	0.84 (0.4-1.9)				63592.5		0.573
N. of Dysplastic Nevi	cum	1169						72134.5		**0.007**
N. of Dysplastic Nevi	0	1169	108 (0.84)	944 (0.91)	1.91	1.09	3.24			**0.019**
	1+		21 (0.16)	96 (0.09)						
N. of Dysplastic Nevi After Inclusion	cum	1167						69823		**0.032**

N. of Dysplastic Nevi After Inclusion	0	1167	118 (0.91)	993 (0.96)	2.06	0.93	4.18			**0.047**
	1+		11 (0.09)	45 (0.04)						
Other Skin Lesions	cum	1183						83172.5		**8.836E-06**
Other Skin Lesions	0	1183	30 (0.23)	432 (0.41)	2.26	1.46	3.6			***1.109E-04***
	1+		98 (0.77)	623 (0.59)						
Other Tumors	0	1124	96 (0.81)	882 (0.88)	1.72	1	2.85			**0.042**
	1+		23 (0.19)	123 (0.12)						
MC1R	score 0-4	754							0.008	0.9308
MC1R	WT	754	22 (0.26)	192 (0.29)	1.15	0.68	2.03			0.702
	any r/R		63 (0.74)	477 (0.71)						

Abbreviations. N= number of patients, CI= confidence interval, T= Wilcoxon test statistic, H= Kruskal-Wallis test statistic, p= p-value, IQR= interquartile range, IS= melanoma in situ, spo= sporadic, fam= familial, cum= cumulative.

Table 5.

Relative frequencies of *CDKN2A* pathogenic variants among patients with single and multiple melanomas

	N. of melanomas	MUT+		MUT-		Row Total
Sporadic	1	28 (0.22)	(0.03)	819 (0.78)	(0.97)	847 (0.715)
	2+	31 (0.24)	(0.24)	99 (0.09)	(0.76)	130 (0.110)
Familial	1	40 (0.31)	(0.25)	123 (0.12)	(0.75)	163 (0.138)
	2+	30 (0.23)	(0.67)	15 (0.01)	(0.33)	45 (0.038)
Column Total		129 (0.109)		1056 (0.891)		1185*

Absolute number of patients is shown outside brackets. Row frequencies are reported on the right, whereas column frequencies are reported at the bottom of each cell.

*Two patients were removed due to missing information on multiple melanomas

Table 6.

Clinical and molecular characteristics of patients selected for survival analysis

		N	MUT	WT	OR	Lower CI	Upper CI	T	H	p
Sex	M	305	48 (0.45)	95 (0.48)	1.1	0.67	1.82			0.719
	F		58 (0.55)	104 (0.52)						
Age	median (IQR)	305	43 (32.25-55.5)	44 (33-56)				11084		0.464
Stage	IS	305	5 (0.02)	22 (0.05)					1.27	0.26
	Ia-Ib		70 (0.27)	122 (0.27)						
	IIa-IIb-IIc		19 (0.07)	37 (0.08)						
	IIIa-IIIb-IIIc		7 (0.03)	15 (0.03)						
	IV		5 (0.02)	3 (0.01)						
Familial Status	fam	305	63 (0.5)	0 (0)						
	spo		43 (0.34)	199 (1)						
N. of Melanomas	cum	305						13914.5		**1.341E-10**
N. of Melanomas	1	305	62 (0.58)	178 (0.89)	5.97	3.2	11.47			**1.086E-09**
	2+		44 (0.42)	21 (0.11)						
Breslow	median (IQR)	305	0.9 (0.36-1.7)	0.9 (0.44-1.7)				10489.5		0.955
N. of Dysplastic Nevi	cum	299						11233		**0.013**
N. of Dysplastic Nevi	0	305	87 (0.82)	176 (0.91)	2.25	1.05	4.87			**0.026**
	1+		19 (0.18)	17 (0.09)						
N. of Dysplastic Nevi After Inclusion	cum	299						10929		**0.018**
N. of Dysplastic Nevi After Inclusion	0	299	95 (0.9)	186 (0.96)	3.06	1.05	9.64			**0.023**
	1+		11 (0.1)	7 (0.04)						
Other Skin Lesions	cum	303						11379.5		0.19
Other Skin Lesions	0	303	23 (0.22)	55 (0.28)	1.4	0.78	2.56			0.271

	1+		83 (0.78)	142 (0.72)						
Other Tumors	0	287	82 (0.83)	171 (0.91)	2.08	0.94	4.58			0.054
	1+		17 (0.17)	17 (0.09)						
MC1R	score 0-4	221							0.1309	0.718
MC1R	WT	221	19 (0.25)	38 (0.26)	1.04	0.53	2.09			1
	any r/R		56 (0.75)	108 (0.74)						

Abbreviations. N= number of patients, CI= confidence interval, T= Wilcoxon test statistic, H= Kruskal-Wallis test statistic, p= p-value, IQR= interquartile range, IS= melanoma in situ, spo= sporadic, fam= familial, cum= cumulative

Figure 7. Overall survival and melanoma-specific survival in MUT+ and MUT- melanoma patients

The Kaplan–Meier curves show similar overall survival (a) and melanoma-specific survival (b) in MUT+ and MUT- patients. Censored patients are shown as vertical lines.

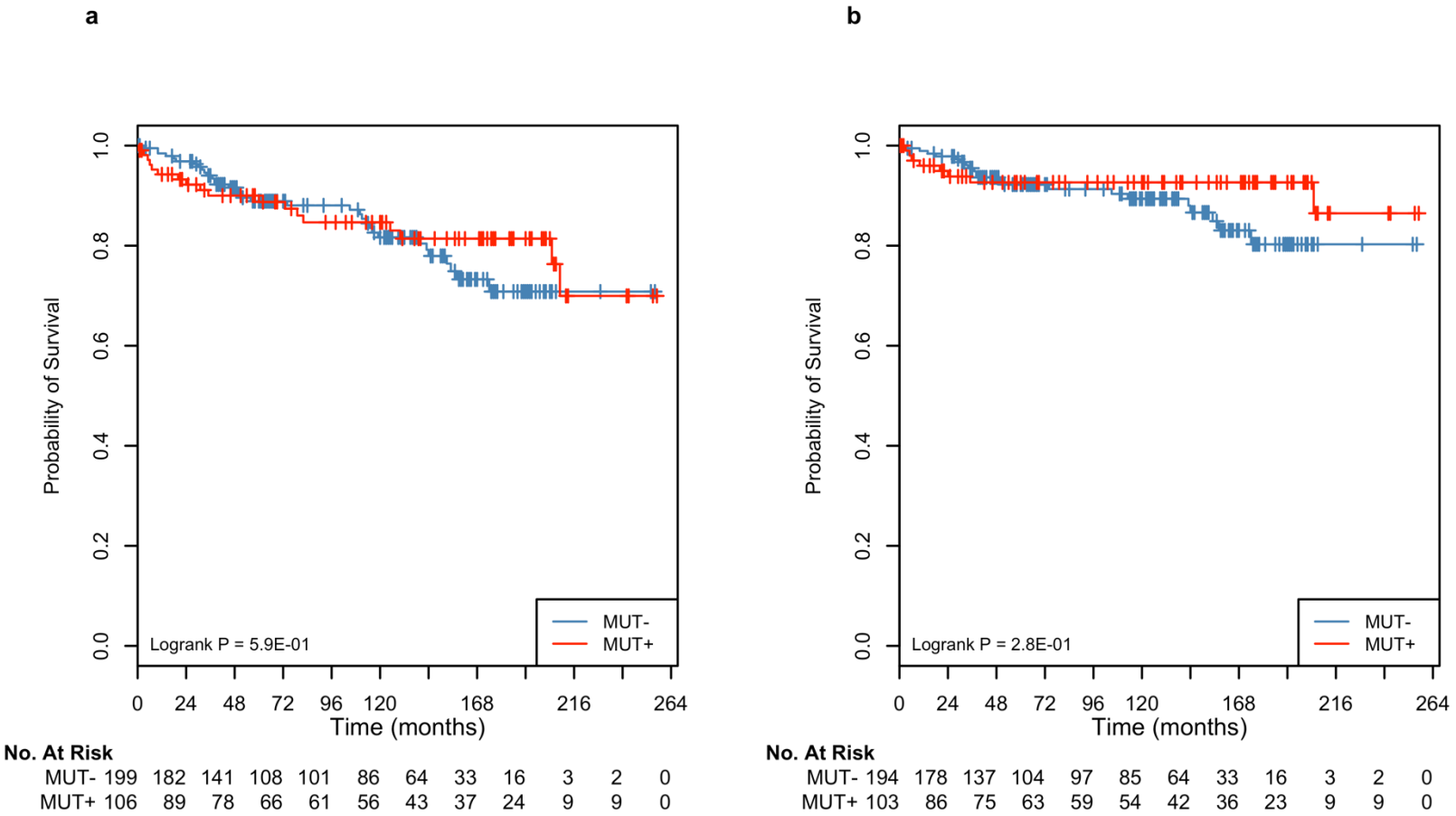
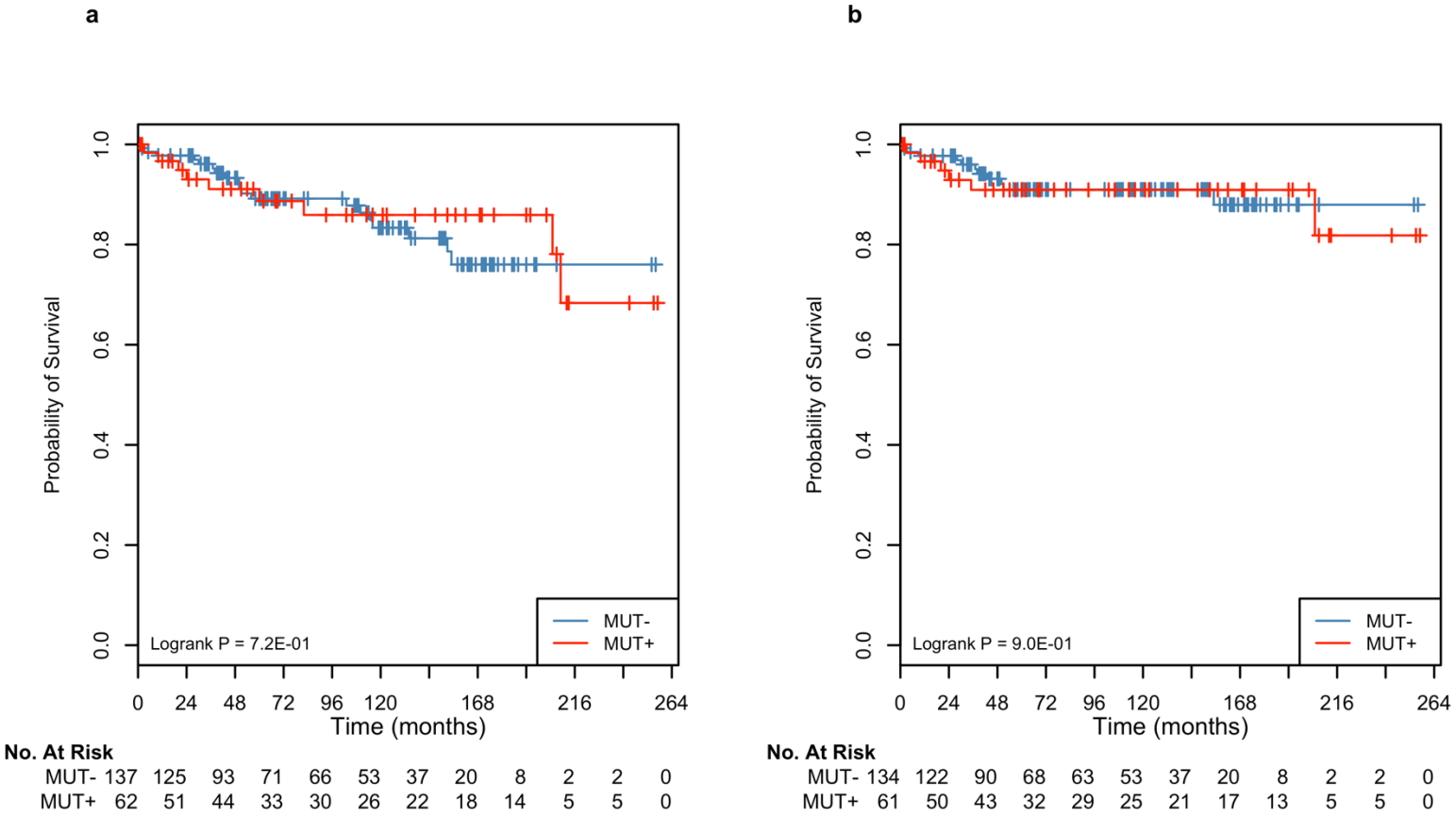


Figure 8. Overall survival and melanoma-specific survival in MUT+ and MUT- incident-only subset of melanoma patients
CDKN2A mutational status did not modify overall survival (a) and melanoma-specific survival (b) in incident cutaneous melanoma patients, as shown by the overlapping Kaplan-Meier curves. Censored patients are shown as vertical lines.



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