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Clinical and genetic characterization of 145 Spanish patients diagnosed with PTEN hamartoma tumor syndrome

Doctoral thesis

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Clinical and genetic characterization of 145 Spanish patients diagnosed with PTEN hamartoma tumor syndrome

Tesis doctoral

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A lo que gané, a lo que perdí y a lo que mantengo

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I know I was born and I know that I'll die. The in between is mine.
(I am mine, Pearl Jam)

ABSTRACT

ABSTRACT

The PTEN hamartoma tumor syndrome (PHTS) is a rare disease characterized by a large phenotypic variability of benign lesions together with a high predisposition to develop several cancer types (breast, thyroid, endometrial, renal, colorectal and melanoma). This hereditary syndrome is associated to germline mutations in the *PTEN* gene, although a considerable proportion of patients are not explained by this gene. Altogether, this results in the lack of awareness on the PHTS, underdiagnosis and poor management of these patients.

The first general objective is to characterize the disease in Spanish patients, both at a genetic level and at a clinical level, together with the comparison of our results with that obtained in other studied populations and the evaluation of the usefulness of the diagnostic criteria. The second objective is to look for other genetic factors that can be involved in the phenotype of the PHTS patients who do not harbor *PTEN* mutations.

To accomplish these objectives, we have gathered a series of 145 Spanish patients diagnosed with PHTS with their respective clinical information and biological samples. The use of a specific checklist allowed us to review the clinical features of interest, and the use of conventional genetic techniques (Sanger, MLPA, aCGH), together with high-throughput procedures (NGS and WES) enabled the molecular characterization, focusing not only in *PTEN* but also searching for other genes. Moreover, the functional studies gave insights into the implications in pathogenicity of different variants of unknown significance in *PTEN*.

The results of this work were used to state several recommendations for the diagnosis: the application of the most useful clinical features to drive genetic testing and the performance of this through multigene panels to detect other possibly altered genes that might confer additional clinical risks. Regarding the follow-up, it is relevant to do obesity check-ups and to anticipate the cancer screenings. Moreover, several findings of this study set the basis for future research.

Overall, this work contributes to accelerate and improve the diagnosis and patient care of the PHTS patients.

RESUMEN

El síndrome de PTEN-tumores hamartomatosos (PHTS) es una enfermedad rara que se caracteriza por una gran variabilidad fenotípica que incluye lesiones benignas, pero también una alta predisposición a desarrollar varios tipos de cáncer (cáncer de mama, tiroides, endometrio, renal, colorrectal y melanoma). Este síndrome hereditario se asocia a mutaciones germinales en el gen *PTEN*, aunque una proporción considerable de pacientes no pueden explicarse por este gen. Todo ello resulta en el desconocimiento de esta enfermedad, la falta de diagnóstico y el mal manejo de estos pacientes.

Los objetivos generales de este proyecto son, en primer lugar, caracterizar la enfermedad en pacientes españoles, tanto a nivel genético como clínico, comparar nuestros hallazgos con los obtenido en otras poblaciones y evaluar la utilidad de los criterios diagnóstico; y en segundo lugar, la búsqueda de otros factores que puedan estar implicados en el fenotipo PHTS de los pacientes que no portan mutaciones en *PTEN*.

Para llevar a cabo estos objetivos, hemos reunido una serie de 145 pacientes españoles diagnosticados con PHTS, con su respectiva información clínica y muestras biológicas. El uso de un cuestionario específico nos permitió revisar las manifestaciones clínicas de interés, y el empleo de técnicas genéticas convencionales (Sanger, MLPA, aCGH), junto con técnicas de mayor rendimiento (NGS y WES) ha permitido la caracterización molecular, centrada no sólo en *PTEN* sino también en la búsqueda de otros genes. Además, los estudios funcionales han arrojado luz acerca de las implicaciones deletéreas de diferentes variantes de significado incierto en *PTEN*.

Los resultados de este trabajo se han empleado para proponer varias recomendaciones para el diagnóstico: el empleo de las manifestaciones clínicas más útiles para derivar al paciente para estudio genético, y realizar este mediante paneles de genes que permitan detectar otros posibles genes alterados que confieran riesgos clínicos añadidos. De cara al seguimiento de los pacientes, es relevante el control del peso y anticipar las revisiones para la detección precoz de cáncer. Por otra parte, varios hallazgos de nuestro trabajo sientan las bases para continuar investigando en esta enfermedad.

En resumen, este trabajo contribuye a acelerar y mejorar el diagnóstico y la atención de los pacientes con PHTS.

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ABBREVIATIONS

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36B4	RPLP0, 60S acidic ribosomal protein P0
aCGH	Microarray-based comparative genomic hybridization
AKT	Serine/threonine kinase 1
ANNOVAR	Annotate Variation
APC	APC regulator of WNT signaling pathway
ASD	Autism spectrum disorder
BAP1	BRCA1 associated protein 1
BMPR1A	Bone morphogenetic protein receptor type 1A
BRRS	Bannayan-Riley-Ruvalcaba syndrome
BWA	Burrows-Wheeler Aligner
CC	Cleveland Clinic
cDNA	Complementary DNA
CEGEN-USC	Genotyping National Centre node of the University of Santiago de Compostela
CI	Confidence interval
CNIO	Centro Nacional de Investigaciones Oncológicas
CONDEL	Consensus Deleteriousness score
COSMIC	Catalogue of Somatic Mutations in Cancer
CS	Cowden syndrome
dbSNP	The Single Nucleotide Polymorphism database
dsDNA	Double stranded DNA
EDTA	Ethylene DiamineTetraacetic Acid
EGFR	Epidermal growth factor receptor
EP400	E1A Binding Protein P400
ERN-GENTURIS	European Reference Network on Genetic Tumour Risk Syndromes
ExAC	Exome Aggregation Consortium
FAP	Familial adenomatous polyposis
FEDER	Federación Española de Enfermedades Raras
FLCN	Folliculin
FPR	False positive rate
GARD	Genetic and Rare Diseases Information Center
gDNA	Genomic DNA
gnomAD	Genome Aggregation Database
H-score	Histo-score
HC	Head circumference

HERC1	HECT and RLD domain containing E3 ubiquitin protein ligase family member 1
HUF	Hospital Universitario de Fuenlabrada
ICC	International Cowden Consortium
IHC	Immunohistochemistry
JPI	Juvenile polyposis syndrome of infancy
KLLN	Killin, P53 Regulated DNA Replication Inhibitor
LDD	Lhermitte-Duclos disease
LOH	Loss of heterozygosity
LOVD	Leiden Open source Variation Database
MAF	Minor allele frequency
miRNA	MicroRNA
MLH3	MutL homolog 3
MLPA	Multiplex ligation-dependent probe amplification
MSI	Microsatellite instability
mut+	Carrier of mutation
MUTYH	MutY DNA glycosylase
NCCN	National Cancer Comprehensive Network
NEDD4	Neural precursor cell expressed developmentally down-regulated protein 4
NF1	Neurofibromatosis type 1
NGS	Next generation sequencing
NMD	Nonsense mediated mRNA decay
NORD	National Organization for Rare Disorders
OR	Odds ratio
PDGFRB	Platelet derived growth factor receptor beta
PHTS	PTEN hamartoma tumor syndrome
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIK3R2	Phosphoinositide-3-kinase regulatory subunit 2
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol(3,4,5)-trisphosphate
PMS2	PMS1 homolog 2, mismatch repair system component
Polyphen-2	Polymorphism Phenotyping v2
PS	Proteus syndrome
PTCH1	Patched 1
PTEN	Phosphatase and tensin homolog
PTENP1	Phosphatase and tensin homolog pseudogene 1
PV-; PV+	Negative predictive value; positive predictive value
qPCR	Quantitative PCR
RET	Ret proto-oncogene

RNF135	Ring finger protein 135
RT-PCR	Reverse transcription PCR
SDH	Succinate dehydrogenase
SEC23B	SEC23 homolog B, coat complex II component
SEM	Standard error of the mean
SIFT	Sorting Intolerant From Tolerant
SMAD4	SMAD family member 4
SNP	Single nucleotide polymorphism
STK11	Serine/threonine kinase 11
SUFU	SUFU negative regulator of hedgehog signaling
TTN	Titin
TSC1; TSC2	TSC complex subunit 1; TSC complex subunit 2
UBN2	Ubinuclein 2
VUS	Variant of unknown significance
WES	Whole exome sequencing
WGS	Whole genome sequencing
WT	Wild type, no known genetic alteration
WWP1	WW domain containing E3 ubiquitin protein ligase 1

INTRODUCTION

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1. Hereditary cancer syndromes

In the wide spectrum of human diseases, there are several conditions that appear with a syndromic pattern; in other words, the affected individual will show a series of alterations or pathogenically related symptoms, which is distinguishable from other groupings or isolated characteristics and whose cause (which can be genetic or not) is known or not (Cohen, 1997).

Germline mutations account as responsible of several human syndromes, and these mutations are susceptible of being transmitted to the offspring, who will be at risk of developing the disease. These are called hereditary syndromes, and some have an associated increased risk to suffer cancer, meaning that the individuals also inherit the cancer predisposition. There are around 200 cancer predisposition syndromes. Some examples are the hereditary breast and ovarian cancer syndrome, the Lynch syndrome or the Li-Fraumeni syndrome. Only 5-10% of the cancers are hereditary, meanwhile 70-80% are sporadic and the remaining 15-20% are multifactorial or familial (Urioste, 2010).

2. The PTEN-hamartoma tumor syndrome (PHTS)

One of the hereditary cancer syndromes is the PTEN-hamartoma tumor syndrome (PHTS), which is inherited in an autosomal dominant way and it is considered a rare disease. The PHTS term gathers together several clinical conditions: Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome (BRRS), *PTEN*-related Proteus syndrome (PS), VATER-associated macrocephaly disease, autism spectrum disorder (ASD)-associated macrocephaly disease, plus the CS-like and PS-like syndromes (where the patients do not meet the full diagnostic clinical criteria for each disorder). All these entities have its cause in common, which is *PTEN* germline alterations, and they share multisystemic clinical features, such as hamartomas, cutaneous lesions, or macrocephaly, but most importantly they have an associated increased predisposition to several cancer types: breast cancer, thyroid cancer, endometrial cancer, colon cancer, renal cancer and melanoma (FIGURE 1) (Tan *et al.*, 2012).

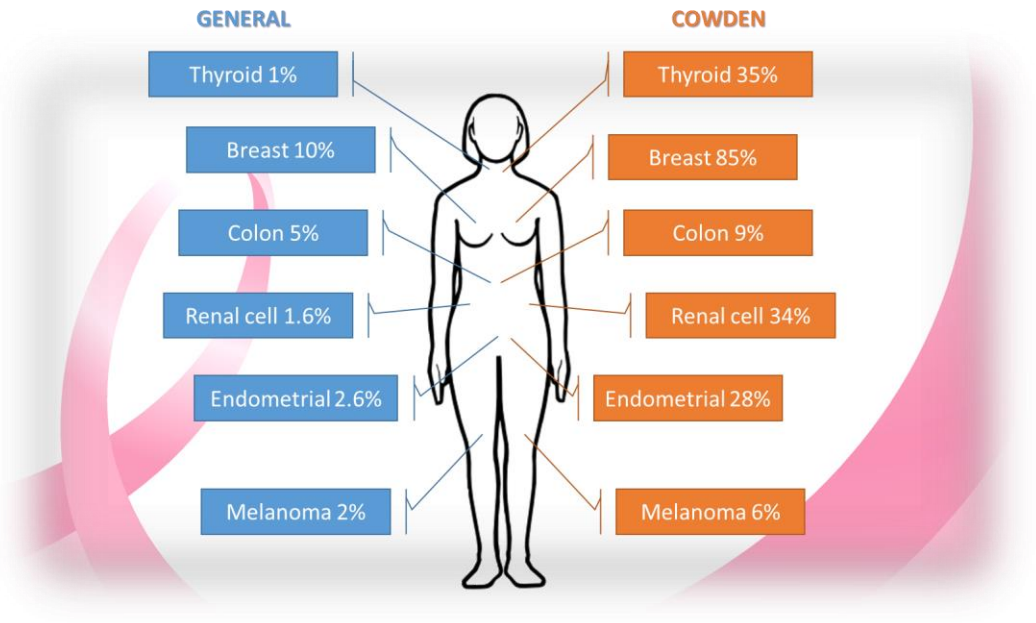

Figure 1. Lifetime cancer risks for PHTS patients compared to general population. Estimations according to Tan *et al.*, 2012.

2.1. Diagnosis

Given the wide and complex spectrum of the clinical features recognized in this multisystemic disease, the PHTS is difficult to diagnose. Plus, some authors define the disease based on the molecular diagnosis (thus only individuals with a germline *PTEN* mutation would be considered PHTS patients) (Mester and Eng, 2013), while others propose a clinical definition (Pilarski, 2019), and on this last purpose different clinical diagnostic criteria have been proposed (Pilarski *et al*., 2013; Tan *et al*., 2011), again without global agreement. Also of note, the clinical expression is highly variable and the same exact mutation in 2 individuals can result in different phenotypes (Marsh *et al*., 1999).

2.1.A. Clinical definition of the PHTS

Since its first description in 1963 (Lloyd and Dennis, 1963), the PHTS is still a rare disease not only because of its frequency in the population but also because of the poor knowledge about it. However, with the years, several studies describing small series of PHTS patients have laid the foundations for the establishment of clinical diagnostic criteria. In this way, in 1996, the International Cowden Consortium (ICC) was created and published the Cowden syndrome diagnostic criteria (Tan *et al.*, 2011) (FIGURE 2). These criteria have been updated in the last decades as new and larger studies came up (Eng, 2016). Also, other authors found discrepancies with the mentioned criteria and therefore proposed new and revised criteria, such as the Pilarski's criteria (Pilarski *et al.*, 2013) (FIGURE 2).

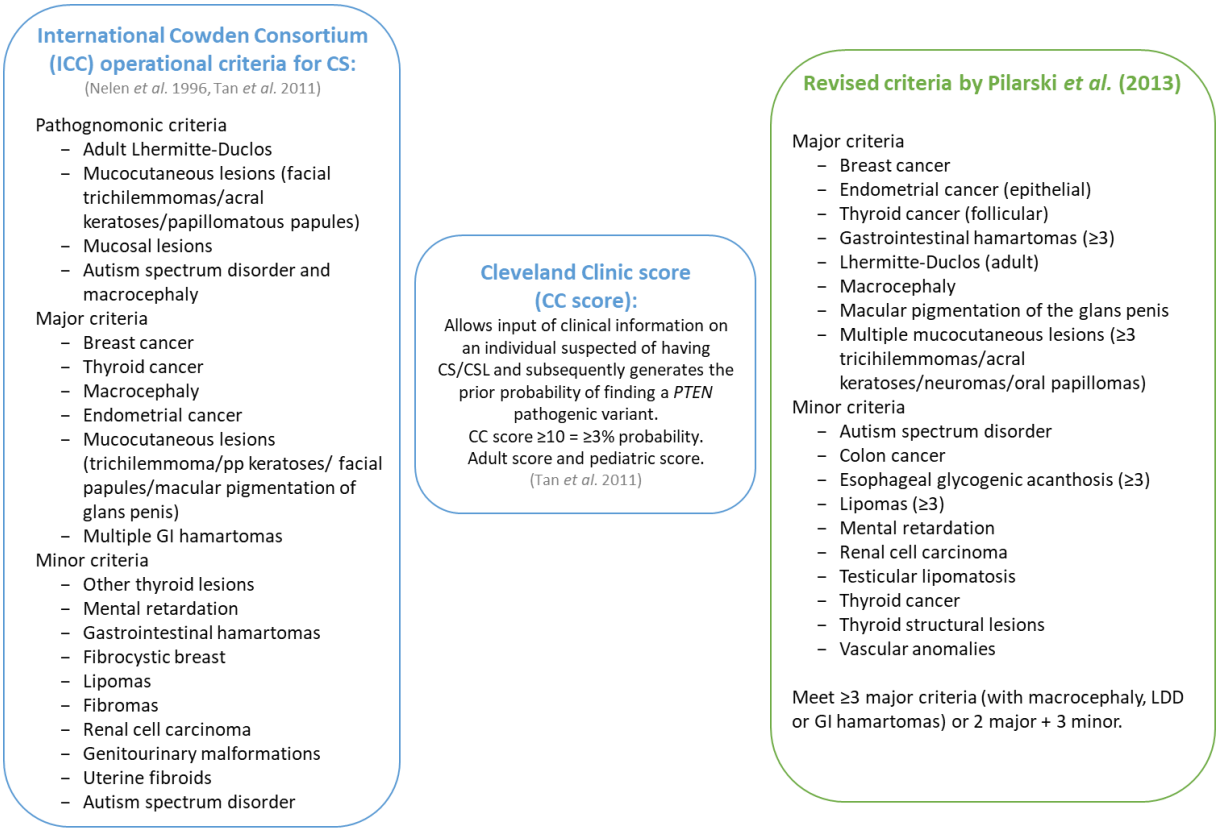

Figure 2. Adapted version of the clinical diagnostic criteria proposed in the literature for PHTS or CS patients. A) ICC operational criteria for CS (Tan *et al*., 2011)**. B) CC score** (Tan *et al*., 2011)**. C) Revised clinical diagnostic criteria for PHTS** (Pilarski *et al*., 2013)**. Complete version of the different criteria can be found in the mentioned references.**

Moreover, the Cleveland Clinic proposed a numerical score (CC score) based on the clinical features present in the patient to estimate the probability of finding a *PTEN* pathogenic mutation (Tan *et al*., 2011) (FIGURE 2).

These criteria and predictive scores have improved the PHTS diagnosis and referral for genetic testing. Nevertheless, they were taken based in a reduced number of patients and selected populations.

According to the clinical definition, an individual is suspect of having the syndrome when he or she meets the diagnostic criteria. However, some clinical criteria (both the ICC's and the CC score) are designed for CS, which is considered the PHTS prototype, but may not be appropriate for every PHTS patient.

2.1.B. Molecular definition of the PHTS

The genetic testing of *PTEN* can be performed by single gene analysis or through gene panel sequencing, a technique that is gaining popularity in the last years. The analysis of *PTEN* should investigate the sequence of all the 9 exons of the gene, as well as the intron-exon boundaries, to detect any point mutations (including the ones that could alter the

correct splicing). A large rearrangement analysis should follow whenever no point mutations are found, and it can be performed through multiplex ligation-dependent probe amplification (MLPA). Some authors have proposed the mutation screening of the *PTEN* promoter, but this additional measure is not established worldwide in the diagnostic laboratories since few cases with variants in that region (and whose deleteriousness is not confirmed) have been described (Tan *et al.*, 2012).

Apparently, germline mosaic mutations in *PTEN* are very rare, with only 4 reports in the literature (Salo-Mullen *et al.*, 2014; Gammon *et al.*, 2013; Pritchard *et al.*, 2013; Zhou *et al.*, 2000).

2.2. Prevalence of PHTS

The prevalence of this rare disease is still unknown, mostly due to the issues described above that result in underdiagnosis. There are only estimations of prevalence for CS (1 in 250,000) (Nelen *et al*. 1999), and these probably correspond to minimum scores.

At national level, the PHTS in Spain is even more unknown. To date, only isolated cases have been described in the medical literature, and the largest published Spanish patient series included 8 CS families (Bussaglia *et al*., 2002).

2.3. Penetrance

Some authors estimated that more than 90% of CS patients show some CS-associated clinical feature by their late 20s and around 99% from age 30 and on, these last showing mucocutaneous manifestations especially (Eng, 2003; Nelen *et al*., 1996). Several of the PHTS-associated symptoms can be evident at a very early age, such as macrocephaly, general overgrowth, mucocutaneous lesions or musculoskeletal aberrations. Therefore, an early diagnosis is possible in a considerable amount of the cases. In the other hand, many other manifestations of the PHTS can appear throughout the lifespan of the individual, with no characteristic age of appearance, and thus difficult the diagnosis.

2.4. Inheritance

PHTS is inherited in an autosomal dominant manner, which means that the offspring of a PHTS patient has a 50% chance of inheriting the mutant allele and developing the disease.

2.5. Clinical entities included in PHTS

The PHTS is kind of an umbrella term that encompasses several disorders with overlapping clinical features and molecular cause.

2.5.A. Cowden syndrome (CS; OMIM 158350)

Among the entities included in PHTS, CS is the most common and the best characterized. It was first described in 1963 by Lloyd and Dennis as a new symptom complex with multisystemic implications and they named it after the proband (and first described PHTS patient): Rachel Cowden, who showed oral papillomatosis, scrotal tongue, thyroid adenomas and several malformations in the head (Lloyd and Dennis, 1963). This event occurred decades before the finding of the responsible locus, in 1996 by Nelen *et al.* (Nelen *et al*., 1996).

The most characteristic clinical features of CS are mucocutaneous lesions (especially oral papillomatosis), macrocephaly, Lhermitte-Duclos (LDD; also called dysplastic gangliocytoma of the cerebellum), gastrointestinal polyposis, benign lesions in the thyroid or breast, and uterine miomas, among others. Of note and as shown in FIGURE 1, CS patients have increased risks to develop several cancer types (Tan *et al*., 2012).

2.5.B. Bannayan-Riley-Ruvalcaba syndrome (BRRS; OMIM 158350)

The name of this syndrome came by the hand of Cohen in 1990 (Cohen, 1990), after deciding to join together the individually described syndromes by: Bannayan, who described the combination of macrocephaly, multiple lipomas and hemangiomas, in 1971 (Bannayan, 1971); by Riley *et al*., who described the combination of macrocephaly, pseudopapilledema and hemangiomas, in 1960 (Riley and Smith, 1960); and by Ruvalcaba *et al.*, who described the combination of macrocephaly, intestinal hamartomatous polyps, genital lentiginosis and intellectual disability, in 1980 (Ruvalcaba *et al*., 1980).

The BRRS is a rare disease, with autosomal dominant inheritance and of unknown prevalence in the population. The most characteristic clinical features are: macrocephaly, lipomatosis, hemangiomatosis, macular pigmentation of the penis, mental retardation, psychomotor retardation and Hashimoto's thyroiditis, together with the hamartomatous polyposis, mainly in tongue and colon (Gorlin *et al*., 1992).

2.5.C. Proteus syndrome (PS)

The first description was by Cohen and Hayden in 1979 (Cohen and Hayden, 1979), but Wiedemann *et al.* were the ones who named this disease after the Greek god Proteus (known for changing form to his will in order to escape from capture) in reference to the large variability of the clinical manifestation of this disease (Wiedemann *et al*., 1983).

The clinical features of PS are not well defined. The most characteristic aspects are the following: progressive, asymmetric, segmental or mosaic overgrowth, from multiple tissues

and from every germ layer, epidermal nevi, hyperostosis, vascular malformations and facial dysmorphia. The symptoms cannot be appreciated in the first years of life and keep developing during childhood (Biesecker *et al*., 1999; Wiedemann *et al*., 1983).

Many PS patients can be explained by somatic mutations in *AKT1* gene. Molecularly, only the fraction of PS with *PTEN* germline mutations is included in PHTS (Zhou *et al*., 2001).

Given the shared clinical manifestations (TABLE 1) and their main molecular cause (*PTEN* mutations) of these three syndromes, it was proposed to encompass all of them together as the PTEN hamartoma tumor syndrome (PHTS) (Marsh *et al*., 1999) and suggested that the study of each entity individually would not be helpful (Lachlan *et al*., 2007). It is probable that the CS and BRRS are allelic since it is described that a same mutation in *PTEN* can lead to any of these phenotypes (even in individuals of the same family) (Marsh *et al*., 1999).

Table 1. Main clinical features shared or not between the 3 principal PHTS syndromes.

	Macrocephaly	Mucocutaneous lesions	GI polyps	LDD	Thyroid/ breast benign lesions	Body overgrowth	Neoplasms	Vascular lesions	Others
CS	Yes	Yes (oral papillomas, trichilemmomas)	Yes	Yes	Yes	No	Yes	Yes	Genitourinary alterations
BRR	Yes	Yes (lipomas, hemangiomas, penile macular pigmentation)	Yes	No	Yes	Yes (general overgrowth)	Yes	Yes	
PS	Yes	Yes (nevi)	No	No	No	Yes (partial body overgrowths)	Yes	Yes	Dysmorphic features, hyperostosis, …

3. Differential diagnosis

Some overlapping clinical features can be found in other diseases caused by alterations in genes of the PI3K/AKT/mTOR downstream pathway of PTEN (sometimes referred as PTEN-opathies) (Yehia *et al.* 2019). Plus, there are several overgrowth syndromes and hamartomatous polyposis diseases which can resemble the PHTS but have a different genetic cause: Peutz-Jeghers syndrome (*STK11*), juvenile polyposis syndrome (*BMPR1A*, *SMAD4*), Birt-Hogg-Dubé syndrome (*FLCN*) or Gorlin syndrome (*PTCH1*, *SUFU*). The ganglioneuromatous polyps can also be present in PHTS patients, although these are more associated to multiple endocrine neoplasia syndromes (*MEN1*, *RET*). The mucocutaneous lesions can resemble Neurofibromatosis type 1 (*NF1*). Last, there is the *AKT1*-related Proteus syndrome which cannot be mistaken for the *PTEN* condition.

4. Genetics of PHTS

4.1. *PTEN* gene and protein functions

The CS susceptibility locus was mapped to the 10q22-23 chromosome region by Nelen *et al*. in 1996 (Nelen *et al*., 1996), and in 1997, the *PTEN* gene was identified by various independent groups: Li *et al*. 1997 named the gene *PTEN* (phosphatase and tensin homolog deleted in chromosome 10) for its homology with the phosphatase and tensin and for being deleted in the chromosome 10 (Li *et al*., 1997). Steck *et al*. also identified the gene in 1997, but they named it *MMAC1* (mutated in multiple advanced cancers-1) and defined more precisely its location: 10q23.3 (Steck *et al*., 1997). Specifically, the *PTEN* gene is located in 10q23.31. Since its finding, *PTEN* has been object of numerous studies, especially regarding its role as a tumor suppressor. Opposite to oncogenes, the tumor suppressor genes counteract cell growth and proliferation; therefore, defects in tumor suppressor function may result in cancer.

PTEN mutations at somatic level are common in many human sporadic cancers, such as endometrial or prostate cancers, glioma, etc. (Hollander *et al.* 2011), but *PTEN* germline defects are more uncommon and are only associated to the heritable PHTS. Given the important role of *PTEN*, it is not a surprise that *PTEN* germline homozygous mutations are embryonic lethal (Di Cristofano and Pandolfi, 2000), therefore, the human germline mutations found in this gene are always heterozygous.

PTEN is the causal gene of PHTS. Germline heterozygous mutations in this gene are found in 80-85% of the CS patients, in 60-65% of BRRS patients and in 10-20% of PS patients. These alterations are also found in CS-like and PS-like cases, as well as in ASD-macrocephaly (<10-20%) and in VATER-macrocephaly (Spinelli *et al*., 2015; Orloff and Eng, 2008; Zbuk and Eng, 2007; Eng, 2003). Therefore, a considerable proportion of the PHTS patients remain with an unexplained cause of their disease.

Germline *PTEN* mutations in PHTS consist in point mutations and large deletions distributed along all the gene sequence, although the number of mutations described is higher between exons 5 and 8. Also, some mutations occur recurrently: c.388C>T (p.R130X), c.697C>T (p.R233X) and c.1003C>T (p.R335X). These are found in higher frequencies in several studied populations, most of them from North America and Europe (Bonneau and Longy 2000; Tan *et al*. 2011; Bubien *et al*. 2013). Moreover, insertion of Alu elements were found at a *PTEN* hotspot in Cowden syndrome (Crivelli *et al*., 2017). Nevertheless, these findings account for a small set of patients and should be validated in larger series.

The *PTEN* gene has 9 exons that encode the PTEN protein of 403 amino acids (aa) and its structure can be simplified in two main regions:

- N-terminal region (aa 1-185), responsible for the lipid and protein phosphatase activity. It contains the consensus sequence HCKAGKGR (aa 123 to 130) which corresponds to the catalytic core.

- C-terminal region (aa 186-403), which contains the C2 domain (aa 186–351) that enables the binding of PTEN to the phospholipid membrane and is relevant for subcellular localization, a PDZ binding domain (relevant for protein-protein interactions), PEST sequences and several phosphorylation sites (relevant for the protein stability). (Waite and Eng 2002).

The PTEN protein is expressed ubiquitously and is implicated in numerous pathways, but in the context of the PHTS disease, its most relevant role is antagonizing the PI3K/AKT/mTOR pathway.

PTEN is a phosphatase with dual specificity of substrates: it dephosphorylates proteins and lipids. Thus, PTEN dephosphorylates focal adhesion kinases (FAK) and also participates in MAPK pathways. Nevertheless, the main substrate of PTEN is one lipid: the phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 is an essential second messenger for AKT activation. In normal conditions, PTEN antagonizes the role of PI3K and inactivates the AKT pathway, resulting in the cell cycle stop or in apoptosis. On the contrary, when PTEN is mutated and cannot make its normal function, PIP3 accumulates in the cell, resulting in the constitutive activation of AKT and favoring in this manner the cell growth and diminishing apoptosis. Therefore, many cancers arise as a result of somatic or germline mutations in *PTEN*. One of the most common sporadic cancers associated to somatic *PTEN* mutations and mostly deletions, is the prostate cancer. Meanwhile, all the germline *PTEN* mutations are associated to PHTS.

Besides promoting apoptosis via downregulation of AKT, many other functions of PTEN have been described. PTEN negatively controls cell growth, invasion and migration and it also prevents genome instability. Otherwise, haploinsufficiency or loss of PTEN increases tumorigenesis and metastasis, genome instability and escaping immune response to cancer (Lee *et al*., 2018). Moreover, PTEN haploinsufficiency, is the cause of constitutive sensitization to insulin in association with an increased risk of obesity and decreased risk of type 2 diabetes (Pal *et al*., 2012).

Given the implication in so many processes, as expected, *PTEN* is tightly regulated by a complex network of genetic, transcriptional, post-transcriptional and post-translational elements (Bermúdez Brito *et al*., 2015).

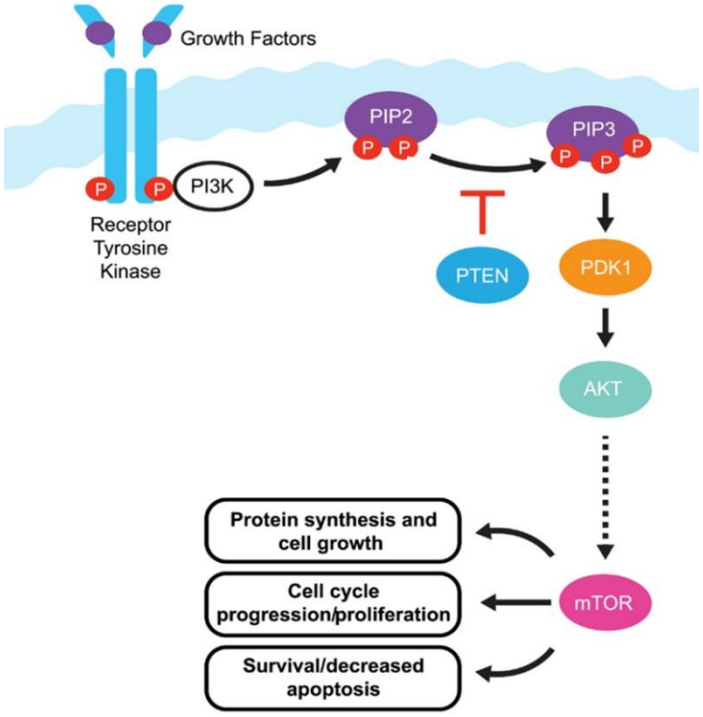

Figure 3. PTEN protein role as tumor suppressor in the inactivation of the PI3K/AKT/mTOR pathway. Adapted with modifications from Phin *et al*. 2013.

4.2. *KLLN*

PTEN shares a bidirectional promoter with the gene *KLLN*, which has only one small exon and is transcribed from the reverse strand. The transcription of both genes is regulated mainly through direct binding of p53 to specific sequence targets of each gene. All this make it safe to hypothesize both genes are co-regulated. The function of the KLLN nuclear protein consists in the inhibition of the DNA synthesis, a required step for cell apoptosis (a process also regulated by p53) (Cho and Liang, 2008).

It was suggested that the hypermethylation of this shared promoter can be involved in the modification of the PHTS phenotype or even have a causal role of the disease like *PTEN* (Nizialek *et al*., 2015), but more studies are needed to confirm this.

4.3. Other genes

As mentioned before, there is a considerable number of patients with a clinical diagnosis of PHTS but for whom no germline *PTEN* mutation was found as responsible agent. Therefore, and thanks to the improvement of the sequencing technologies (emergence of whole exome sequencing and next generation sequencing), several researchers have looked for other possible factors implicated in the development of this disease. As an

example, germline heterozygous variants in *SEC23B* have been described to be associated with Cowden syndrome and enriched in apparently sporadic thyroid cancer (Yehia *et al*., 2015), and germline variants in *TTN* have been found enriched in patients with classic BRRS features (Yehia *et al.* 2017).

5. Carcinogenesis in PHTS

Mouse models suggest that *PTEN* behaves as an haploinsufficient gene (Alimonti *et al*., 2010), meaning that the germline mutation and the consequent partial loss of activity of the protein are enough for cancer development. Thus, in PHTS it is feasible that subtle levels of the tumor suppressor activity would lead to milder or more severe phenotypes and carcinogenesis depending on the protein dosage (Carracedo *et al*., 2011; Alimonti *et al*., 2010).

6. Genotype-phenotype correlations in PHTS

To date, no research has been able to find any strong correlation between the PHTS genotype and phenotype. Even in the same family, one individual can have the phenotype of BRR, while a relative carrying the exact same mutation can have CS (Marsh *et al*., 1999). Many efforts have been made to try to predict the clinical outcome depending on the genotype, through *in vitro* experiments in human cell lines (Mingo *et al*., 2018), in humanized yeast models (Mingo *et al*., 2018) and *in vivo* mouse neurons (Mighell *et al*., 2018), but to date there are only hypotheses and non-consistent observations in small-scale studies (Smith *et al*., 2018). Some authors proposed that the expected phenotype could be inferred considering the dose of protein activity retained: mutations leading to a partial activity of PTEN would cause milder phenotypes (especially ASD-related phenotypes) and mutations affecting the PTEN catalytic core leading to loss of the lipid phosphatase activity would cause the most severe phenotypes (including cancer) (Leslie and Longy, 2016; Spinelli *et al*., 2015).

The Pten heterozygous mice models resemble but do not reproduce completely the features of the PHTS disease (Carnero and Paramio, 2014). Pten conditional knock-out mice are viable and are used for the study of the *PTEN*-carcinogenesis and therapeutic strategies, in organs like the thyroid, endometrium and prostate (Mirantes *et al*., 2013). Also, several other mice models have been useful for the research on specific aspects of the disease, such as cutaneous hamartomas (Wang *et al*., 2013), retinal hamartomas (Tachibana *et al*., 2018), or LDD (Backman *et al*., 2001; Kwon *et al*., 2001).

7. Genetic counseling and patient management: surveillance and therapeutic options

Given the high variability of expression of the disease and the unawareness of it, there are no consensus guidelines regarding PHTS patient management.

Genetic counseling is an essential step in the management of PHTS patients. The key implications of having this disease should be transmitted clearly to the patient at the consultancy. First, to understand that PHTS is a disease with increased risks to several cancer types. Second, that there are some measures of follow-up to detect any malignancy at the earliest stage possible. Third, that the disease is heritable, therefore it can be passed to the offspring. It is also recommended to perform genetic testing in first grade relatives and other family members at risk, as well as in those whose clinical history suggests a suspect of PHTS. Also, preimplantation genetic diagnosis can be an option in affected individuals.

Once a patient has been diagnosed of PHTS, the surveillance of several symptoms is recommended (TABLE 2), especially to detect cancer at the earliest stage possible.

Table 2. Recommendations on follow-up strategies. (Information based on Eng, 2016**).**

Clinical examination	Children (under 18)	Adult women	Adult men
Thyroid ultrasound	Yearly		
Dermatologic evaluation	Yearly		
Breast self-examination	-	Monthly (beginning at 30)	-
Breast screening	-	Annual (beginning at 30)	-
Endometrial screening	-	Annual (beginning at 30)	-
Colonoscopy	-	Depending on degree of polyposis (beginning at 35)	
Renal imaging	-	Biennial (beginning at 40)	
Cancer screening in case of family history	Beginning 5-10 years before the youngest age of onset in the family		

It is also important to note that the follow-up of PHTS should imply a multidisciplinary team, given the multisystemic expression of this disease. Moreover, since the phenotype cannot be predicted from the genotype, all patients should follow similar surveillance recommendations.

Currently, there is no cure for the PHTS disease. The therapeutic options consist on the individual treatment of the different symptoms as their sporadic counterparts, for example, specific breast cancer therapy or surgery for the excision of mucocutaneous lesions.

Since the causal factor of the disease are the defects in PTEN function at germline level, several research efforts have been made to find a molecular treatment that can restore the PTEN tumor suppressor pathway in every cell of the organism. In this manner, PI3K, AKT and mTOR inhibitors such as rapamycin (also known as sirolimus) are some of the promising options. The usefulness of some derivatives of the rapamycin, such as everolimus, is currently being studied in clinical trials to treat PHTS. Nevertheless, all these agents have a considerable associated toxicity, which is proportional to their spectrum of activity, and thus, there are no solid recommendations on their use yet (Noorolyai *et al*., 2019; Dillon and Miller, 2014; LoPiccolo *et al*., 2007). However, research on this field continues to produce exciting findings. Very recently, some authors showed that pharmacological inhibition of WWP1 (an ubiquitin E3 ligase of PTEN protein that suppresses its dimerization, membrane recruitment and function), with indole-3-carbinol resulted in PTEN "reactivation" and downregulation of the PI3K/AKT/mTOR axis *in vivo*; therefore, this could be an effective therapeutic manner to restore the tumor suppressor functions of PTEN in PHTS patients (Lee *et al*. 2019).

8. PHTS awareness in the scientific field, in the clinic and in the society

The knowledge on the PHTS disease is very limited for several reasons. Few studies regarding PHTS have been published in scientific journals (FIGURE 5A). Most of these papers come from selected groups, namely the groups directed by Dr. Eng, Dr. Pilarski or Dr. Leslie (some of which correspond to the largest patient series published; FIGURE 5B), meanwhile many other publications account for medical reports of single cases. Estimations of cancer risks, incidence, etc. are based on literature reports, many of which were published before the establishment of the diagnostic criteria. The cohorts are rarely followed-up during their lifetime, so it's possible that some patients did not have cancer at the moment of the study but could have developed it later, leading therefore to underestimates of the cancer risks. On the other hand, it is possible that there is an enrichment in cancer patients accounting for the individuals referred from the oncologists' consultancies.

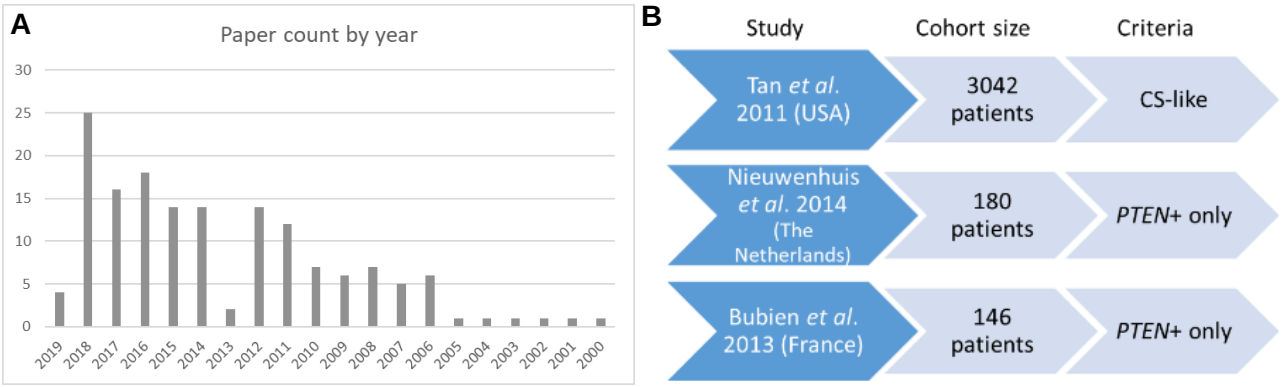

Figure 4. A) Count of papers by year indexed in PubMed with the term "pten hamartoma" in its title or abstract. (Date checked: 12/02/19). B) Largest series of PHTS patients in the literature at international level to date, indicating the cohort size and the patient selection criteria.

After its description, the PHTS was registered as a rare disease in several specialized web portals that contain general information about the disease but also links to associations and clinical trials, and therefore are useful both for experts and patients: European portal Orphanet (ORPHA:306498), the National Institutes of Health's GARD (Genetic and Rare Diseases Information Center), NORD (National Organization of Rare Diseases), ERN GENTURIS (European Reference Network for all patients with one of the rare genetic tumour risk syndromes) and FEDER (Federación Española de Enfermedades Raras). Besides the international recognition of the disease as so, several associations started to give attention to this pathology in the last decade. One example is the PTEN Research Foundation, established in 2017 as an international network that gathers professionals of the academic research, clinical research, and drug development expertise with the objective of finding a therapy for PHTS.

Currently, there are no consensus guidelines for the management of PHTS, with the consequent unawareness of the syndrome in the clinic and therefore underdiagnosis. This situation is even worse in Spain, where there are no registries of the affected patients, and the publication with the largest number of Spanish PHTS patients studied only 8 families (Bussaglia *et al*., 2002) (versus hundreds in the mentioned foreign papers), and so, this disease is still unknown for many national healthcare professionals. Thus, the patients face an uncertain future, which is especially unfortunate having in mind the high cancer risks they face. Therefore, our work on the characterization at a clinical and genetic level of a relevant series of Spanish patients diagnosed with PHTS will contribute to improve this scenario.

OBJECTIVES

OBJECTIVES

1. Clinical and molecular characterization of the PTEN-hamartoma tumor syndrome (PHTS) in Spanish patients.

1.1 Assessment of the *PTEN* implication in the disease.

1.2. Comparison with other population studies.

1.3. Evaluation of the genotype-phenotype correlations and the diagnostic criteria.

2. Search for other genetic factors involved in the disease as phenotype modifiers or as causal elements.

MATERIAL AND METHODS

MATERIAL AND METHODS

1. Patients and clinical evaluation

1.1. Patient recruitment and selection criteria

145 patients of Spanish origin and with suspect of PHTS referred to the CNIO Familial Cancer Clinical Unit from the year 2000 to 2017 were selected for inclusion on this project. We applied relaxed clinical criteria based on the ones from the ICC (Tan *et al.* 2011), including patients who met the "pathognomonic" criteria, who met 1 major criterion and 2 minor criteria, or who suffered any 2 cancers within the spectrum of the PHTS. When the patients were retrospectively selected, the responsible physician and hospital was contacted through e-mail or phone call to ask for collaboration on this project and provide the updated and revised clinical information, the signed informed consent and new biological samples (blood or DNA samples and paraffin-embedded tumor tissues).

1.2. Derivation

Patients were visited either at the HUF or at any of the collaborating hospitals (TABLE S1). Samples of peripheral blood (15 cm^3 in EDTA tubes) were obtained from the patients seen at the HUF and referred to our laboratory. The collaborating hospitals referred either DNA or blood samples. The blood samples were received in our laboratory in a maximum of 24 or 48 hours after blood extraction for RNA extraction and DNA extraction respectively.

1.3. Information gathered from the patients

Three pieces of information were requested to the physician from each proband.

1.3.A. Pedigree

At the consultancy, the physician interviewed the proband asking for information on the personal and family relatives clinical records: dates of birth and death, dates of diagnosis of the clinical features, cancer cases, ages of onset, consanguinity, etc., and the pedigree is drawn with these data.

1.3.B. Checklist

In order to obtain detailed and standardized phenotypic information, we distributed a clinical questionnaire (checklist) to the collaborating physicians. The checklist was designed by us in purpose for the PHTS project with the objective that the clinician would fill the checklist at the moment of consulting the patient. It included a list of features relevant for PHTS diagnosis, selected based on the literature (Tan *et al.*, 2011; Bubien *et al.*, 2013; Mester and Eng, 2013; Pilarski *et al.*, 2013; Nieuwenhuis *et al.*, 2014) and

grouped in categories for a more user-friendly pattern. We were interested not only in the presence or absence of the features (which was asked with “yes” and “no” options) but also in the age at diagnosis, number of lesions, histologic type, etc (free answer fashion). There were also designated spaces to mention any other feature of the proband as well as clinical family history the clinician could consider relevant. Spanish version of the checklist sent to the corresponding physicians is available in the APPENDIX I. The data from the checklists was then incorporated (with anonymization) to Excel files for the further description of the whole series.

1.3.C. Informed consent

The patients received information about the research project and signed informed consent (APPENDIX I). This study was approved by the ethics committee of the HUF.

2. DNA extraction

At our laboratory, genomic DNA from the peripheral blood’s leukocytes was extracted through automated processes: MagNA PURE LC Instrument procedure (Roche) until 2017 and MaxWell RSC Whole Blood DNA extraction kit in the MaxWell RSC instrument (Promega) since then, following the manufacturer’s instructions. DNA extraction from paraffin embedded tissues was performed following the Covaris protocol (option B) to obtain large fragments (>2 kb).

3. DNA quality control and concentration quantification

Several methods were used to check integrity of double-stranded DNA (dsDNA) and to quantify its concentration: agarose gel electrophoresis, NanoDrop spectrophotometer (ND-1000 V3.7.1; Thermo Fisher), Quant-iT PicoGreen dsDNA reagent (Thermo Fisher), Quantus fluorometer (Promega) or BioAnalyzer (Agilent). The choice of one or another was based on the requirements of the further protocol.

4. Point mutation screening: *PTEN* sequencing (PCR, multiplex-PCR and blood-PCR)

The presence of germline point mutations was evaluated in the 9 exons of *PTEN* (studying also the intron-exon boundaries) by PCR and Sanger sequencing. Roche’s reagents were used for the PCR reactions performed before 2017 (Roche Taq enzyme, Roche dNTPs and Roche PCR buffer) and with TaKaRa’s reagents (TaKaRa Taq enzyme, TaKaRa dNTPs and TaKaRa buffer) since then. Exon 8 and the promoter of *PTEN* were amplified using QIAGEN multiplex PCR buffer.

Reactions were performed in Eppendorf and Nexus thermocyclers. Amplification of the PCR products and lack of contamination was checked through gel electrophoresis. Unincorporated primers and dNTPs were removed from the PCR products using illustra ExoProStar (Sigma-Aldrich) prior sequencing. Sanger sequencing was performed in an ABI Prism 3700 sequencer (Thermo Fisher). Chromatograms were analyzed with FinchTV v.1.3.0 (Geospiza Inc.) and the sequences were compared with the reference sequence of *PTEN* gene NM000314.4 transcript ENST00000371953 (GRCh37).

Primers were designed with Primer3Plus (Rozen and Skaletskty, 2000) and their specificity was checked with the UCSC *in silico* PCR tool (https://genome.ucsc.edu/cgi-bin/hgPcr). The list of primers can be found in the TABLE S2. The amplification conditions for each primer pair were defined after testing temperature gradient, time of melting, cycles number, addition of DMSO, etc.

5. *PTEN* large rearrangement screening

5.1 Multiplex ligation-dependent probe amplification (MLPA)

In case no point mutations were found, DNA samples were submitted to large rearrangement analysis through multiplex ligation-dependent probe amplification (MLPA) with SALSA P225D1 (MRC Holland) following manufacturer's instructions. In summary, the MLPA has 4 steps: DNA denaturation, hybridization with the probes, ligation and multiplex PCR. MLPA products were subjected to gel electrophoresis to check for multiple fragment amplification, loaded in a 96 well optical plate with formamide and GeneScan 500 LIZ Size Standard, and sequenced in a 3730 DNA Analyzer (Thermo Fisher). Fragment analysis was performed by normalizing against a non-affected control sample using the Peak Scanner v.1.0 software. Deletion was considered when the amplification peak for a specific probe had 50% less height than the correspondent peak of the control sample.

5.2. Microarray-based comparative genomic hybridization (aCGH)

A SurePrint G3 Unrestricted CGH 4x180K microarray (Agilent) was used in the cases with large deletions in *PTEN* sequence's ends, to interrogate the extent of the deletion and the location of the breakpoints. The protocol used was the Agilent Oligonucleotide array-based CGH for Genomic DNA Analysis version 7.3. Briefly, gDNA samples were digested with restriction enzymes, labeled with fluorescent dyes and purified before hybridization reaction. The microarray was then processed and scanned. Agilent CytoGenomics v4.0.3 software was used for visualization of the aCGH results.

6. Immunohistochemistry (IHC)

5 available paraffin-embedded tumor tissues were cut in 5 µm sections and stained for PTEN and p-AKT using rabbit monoclonal antibodies 138G6 and D9E respectively (Cell Signaling Technology), using an automated stainer (Autostainer). Positive or negative staining was checked in a bright-field microscope under the supervision of a pathologist. Tumor versus adjacent normal tissue was ascertained by a pathologist using hematoxylin-eosin slides. Histo-score (H-score) was calculated following the next equation: H-score=staining intensity x (% of stained cells).

7. Next Generation Sequencing (NGS; gene panel)

A total of 131 DNA samples (127 from blood and 4 from paraffin-embedded tumor tissue samples) were included in a custom NGS panel of Nimblegen (Roche) (see list of the 46 targeted genes in TABLE 3) to look for other possible genetic factors implicated as phenotype modifiers of the disease or even with a causal role. In order to obtain good yields in NGS, the DNA samples were prepared differently as follows. Whenever the samples were eluted in EDTA and correspondent blood samples were available, the DNA extraction was performed again using MaxWell and eluting now in Tris-HCl 0.1 M pH=8. The DNA samples referred from outside our laboratory, with no knowledge on their elution buffer, were purified using a ratio of 1.6X Promega magnetic beads. PicoGreen was used to quantify the dsDNA concentration for each sample. The input consisted in 250 ng of germline genomic DNA (gDNA).

The library was prepared following the SeqCap EZ HyperCap Workflow User's Guide (Roche). Briefly, the steps are the followings:

- Preparation of the sample library using the KAPA HyperPlus Library Preparation Kit:

  gDNA samples are enzymatically fragmented. The next step is the end repair and A-tailing reaction. Then, the KAPA Dual-indexed Adapters (15 µM) are ligated to the samples. These adapters allow for the unique identification of each DNA sample (which is relevant for the further pool). A post-ligation cleanup is performed using AMPure XP Beads and the DNA is then eluted in PCR-grade H_2O. Finally, for the DNAs coming from blood samples, the double-sided size selection is performed. This step allows an increase of the on-target as it allows the discrimination of long DNA fragments (which are not useful for the further steps and can give rise to overamplification).

- Library amplification: Pre-Capture Ligation Mediated PCR:

The library is amplified through a ligation mediated PCR (LM-PCR), for which the master mix and primer mix are provided also in the KAPA HyperPlus Library Preparation Kit. The number of PCR cycles for DNAs from bloods was 5 and 7 for tumors.

Before continuing the protocol, a quality control is performed through PicoGreen (optimal expected concentration yield is ≥1 µg), followed by the use of Agilent Bioanalyzer DNA 1000 assay to check the fragment size distribution is correct (DNA fragments from 150 to 500 bp) in the samples of our pre-capture library.

- Preparation of the multiplex DNA sample library pool:
  Equal amounts of the DNA samples are mixed together in a single tube to obtain a single pool with a combined DNA mass of 8 µg. COT Human DNA and HyperCap Universal Blocking Oligos are added to the pool. The pool is then dried in a DNA vacuum concentrator on high heat (SpeedVac, >60°C).

- Hybridization:
  The target regions are captured by hybridizing the gDNA sample library with the custom SeqCap EZ probe pool (see list of the 46 genes targeted in TABLE 3). Hybridization reagents are provided in the kit and the reaction is performed in the thermocycler for 16 to 20 hours.

- Wash and recover of the captured DNA:
  The hybridized sample is bound to the Capture Beads. These capture beads are washed with specific buffers prior and also after the addition to the DNA sample. No elution is performed in this step as the beads plus the captured DNA will be used as template in the following LM-PCR.

- Amplification of the captured DNA: Post-Capture LM-PCR and clean-up:
  Using the post-capture LM-PCR master mix, the captured DNA sample is amplified and then purified using AMPure XP Beads. Elution is performed in PCR-grade H_2O. Another quality control is performed before proceeding with the sequencing. This time, since all the samples are now in a single tube, the concentration is checked using Quantus. An expected yield around 38 ng/µL is adequate. BioAnalyzer is also performed to check the fragment size distribution is also correct. Considering these data, the captured and amplified DNA sample pool is then diluted to 10 nM to proceed with the sequencing.

Library sequencing was performed in a HiSeq 2500 Illumina Sequencing Instrument at the Genomics Unit of the CNIC with a coverage up to 900X for blood samples and 700X for the tumors, 4 million paired-end reads and 15% of duplicates approximately for each

sample. Another quality control is performed using FASTQC software to check correct sequencing of every sample (coverage, duplicates, phred, etc.). Germline variant annotation was performed separatedly for SNVs and indels, using several tools: BWA, SAM, PICARD, GATK, HaplotypeCaller, VEP, etc. Variant filtering consisted in maintaining only the variants in canonical transcripts (APPRIS), with high or moderate impact, an allele frequency between 0.3-0.6 for heterozygotes and around 0.9 for homozygotes, and MAF under 0.1% in general population (gnomAD). Somatic variants were annotated with Mutect2, and prioritized similarly, excluding the ones present in the correspondent paired-blood sample (germline variants) and/or with allele frequency under 15%. Similarly, mosaicisms were analyzed with Mutect2 and artifacts were excluded considering occurrence in multiple samples or highly variable allele frequencies. Integrative Genomics Viewer (IGV; Broad Institute) was also used to visually check the NGS data for possible artifacts (duplicates, repetitive regions, low read depth or poor coverage).

Table 3. List of the 46 genes included in the NGS panel

Nr	Gene	Nr	Gene
1	*AKT1*	24	*FLCN*
2	*AKT2*	25	*TP53*
3	*AKT3*	26	*FH*
4	*DEPDC5*	27	*MET*
5	*MTOR*	28	*CDKN2A*
6	*PIK3C2B*	29	*AR*
7	*PIK3CA*	30	*KRAS*
8	*PIK3R1*	31	*BRAF*
9	*PTEN*	32	*NRAS*
10	*RICTOR*	33	*MEN1*
11	*RPTOR*	34	*DAXX*
12	*STK11*	35	*ATRX*
13	*TSC1*	36	*CUL3*
14	*TSC2*	37	*NF1*
15	*NF2*	38	*ARID1A*
16	*SETD2*	39	*CDKN1B*
17	*SMARCB1*	40	*EIF1AX*
18	*USP9X*	41	*NFE2L2*
19	*BAP1*	42	*STAG2*
20	*KDM5C*	43	*TERT*_region ATG (250 nt of the promoter)
21	*KDM6A*	44	*TFE3* fusion (ex2-ex6; incl. intr2-5; ENST00000315869.7)
22	*PBRM1*	45	*TFEB* fusion (1 kb intr2-ex3; ENST00000230323.8)
23	*VHL*	46	*TFEB* fusion (ex9, intr9, ex10; ENST00000230323.8)

8. Whole exome sequencing (WES)

11 *PTEN*-wt probands (7 meeting Pilarski's clinical diagnostic criteria, 3 with LDD, and 1 pediatric case) were selected for whole exome sequencing. Germline genomic DNA samples from these individuals were quantitated using Quant-iT PicoGreen dsDNA reagent (Thermo Fisher), their quality was checked using the NanoDrop spectrophotometer (ND-1000 V3.7.1; Thermo Fisher) and degradation was excluded after

agarose gel electrophoresis. DNA samples were sent frozen to Novogene (China), to perform the whole exome sequencing (WES) with 100X coverage (12 Gb) and primary bioinformatics analysis. At Novogene, the first step was the quality control (confirm again the concentration of DNA, purity and lack of degradation). Second, a total amount of 1 μg genomic DNA per sample was used as input material for the DNA library preparation. Sequencing libraries were generated using Agilent SureSelect Human All Exon kit (Agilent Technologies). The next step was the bioinformatics analysis. The original fluorescence images obtained from Illumina high throughput sequencing platform were transformed to short reads by base calling. These short reads (raw data) were recorded in FASTQ format, which contains sequence information (reads) and corresponding sequencing quality information. The raw data were cleaned, eliminating artifacts. Burrows-Wheeler Aligner (BWA) was used to map the paired-end clean reads to the human reference genome. Then it followed the variant detection of SNPs and InDels, and annotation with the tool ANNOVAR (Wang *et al*., 2010) in multiple aspects, including protein coding changes, affected genomic regions, allele frequency reported in databases, deleteriousness prediction, etc. The probands' WES data were grouped according to the clinical manifestations as mentioned before in order to search for shared variants, genes or pathways affected. In the variant filtering, we assumed a monogenic model of the disease and excluded the variants that were in non-canonical transcripts, non-exonic regions, synonymous, low read depth or MAF>0.001 in European population. We prioritized variants with *in silico* predicted deleteriousness (through SIFT, Polyphen2, Condel, FATHMMM, MutationTaster, MutationAssessor) and that appeared in genes related with PTEN function or pathway (using STRING, Reactome and Kegg pathway), or in known cancer genes, or with the patient phenotype, such as autism genes, (using ClinVar, OMIM and HPO).

9. Variant interpretation

Mutations were considered deleterious if they were previously described as so in public databases (ClinVar, HGMD, ExAC, dbSNP, gnomAD, LOVD), or when an altered cDNA sequence supported the pathogenicity.

10. Variant validation

The presence of mutations or VUS was confirmed on a second sample and through a different method: MyTaq Blood PCR (Bioline) to validate variants found through point mutation screening, and Sanger sequencing to validate variants found through NGS or WES.

11. Total RNA extraction

RNA was extracted manually starting from 7 mL of peripheral blood. Briefly, first, ficol-hystopaque density gradient was used to isolate the leukocytes, which were then washed with PBS 1X-DEPC 0.1% to avoid contamination of erythrocytes. TRIzol reagent (ThermoFisher) is added to lyse the cells while inhibiting RNAses. Then, chloroform is added to separate the RNA in a density layer which is recovered through pipetting. Finally, the isopropanol is added to precipitate the RNA. For long-term storage, RNA samples were kept in isopropanol at -80 °C. Before use, isopropanol was cleaned out from each RNA sample through wash steps with 70% ethanol and DEPC H_2O, and finally resuspending the RNA in RNAse-free water. Concentration and integrity of the RNA were checked using NanoDrop (ND-1000 V3.7.1; Thermo Fisher). The adequate ratios for RNA are the followings: concentration: 500-1000 ng/µL, A260/280: ~2.0 and A260/230: 1.8-2.2.

12. cDNA synthesis

The High Capacity cDNA reverse transcriptase kit (Applied Biosystems) and the manufacturer's guide was used for the cDNA synthesis through reverse-transcription PCR (RT-PCR), starting from 1000 ng of RNA.

13. qPCR and mRNA expression analysis

Control samples consisted on cDNAs from non-affected individuals (neither PHTS nor any cancer history) who visited the HUF consultancy and altruistically donated a blood sample for this study after being informed of the project. Each sample was analyzed in triplicate. qPCR reaction was performed for each sample in triplicates with Promega's Master Mix Go Taq, following manufacturer's instructions, in an ABI QuantStudio S6 Flex System (Applied Biosystems), and the results were analyzed with the QuantStudio Real-Time PCR software. *36B4* was used as reference gene (Akamine *et al*., 2007) to calculate *PTEN*, *KLLN* and *PTENP1* relative mRNA expression using $2^{-\Delta\Delta Cq}$ method. Primer pairs are shown in TABLE S2.

14. Functional studies of the *PTEN* VUS

The experiments were carried out by the groups led by Dr. Pulido (Cancer Biomarkers group at BioCruces Health Research Institute, País Vasco) and Dr. Molina (Signal transduction in *Saccharomyces cerevisiae* group at the Universidad Complutense de Madrid). Cell culture conditions, transformation and plasmids (pYES2 PTEN, YCpLG myc-p110α-CAAX, pRK5 PTEN-GFP, pSG5 HA-AKT1) accord to previously described in

Coronas-Serna *et al*., 2018; Mingo *et al*., 2018; Gil *et al*., 2015. Immunofluorescence and microscopy were performed as described in Mingo *et al*., 2018.

15. Statistical analyses

Chi-square or Fisher's exact tests were used to evaluate differences between our cohort and others previously published (Tan *et al.*, 2011; Bubien *et al.*, 2013; Nieuwenhuis *et al.*, 2014). Logistic regression was used to evaluate associations and risks. qPCR figures and analyses (t-test, Mann-Whitney) were done using GraphPad Prism (GraphPad Software, Inc.). *P* values under 0.05 were considered statistically significant: **P* < 0.05; ***P* < 0.005; ****P* < 0.0005.

16. Descriptive analyses

Several considerations were made to describe our patient series and are mentioned here.

The macrocephaly is considered when the head circumference (HC) measurement is above 2 standard deviations, or which is the same, above the 97th percentile. The standard HC values and percentiles are defined according to age, gender and ethnic origin. In our checklist we asked that the clinician indicated the HC measurement of the proband at birth and at the moment of the consultancy, and we then considered a macrocephalic status using the pediatric and adult HC tables (Lapunzina and Aiello, 2002) as reference. The same strategy was followed to consider general body overgrowth through the weight and height measurements.

Although breast cancer can affect also men and at least 2 cases have been described driven by germline *PTEN* mutations (Fackenthal *et al*., 2001), none of our male patients developed breast cancer and therefore in our study we considered the breast cancer as a sex-linked cancer related only with women.

To decide if our patients met or not the clinical criteria proposed in the literature, we took the following decisions. Patients met the relaxed ICC criteria if they met any of the pathognomonic, or 2 or more major criteria or two or more minor criteria. In order to be stricter and because we had a wide spectrum of mucosal lesions reported that can be also easily found in general population, this feature was not considered as enough criterion. CC scores, both pediatric and adult, were calculated for each individual using the Cleveland Clinic PTEN Risk Calculator, available at https://www.lerner.ccf.org/gmi/ccscore/. Criteria to meet Pilarski's revised clinical criteria was in accordance to that proposed by the authors: any two major with or without minor criteria, or one major and two minor criteria, or three minor criteria (Pilarski *et al*., 2013).

Calculation of the measures of the diagnostic criteria was as follows: specificity=true negative/(true negative+false positive); sensitivity=true positive/(true positive+false negative); accuracy=(true positive+true negative)/(positive+negative); false positive rate=false positive/(false positive+true negative); positive predictive value=true positive/(false positive+true positive); negative predictive value=true negative/(true negative+false negative). We considered as positive cases the *PTEN*-mut+ individuals who met the diagnostic criteria (true) or not (false), and as negative cases the *PTEN*-wt who met the diagnostic criteria (false) or not (true).

RESULTS

RESULTS

1. Patient characteristics

1.1. Recruitment and sample collection

Our series is composed by a total of 145 patients. 26 patients (18%) were visited in our consultancy of the CNIO Familial Cancer Clinical Unit in the Hospital Universitario de Fuenlabrada (HUF) to receive genetic counseling, and were evaluated by a geneticist with a thorough knowledge of PHTS and by a dermatologist; while the remaining (n=119; 82%) were referred to our laboratory from around 35 different hospitals, accounting for 8 Spanish autonomous communities of origin and with the collaboration of around 37 physicians of diverse medical specialties (FIGURE 5-A).

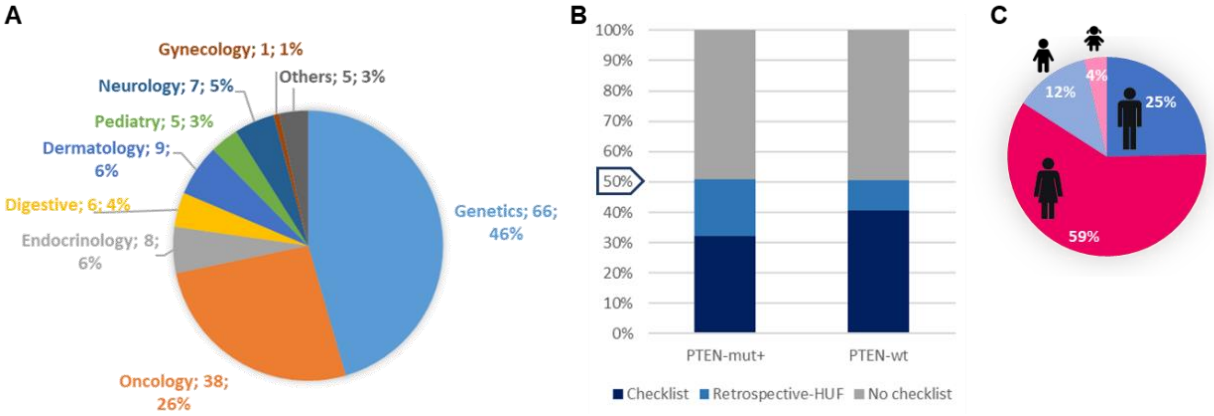

Figure 5. A) Medical specialties from which the patients were referred for PHTS diagnosis. B) Obtained checklists or consultancy at HUF. C) Patient series demographic composition according to age and sex. (VUS carriers are excluded of these graphs).

1.2. Demographic characteristics

The series of probands studied in this work, consisted mostly in adults (84%) but also included 25 patients (16%) under the age of 18 years old. 59% of the adults were women and 25% men, plus 12% young men and 4% young women (FIGURE 5-C). Overall age range was 1-76 years. Median age was 30 years in the mutation carriers (*PTEN*-mut+) and 46 in the *PTEN*-wt. All individuals were Caucasian with Spanish origin.

1.3. Clinical review through checklists

In order to obtain complete revised clinical information in a homogeneous way, we created a specific clinical questionnaire (checklist) for the PHTS clinical characterization of the patients and sent them to the corresponding physicians. Complete data were obtained through checklists for 51 patients (15 *PTEN*-mut+, 4 VUS carriers and 32 *PTEN*-wt) (FIGURE 5-B). Nevertheless, we can assume we also have exhaustive clinical data for the patients recruited retrospectively who had visited the consultancy in the HUF years before

the creation of the checklists but who were also evaluated by a clinical geneticist with expertise in PHTS (n=21). Therefore, we have good clinical information from at least 72 patients (50% of the total series). For the remaining cases, we reviewed the medical records referred by the corresponding physician.

2. *PTEN* mutation spectrum

All the patients included in this project were screened for the presence of mutations in the *PTEN* gene. Genetic testing of *PTEN* currently consists in the analysis of the exons and intron-boundaries, together with the screening for large deletions. Almost half of the patients (46% of the total series) harbored some alteration in the *PTEN* gene. 52 patients (36%) were positive for pathogenic germline point mutations in *PTEN* and 7 individuals (5%) harbored large deletions in the mentioned gene. 7 additional patients (5%) carried variants of unknown significance (VUS) (FIGURE 6). We will refer to the carriers of pathogenic mutations (either point mutations or large deletions) as *PTEN*-mut+, excluding the VUS carriers due to the complexity of interpreting the consequences of these genetic variants. (The complete list of *PTEN* alterations are shown in TABLE S3, S4 AND S5). The remaining 79 probands (54%) were negative for point mutations, large rearrangements or VUS in *PTEN* gene. We will refer to these patients as *PTEN*-wt.

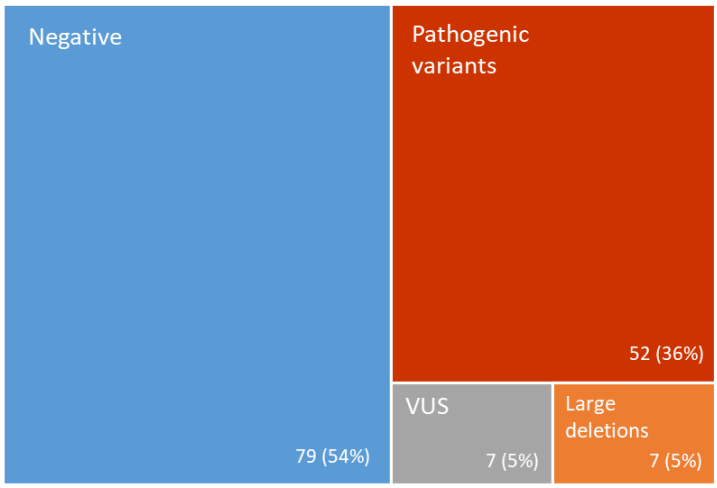

Figure 6. *PTEN* germline status of the 145 patients. Size of rectangles corresponds to the number of individuals in each category (number and proportion of the total series are indicated). Negative: no mutation found in *PTEN*; VUS: variant of unknown significance.

2.1. *PTEN* point mutations

We found 43 different *PTEN* point mutations (TABLE S3). All types of mutations were found, from missense, to splicing or frameshift, among others (FIGURE 7-B). Several mutations appeared recurrently in our series: c.1003C>T p.(Arg335Ter) located in exon 8 (n=5 patients), c.388C>T p.(Arg130*) in exon 5 (n=3); c-39_40del p.(Arg14Glufs*29) in exon 1 (n=2), c.406T>C p.(Cys136Arg) in exon 5 (n=2) and c.697C>T p.(Arg233*) in exon 7 (n=2).

The point mutations appeared at higher frequencies between exons 5 and 8 of *PTEN*, but we also found a considerable number of patients carrying non-recurrent mutations located in exon 1 of *PTEN* (FIGURE 7-A). From the patients with exonic mutations, 5 (11%) had mutations affecting the catalytic core of the PTEN protein (residues 123 to 130), 21 (47%) affecting the phosphatase domain (PTP) amino acids (residues 1 to 185; other than the catalytic core) and 19 (42%) the C2 domain (residues 186 to 351), but none had mutations in the C-terminal end (residues 352 to 403) (FIGURE 7-C). The missense mutations appeared more in the phosphatase domain of PTEN protein, meanwhile the mutations with a putative truncating effect in the protein (either frameshift or nonsense mutations) affected mostly the C2 domain (FIGURE 7-D).

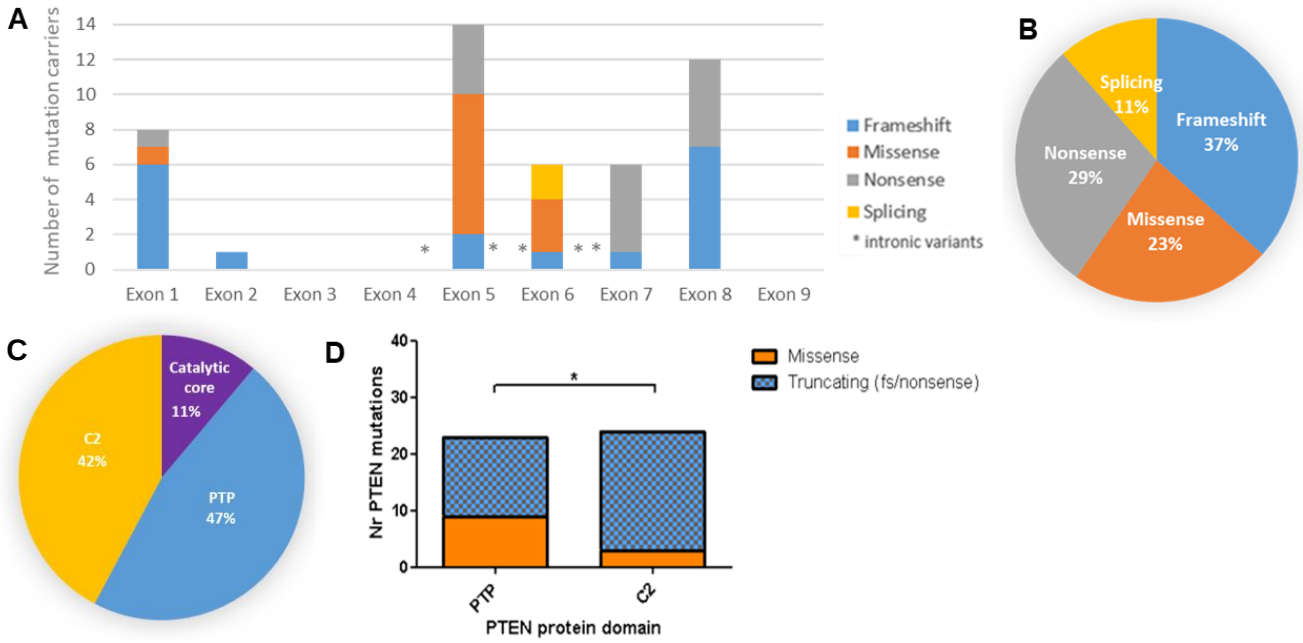

Figure 7. *PTEN* point mutations: distribution, types and protein domains affected. (VUS are excluded from this figure). A) Distribution of the point mutations in the *PTEN* exons. Mutation types are also shown. B) Proportion of each type of mutation in our study. C) PTEN protein domains affected by the exonic mutations. D) *PTEN* mutations (either frameshift or nonsense) accumulated in the phosphatase (PTP) and C2 domains.

2.2. *PTEN* large rearrangements

Using MLPA, we found 7 (5%) patients harbored large deletions in *PTEN* and in 5 of them the rearrangement included one or more neighboring genes (FIGURE 8). The aCGH allowed the exhaustive characterization of these 5 large deletions, where 3 extended to the proximal gene *KLLN* and the other 2 cases involved a region of 8 and 10 Mb, therefore affecting many other genes such as *BMPR1A* (FIGURE 8, TABLE S5).

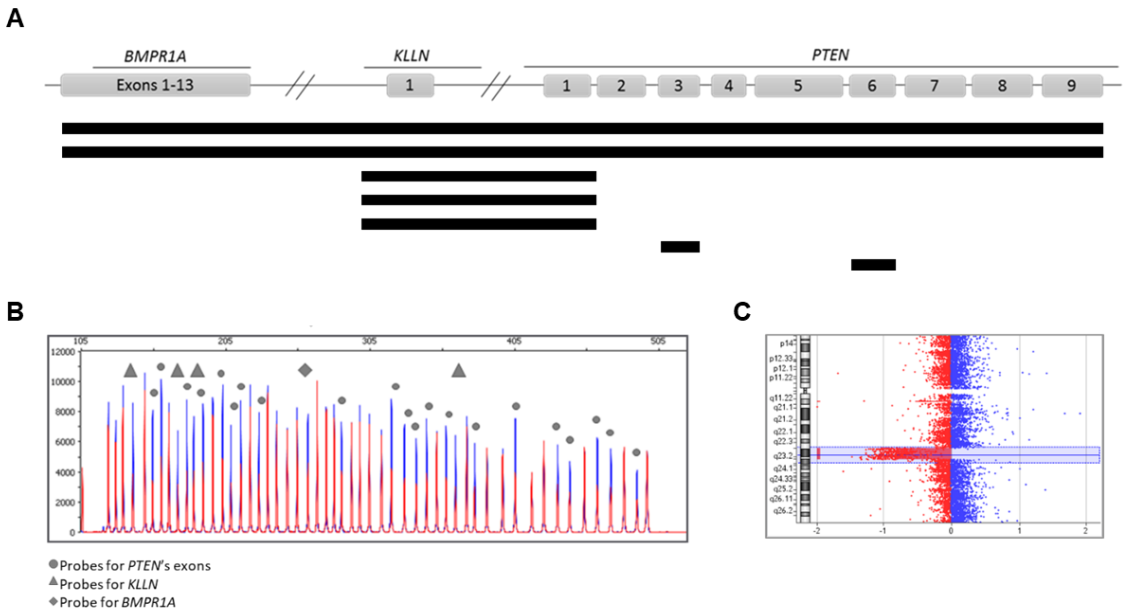

Figure 8. Large deletions found involving *PTEN* locus. A) Schematic representation of the gene regions affected by each of the 7 deletion cases. B) MLPA output of one sample showing the deletion of *PTEN*, *KLLN* and *BMPR1A* genes. C) aCGH output of the same sample as in B, showing deletion of 8 Mb in chr10q22.3-q23.31. Detailed figures of the total deleted regions are shown in TABLE S5.

2.3. Functional studies of the *PTEN* VUS

Seven of our patients were carriers of VUS (listed in TABLE S4). From these, we decided to explore the ones putatively causing an amino acid change, either in the phosphatase or in the C2 domain of the PTEN protein, and which were related with a strong phenotype of PHTS in the carriers (TABLE 4), therefore supporting our hypothesis that these variants might be pathogenic. For this purpose, we carried out *in vitro* functional studies in a humanized yeast model and in the mammalian cell line COS-7, in collaboration with the group of Dr. Pulido and Dr. Molina to study the impact of each variant individually on the different PTEN protein roles.

Table 4. Selected VUS for the functional studies and brief description of the clinical features found in the carriers. (Complete clinical description can be found in TABLE S4). CLS: cytoplasmic localization signal, NLS: nuclear localization signal, Ppase: phosphatase.

Exon	DNA change	Protein change	Protein domain	Clinical features
1	c.77C>T	p.(Thr26Ile)	Ppase (NLS and CLS)	(Pediatric case). Macrocephaly, obesity, macular pigmentation penis, bilateral gynecomastia, autism, mental retardation, musculoskeletal alterations.
5	c.284C>G	p.(Pro95Arg)	Ppase	Macrocephaly, overweight, papules, palmoplantar keratoses, trichilemmomas, oral papillomatosis, lipomas, macular pigmentation penis, goiter, Hashimoto's thyroiditis, colonic and gastrointestinal polyps, cerebral hamartomas.
6	c.529T>A	p.(Tyr177Asn)	Ppase	(Pediatric case). Macrocephaly, motor delay.
7	c.781C>G	p.(Gln261Glu)	C2	Macrocephaly, lipomas, macular pigmentation penis, colorectal polyps, general overgrowth.
8	c.929A>G	p.(Asp310Gly)	C2	Macrocephaly, papules, palmoplantar keratoses, thyroid adenomas, papillary-follicular thyroid cancer, colorectal polyps (inflammatory, lymphoid and hamartomatous), testicular cancer.
8	c.829A>G	p.(Thr277Ala)	C2	(Pediatric case). Macrocephaly, general developmental disorder, speech delay, general overgrowth.

First, the phosphatase activity of the different PTEN mutant proteins was explored in the COS-7 overexpressing the mutant or PTEN WT protein together with expression of the p.K179M AKT1 protein (catalytically inactive) and the ratios of the active form of AKT (AKT phosphorylated at Ser473) and the total AKT were measured. Cells transfected with empty plasmid of PTEN (mock) and cells transfected with the PTEN mutant p.C124S, which is completely phosphatase inactive, were also used as controls. As result, the P95R was the only mutant with a complete loss of catalytic activity, as these cells showed high levels of p-AKT, similarly to the mock cells and the PTEN C124S cells. Meanwhile, the other PTEN mutants showed similar activity as the PTEN WT (FIGURE 9).

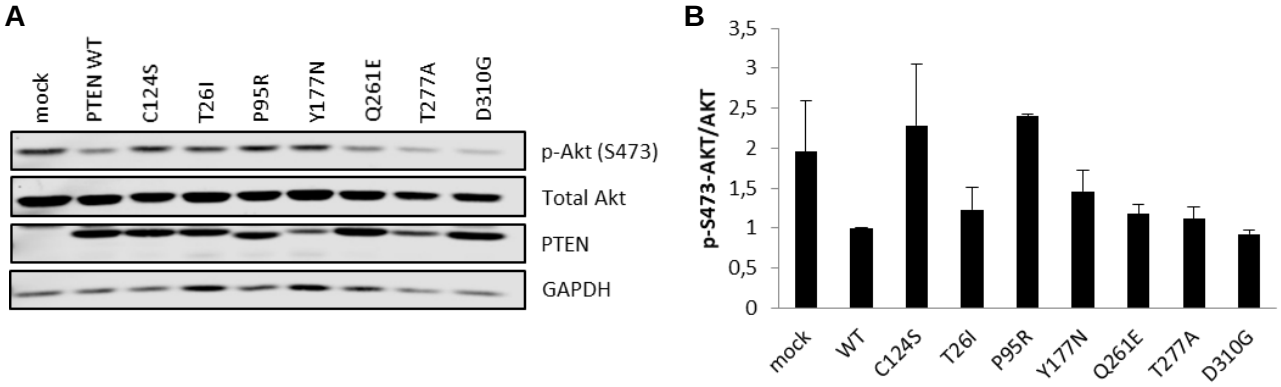

Figure 9. PTEN catalytic activity assessed using AKT levels as surrogate, in COS-7 cells co-expressing PTEN and AKT. A) WB of one representative experiment. GAPDH was used as loading control. B) pAKT(Ser473)/AKT ratio for each condition. Bars represent mean quantification of the bands from 2 experiments ± SD. Mock: empty vectors. (Data provided by Dr. Pulido).

These observations were replicated with the toxicity rescue assay in a humanized yeast model. The artificial hyperactive catalytic subunit of PI3K: p110α-CAAX, depletes the PIP2 levels at the plasma membrane and consequently generates more PIP3 which activates AKT1. This situation is toxic for the yeast and results in growth inhibition. Yeasts were transfected with a plasmid encoding this p110α-CAAX and another encoding the PTEN WT or mutant protein, and their expression was repressed with glucose addition or induced with galactose addition. The activity of PTEN was ascertained considering the survival ability of the yeasts (FIGURE 10-A) and through the levels of AKT at the plasma membrane (FIGURE 10-B). Again, the P95R was the only mutant totally inactive as lipid phosphatase. The mutants T26I and Y177N showed apparently partial activity and the remaining (Q261E, T277A and D310G) were functional as the WT protein.

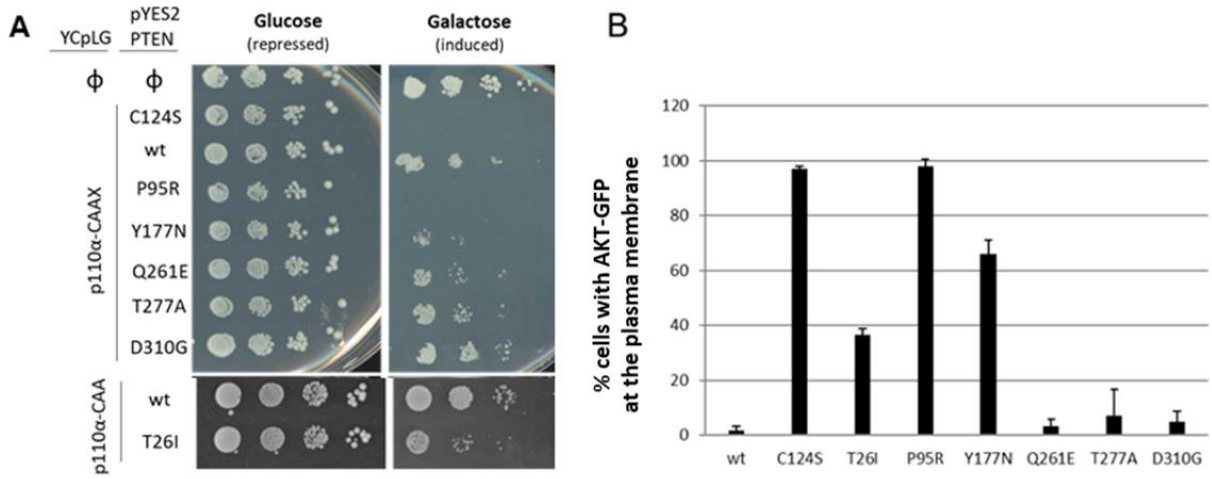

Figure 10. PTEN catalytic ability measured in the humanized yeast model. A) Yeast growth drop assay with cells transformed with the combination of plasmids, one encoding the p110α-CAAX and the other encoding the different PTEN mutants. Growth was evaluated under glucose (heterologous proteins not induced) or galactose addition (induced). B) AKT-GFP microscopical quantification in yeasts co-transformed with plasmid encoding the AKT-GFP reporter. Bars correspond to the mean ± SD. (Data provided by Dr. Pulido).

We hypothesized these VUS could also affect PTEN protein subcellular localization. No differences were observed through immunofluorescence in the localization of the different mutants compared with the WT form when looking at a context of entire PTEN protein (residues 1 to 403) (FIGURE 11-A). However, in the context of the truncated PTEN protein form (aa 1-375), which tends to accumulate in the nucleus, (FIGURE 11-B) and when tagging the C-terminal domain of PTEN with GFP (FIGURE 11-C), some of the mutant proteins displayed altered localization. The protein variants P95R, Y177N and T277A had a defective nuclear accumulation.

Altogether, the results suggest a connection between the VUS in the N-terminal domain of PTEN (P95R, T26I and Y177N), the consequent impaired catalytic activity of the PTEN

mutant proteins and their aberrant nuclear localization, supporting its pathogenic role. On the other hand, the VUS located in the C-terminal region will require further research as in these studies they behaved similar to the WT protein, with exception of T277A, which also showed a reduced accumulation in the nucleus (FIGURE 11-B AND C).

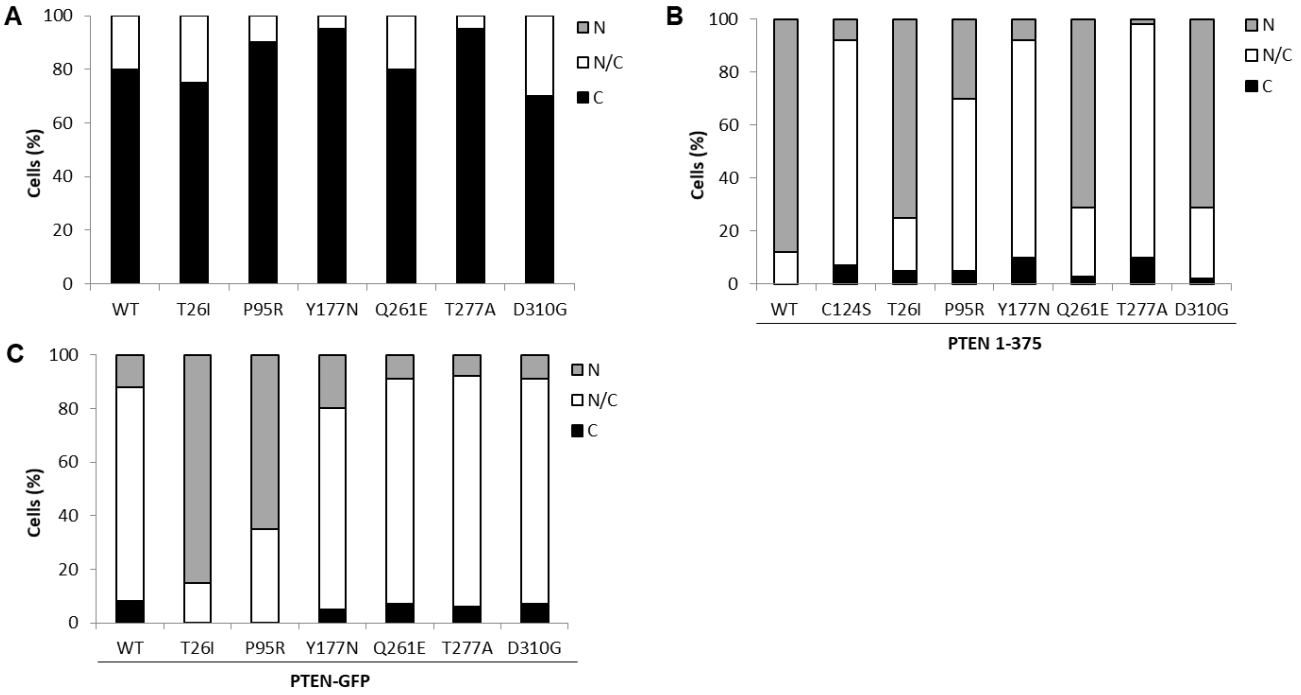

Figure 11. Subcellular localization of PTEN variants in COS-7 cells. A) Localization assessed through IF in the background of entire PTEN protein form (1 to 403 aa). B) Localization assessed through IF in the background of truncated PTEN protein 1-375. C) Localization assessed through GFP fluorescence measure in the context of PTEN tagged with GFP in its C-terminal domain. C: cytoplasmic, N: nuclear, N/C: nuclear/cytoplasmic. (Graphs provided by Dr. Pulido).

2.4. Origin of the *PTEN* variants

We were able to explore the origin of the *PTEN* variants in 21 cases through genetic testing in their family relatives. Thus, we confirmed the presence of 14 *de novo* (24% of the *PTEN* variant carriers) and 7 familial variant cases (12%). There were other 12 *PTEN*-mut+ cases with first-degree relatives showing PHTS features, as determined through family history review, that could be additional familial cases. From the pediatric cases who were *PTEN*-mut+, 7 were confirmed to have a *de novo* variant.

3. Clinical characterization and clinical criteria for *PTEN* study

The most frequent clinical manifestations for patient referral for *PTEN* mutation analysis were mucocutaneous, thyroid lesions and macrocephaly (FIGURE 12).

We compared the two groups of patients, *PTEN*-mut+ and *PTEN*-wt, in order to establish clinical criteria that improve the selection of candidate patients to the molecular study. VUS carriers were excluded of these analyses.

We analyzed the presence or absence of the clinical features already considered as characteristic of PHTS, such as macrocephaly, but also others not so straightforward associated, such as obesity, in our patient series. We could see that, as expected, all the features were more present in the *PTEN*-mut+ patients (FIGURE 12). The 3 more common traits in the *PTEN*-mut+ group of patients were the mucocutaneous lesions (any type, in general), thyroid lesions and macrocephaly, followed very closely by oral papillomas and gastrointestinal polyposis. On the other hand, the 3 more common clinical features among the *PTEN*-wt patients, were the mucocutaneous, thyroid and gynecological lesions, and papules.

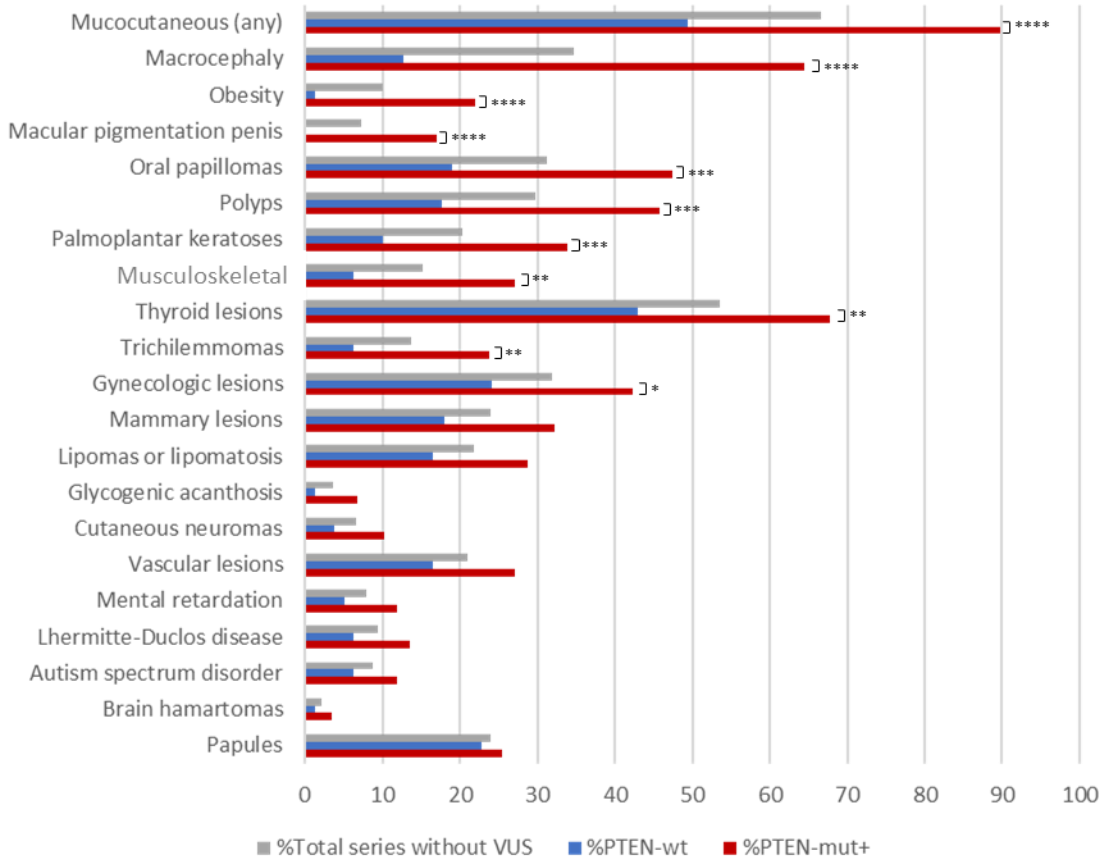

Figure 12. Proportion of individuals showing each clinical manifestation in our PHTS series, excluding the VUS carriers. Chi-square or Fisher test significance is shown for comparisons on absolute numbers of the *PTEN*-wt and *PTEN*-mut+ groups. (The presence and absence of all the reported clinical features can be found in the SUPPLEMENTARY FIGURES for every patient).

When comparing the clinical findings in the *PTEN*-mut+ and *PTEN*-wt we found that some features were significantly more common among the first ones, namely the macrocephaly, obesity, mucocutaneous lesions (in general), macular pigmentation of the penis, oral papillomas, gastrointestinal polyposis, palmoplantar keratosis, musculoskeletal lesions, thyroid lesions, trichilemmomas and gynecologic lesions, suggesting its usefulness as diagnostic criteria. Of note, the overall mucocutaneous lesions were reported for 90% of the *PTEN*-mut+, but only accounted for 49% of the *PTEN*-wt, and in the same way, the

macrocephaly was present in 64% of the *PTEN*-mut+ patients, but only 13% of the *PTEN*-wt were referred with this feature. On the other hand, other classical clinical features of PHTS, such as Lhermitte-Duclos, autism and vascular lesions, were not able to significantly discriminate the *PTEN*-mut+ individuals from the *PTEN*-wt (FIGURE 12).

Regarding the cancer susceptibility, we found that 69 individuals (50% of the total series, excluding VUS carriers) had developed some cancer. The incidence of cancer was higher in the *PTEN*-wt (n=46; 58%; p=0.0017) compared to the *PTEN*-mut+ patients (n=23; 39%) (FIGURE 13-C).

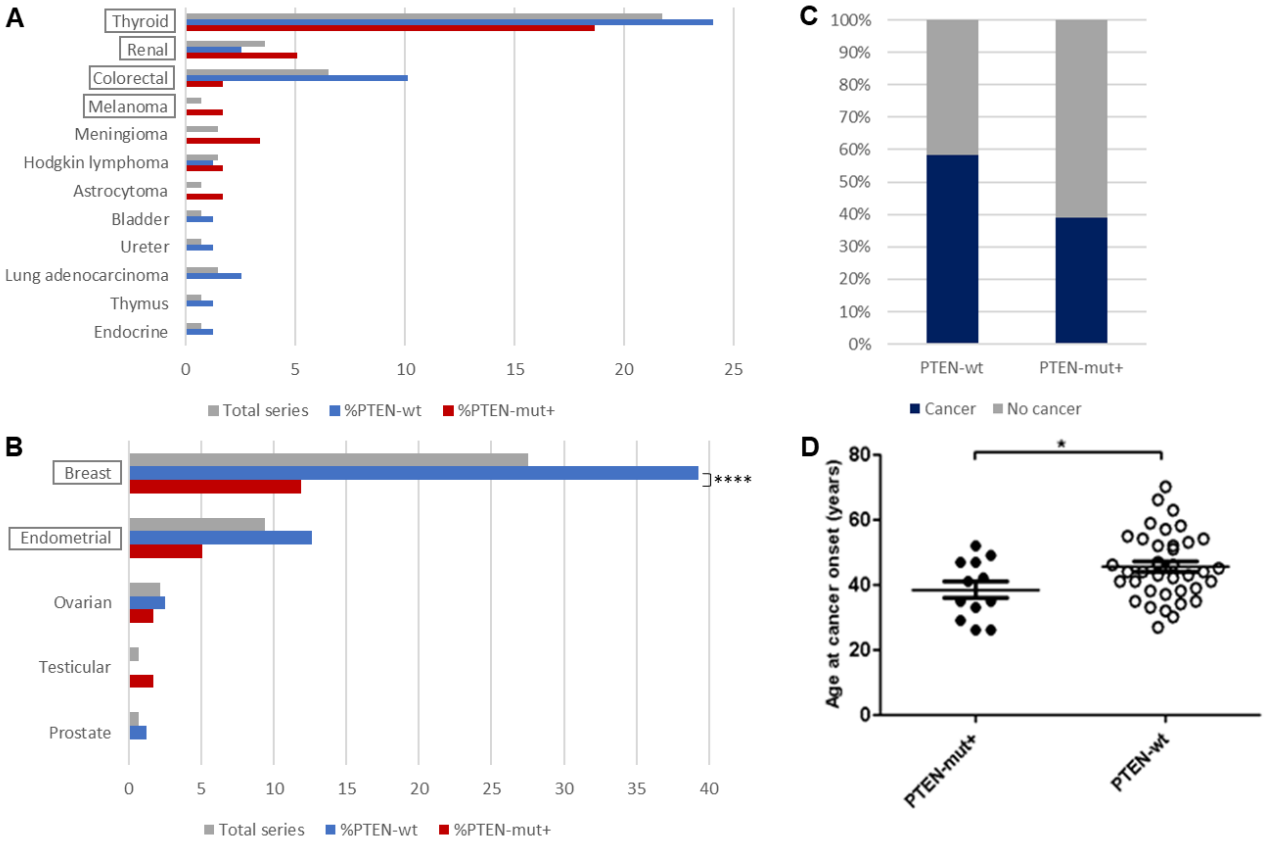

Figure 13. A) Occurrence of the different cancer types (not linked to sex) in the patient series. PHTS-associated cancer types are indicated in squares. B) Occurrence of the sex-linked cancer types in the patient series. PHTS-associated cancer types are indicated in squares. C) Occurrence of cancer in the patient groups. D) Age at cancer onset for the two groups of patients (PTEN-wt and PTEN-mut+). Each dot corresponds to one individual. Mean age and SEM are indicated. A total of 50 patients with known age at cancer onset are represented, excluding the pediatric cases. Two tailed t-test P-value=0.034.

The 3 most frequent cancer types in the whole series (excluding the VUS carriers) were the thyroid, colorectal and renal cancer, while regarding the sex-linked cancers, the most frequent were the breast, endometrial and ovarian cancers (FIGURE 13-A AND B).

64 individuals had suffered some cancer within the spectrum of PHTS (PHTS-cancer), which consist in breast, thyroid, endometrial, renal cell, colorectal cancers and melanoma: 20 *PTEN*-mut+ (31% of the PHTS-cancer patients) and 44 *PTEN*-wt (69% of the PHTS-

cancer patients). Even though the PHTS-associated cancer types can also be found commonly among general population, the abundance of these cancers among our *PTEN*-wt patients was higher than expected and this is probably due to a selection bias. We also found other cancer types not so frequently associated to PHTS, like meningioma, astrocytoma or testicular cancer, in individuals of both *PTEN*-wt and *PTEN*-mut+ (FIGURE 13-A AND B). The spectrum of cancer types was not wider in the *PTEN*-wt or in the *PTEN*-mut+.

We explored how many of the PHTS-cancer patients also had other PHTS-features, specifically macrocephaly, LDD, mucocutaneous lesions or gastrointestinal polyposis (1 or more of any of these features). All the *PTEN*-mut+ patients with a PHTS-cancer also showed other PHTS-features, but only 26 *PTEN*-wt patients did so. Of note, a portion of our series (n=18) was referred for presenting only PHTS-associated cancers, with apparently no other feature of the disease. None of these individuals were carriers of *PTEN* mutations and accounted for 23% of the whole group of *PTEN*-wt patients, suggesting that the only presentation of cancer might not be enough criterion to perform *PTEN* genetic testing.

Although *PTEN* mutation carriers have risk to develop multiple cancers, we found that the manifestation of two or more cancers per patient was more frequent in the *PTEN*-wt group of our series (p=0.026) and only a few of these individuals were *PTEN*-mut+ (FIGURE 14-A). The most frequent combination of cancer types in both patient groups was breast cancer together with thyroid cancer and breast cancer together with endometrial cancer, accounting for the classical PHTS-cancer types (FIGURE 14-B).

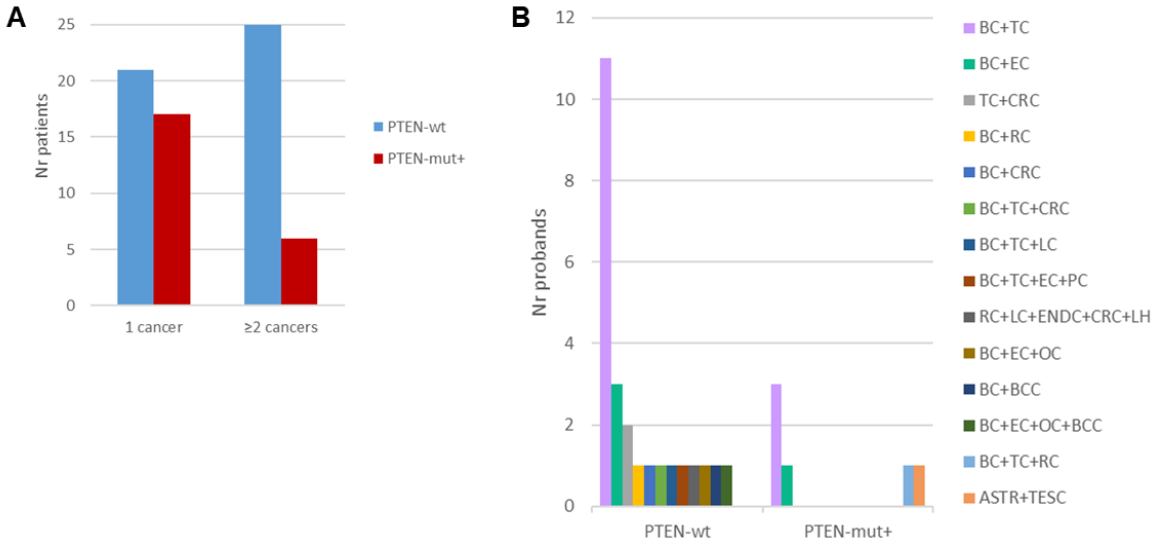

Figure 14. Development of multiple cancers in our PHTS patients. A) Occurrence of 2 or more cancers in *PTEN*-wt versus *PTEN*-mut+ individuals. B) Spectrum of the combination of cancer types found in each patient that suffered 2 or more cancers. ASTR: astrocytoma; BC: breast cancer; BCC: basal cell carcinoma; CRC: colorectal cancer; EC: endometrial cancer; ENDC: endocrine cancer; LC: lung cancer; LH: Hodgkin lymphoma; OC: ovarian cancer; PC: parathyroid cancer; RC: renal cancer; TC: thyroid cancer; TESC: testicular cancer.

The range of age at cancer diagnosis was 6-54 years in *PTEN*-mut+ and 6-70 years in *PTEN*-wt (mean age in *PTEN*-mut+ was 31 years; and 46 years in *PTEN*-wt). When considering only the adult cases of cancer, we found that the cancer had an earlier onset in the individuals carrying mutations in *PTEN* (p=0.034) (FIGURE 13-D).

Although in PHTS the cancer usually appears in adulthood, we found a considerable proportion of our patients (14% of the *PTEN*-mut+ and only 0.02% individuals of the *PTEN*-wt; p=0.013) that had developed cancer at childhood or adolescence (TABLE 5). This suggests a possible risk to develop cancer at early age for germline *PTEN* mutation carriers. Some of the cancer types we encountered in these young patients are very rare in these age range, such as the endometrial carcinoma or the clear cell renal cell carcinoma.

Table 5. Cancer occurrence in individuals ≤18 years old from our series.

Cancer type	nr probands	Age of onset	*PTEN* germline status
Papillary thyroid cancer	1	16	p.(Lys6Argfs*4)
	1	16	p.(Arg335*)
Follicular thyroid cancer	1	14	p.(Arg335*)
Endometrial adenocarcinoma	1	15	p.(C136R)
Ovarian endodermal sinus tumor	1	6	p.(C136R)
Clear cell renal cell carcinoma	1	18	p.0?
Hodgkin lymphoma	1	18	p.(Gln17*)
Testicular mixed germ cell tumor	1	18	p.(Thr277Asnfs*21)
Hodgkin lymphoma	1	6	WT
Thyroid cancer (unspecified type)	1	14	WT

4. Genotype-phenotype correlations

Given the high clinical heterogeneity and the high risk to develop cancer in the PHTS, it is relevant to predict the phenotype, and this might be possible through the genotype. Thus, we found some significant associations between certain genotypes and phenotypes. Carrying a mutation in *PTEN*'s exon 1 was positively associated with developing renal cancer (p=0.045), palmoplantar keratosis (p=0.01) and papules (p=0.02), and mutations in exon 8 with less musculoskeletal lesions (p=0.03). On the other hand, large deletions in *PTEN* were associated with a low frequency of polyposis (at gastric level p=0.047, and at colorectal level p=0.017) and neurological disorders (autism p=0.030 and mental retardation p=0.030).

Using the CC score as a surrogate of the phenotype burden of the patients, we assessed if the patients had a more severe phenotype depending on the origin of the mutation, the location of the mutation and the protein domain affected by the variant. However, no significant differences were observed for any of the mentioned aspects (FIGURE 15). The phenotype burden was only significantly higher between *PTEN*-mut+ and *PTEN*-wt, given that the CC score was created to discern these two groups

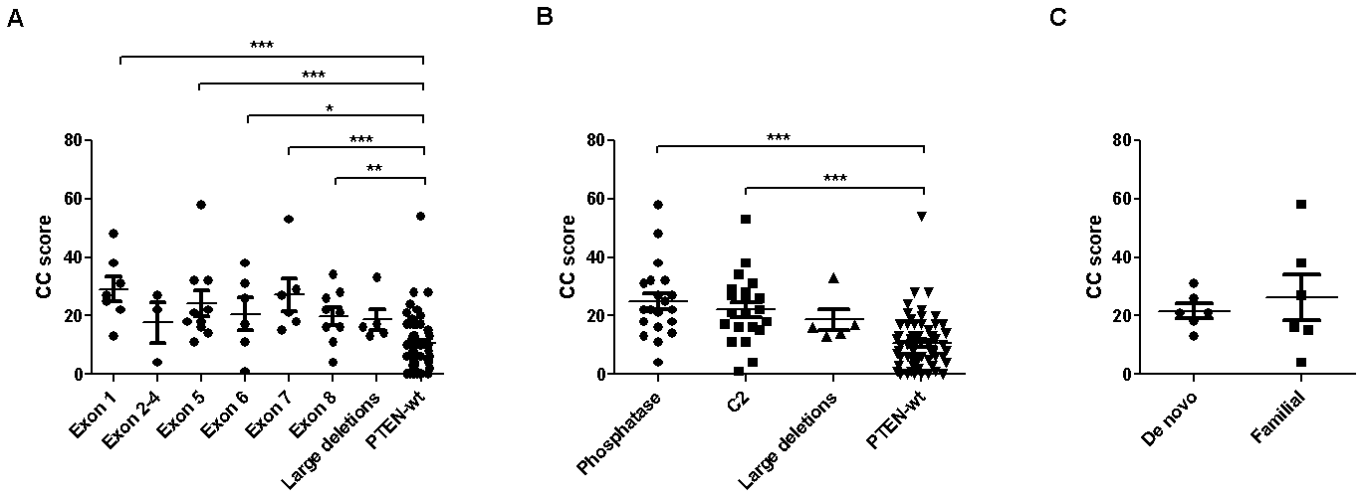

Figure 15. Phenotype burden assessed with the CC scores for each adult patient. Pediatric cases and VUS carriers were excluded. 46 *PTEN*-mut+ and 60 *PTEN*-wt had complete clinical data and their CC scores were plotted according to the location of the mutation (A) and the altered protein domain (B). C) CC scores of the 12 adult *PTEN*-mut+ in which we could confirm the origin of the *PTEN* mutation.

We also explored the usefulness of the phenotypic features of the disease in predicting the presence of a germline *PTEN* mutation, which would be useful to select the patients according to their clinical features prior referral for *PTEN* genetic testing. In this manner, we found 3 clinical features that increased significantly the probability of carrying a germline *PTEN* mutation: macrocephaly, mucocutaneous lesions and gastrointestinal polyposis (TABLE 6). Again, obesity also increased strongly the risk of being a *PTEN*-mut+ individual. Meanwhile, the breast cancer, the number of cancers per patient or the age at cancer onset, had the opposite effect. We also noticed that a family history suggestive of PHTS did not contribute to a higher risk of finding a *PTEN* germline mutation in the case of study.

Table 6. Risk of carrying a *PTEN* germline mutation according to different clinical features, based in our patient series analysis. Odds ratios obtained with logistic regression.

Clinical feature	OR	CI95	p-value
Macrocephaly	**11.186**	4.88 - 25.67	**1.21E-08**
Mucocutaneous lesions	**7.619**	3.09 - 18.82	**1.07E-05**
Gastrointestinal polyps	**4.488**	2.08 - 9.7	**0.1E-03**
Lhermitte-Duclos	2.322	0.72 - 7.50	0.159
Obesity	**23.045**	2.91 - 182.14	**0.003**

Breast cancer	0.208	0.08 - 0.52	0.001
Thyroid cancer	0.724	0.31 - 1.67	0.447
Endometrial cancer	0.502	0.15 - 1.69	0.265
Renal cancer	2.062	0.33 - 12.76	0.436
Colorectal cancer	0.153	0.02 - 1.26	0.081
Family history	1.477	0.64 - 3.40	0.359
Nr cancers per patient	0.546	0.36 - 0.82	0.004
Cancer onset age	0.907	0.86 - 0.96	0.001

Because we noticed that we had many cancer patients among the group of *PTEN*-wt individuals (FIGURE 13-C), we decided to analyze what was the contribution of presenting cancer alone (with no other feature of the disease) to find a germline *PTEN* mutation in the patient, and we observed that it did not confer risk (risk=0.4). Instead, the presence alone of any of the following classical features: macrocephaly, mucocutaneous lesions and gastrointestinal polyposis, implied a significant increased probability to harbor a *PTEN* mutation, supporting once more its usefulness as diagnostic criteria (TABLE 7).

Table 7. Risk of carrying a *PTEN* germline mutation when the patient presents only a certain clinical feature (for example, a patient who shows only LDD and no other manifestation). Odds ratios obtained with logistic regression.

Clinical feature	OR	CI95	p-value
Cancer	0.399	0.16 - 1.01	0.052
Macrocephaly	**11.385**	**4.29 - 30.21**	**1.04E-06**
Mucocutaneous lesions	**6.861**	**2.18 - 21.60**	**0.001**
Gastrointestinal polyps	**4.220**	**1.60 - 11.16**	**0.004**
Lhermitte-Duclos	3.776	0.74 - 19.29	0.110

We also wanted to explore the risks of carrying a *PTEN* mutation depending on the combination of multiple clinical features, such as the combination of macrocephaly, mucocutaneous lesions, LDD and gastrointestinal polyposis together with or without cancer. However, our sample size did not allow these analyses. Nevertheless, we were able to analyze the combination of presenting cancer together with another PHTS-related features. Only the combination of cancer together with mucocutaneous lesions reached significance, and this phenotype still gave a low risk to harbor a *PTEN* mutation (TABLE 8). Again, this is in support that patients who only have cancer, regardless of the number of cancers and even if these are PHTS-cancer types, but have no other manifestations of the syndrome, it is unlikely that they will harbor a *PTEN* mutation.

Table 8. Risk of carrying a germline *PTEN* mutation when the patient presents cancer together with another classical feature of PHTS. Odds ratios were obtained with logistic regression.

Clinical feature	OR	CI95	p-value
Cancer + macrocephaly	1.044	0.12 - 9.41	0.969
Cancer + mucocut.	0.057	4.78E-03 - 0.67	0.023
Cancer + polyps	1.270	0.16 - 9.98	0.820
Cancer + LDD	3.151	0.11 - 92.67	0.506

Moreover, we explored the usefulness of immunohistochemistry (IHC) in identifying PHTS patients who are *PTEN* germline mutation carriers. Thus, we performed IHC of PTEN and IHC of the active phosphorylated form of AKT (p-AKT) in the 5 available paraffin-embedded tumor tissues from different cancer types of 4 patients. However, the results did not correlate with the germline genotype (TABLE 9).

Table 9. PTEN and p-AKT IHC in tumor samples and corresponding *PTEN* genotypes. NGS: next generation sequencing, WT: wild-type.

Patient ID	Germline *PTEN* status	*PTEN* status in the tumor	Sample	Cancer tissue	PTEN staining (H-score)	p-AKT staining (H-score)
189F	c.634+5G>A	c.634+5G>A (NGS)	3T179	Thyroid	Positive ++90% (180)	Negative (0)
308F	WT	WT (NGS)	4T158	Rectum	Positive ++70% (140)	Negative (0)
8S356	WT	c.635-1G>A (NGS)	9T40	Lung	Negative (0)	Weak <1% (1)
11S1087	Large deletion (*KLLN* and *PTEN* exon 1)	*	16T122-1	Thyroid (papillary-follicular)	Positive ++ 90% (180)	Weak <1% (1)
		**	16T122-2	Invasive ductal carcinoma of the breast	Positive +++100% (300)	Negative (0)

*Large deletions are not called in the NGS pipeline. **Insufficient tumoral DNA for analysis.

The lung adenocarcinoma was the only sample that showed complete loss of PTEN expression, possibly accounting for the somatic splicing variant in *PTEN*, even though the patient was *PTEN*-wt at germline level. However, p-AKT was not accordingly overexpressed, instead it had a weak staining (TABLE 9).

5. Search for other genetic factors involved as phenotype modifiers or as cause of the PHTS disease

We decided to extend the study beyond the conventional clinical and molecular characterization of the PHTS in our series, by searching for other genetic factors that might be involved in modifying the phenotype or as the cause of the disease. For this purpose, we studied both the *PTEN*-mut+ and the *PTEN*-wt patients through several approaches.

5.1 *PTEN* promoter screening for point mutations

In the group of the patients who were negative to carry *PTEN* mutations (*PTEN*-wt; n=79), we decided to screen for mutations in the *PTEN* promoter that could alter the gene expression. We analyzed the region chr10:89,622,837-89,623,519, which includes the putative p53 binding site that regulates *PTEN* transcription (Zhou, Waite, *et al*., 2003; Stambolic *et al*., 2001), in 31 *PTEN*-wt patients selected for meeting strict clinical criteria, but we found no mutations. The only variant we found was c.-903G>A (rs1044322) in 3 unrelated patients, but we consider it a polymorphism as it is a frequent event in general population (MAF>0.01) and it is classified as benign in ClinVar.

5.2. Evaluation of the role of *KLLN*

The *KLLN* promoter hypermethylation has been described as a potential additional risk for both benign features and cancer in the context of the PHTS disease and depending on the *PTEN* status (Nizialek *et al*., 2015). In collaboration with the Genotyping National Centre node of the University of Santiago de Compostela (CEGEN-USC), we performed an exploratory analysis of the methylation status of multiple CpG islands located in the region of the shared promoter between *KLLN* and *PTEN* through EpiTyper. Despite the effort, we were not able to obtain informative results (FIGURE S2). So instead, we approached this objective through the study of the mRNA expression levels. For this purpose, we performed qPCR analysis of 23 patients (both *PTEN*-mut+ and *PTEN*-wt) and 23 control samples (unrelated healthy individuals), using the *36B4* gene expression levels as reference.

In our series, we saw that the expression levels of *KLLN* mRNA were similar between patients and controls, with exception of the patients harboring *PTEN* variants in exon 1 (one nonsense and another truncating) of *PTEN*, who showed overexpression of *KLLN* (FIGURE 16-A).

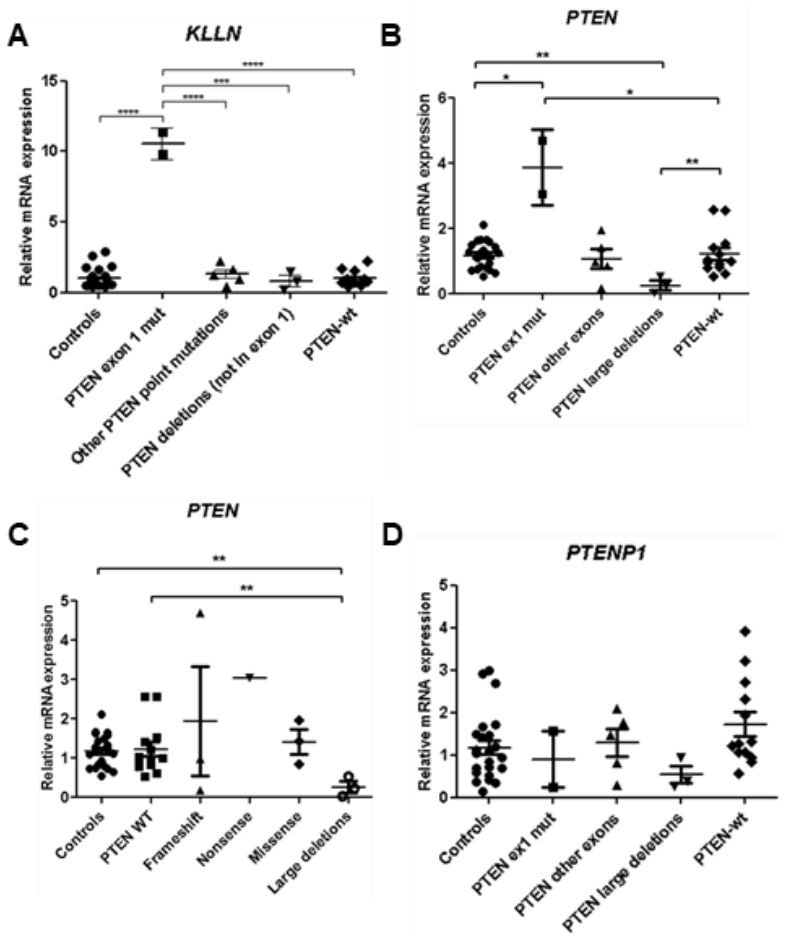

Figure 16. mRNA expression levels of *KLLN* (A), *PTEN* (B-C) and *PTENP1* (D), determined by qPCR using as reference expression gene *36B4*. Control individuals were compared to subgroups of PHTS patients according to the mutation status of *PTEN*. Only significant differences are indicated (two tailed t-test, P <0.05). Each dot represents the mean value of the expression the gene of study for each patient (assessed in triplicates). The mean value of each group ± SEM is indicated.

We also checked the expression levels of *PTEN* using specific primers located in a non-homologous region of the 3'UTR with respect to its pseudogene (*PTENP1*) (Poliseno *et al*., 2010), and in this manner we also captured entire products of *PTEN* mRNA and not truncated ones that can be further degraded. Although we expected to find reduced levels of *PTEN* among the patients, at least the ones with germline *PTEN* mutations, we found that only the cases harboring *PTEN* large deletions showed reduced levels of *PTEN* expression (FIGURE 16-B AND C). Surprisingly, the carriers of mutations in exon 1 also showed the highest levels of expression of *PTEN* (FIGURE 16-B), as it happened with *KLLN* (FIGURE 16-A). No significant differences were found for the expression of *PTEN* regarding the type of mutation, with exception of the large deletion carriers (FIGURE 16-C), neither regarding the clinical manifestations in the patients (FIGURE S4).

PTENP1 is a pseudogene that gets transcribed and it has been described to prevent *PTEN* inhibition through miRNAs by acting as a sponge for them (Poliseno *et al*., 2010). Considering this, we also explored the expression of this pseudogene in our series of

patients and controls. No differences were found between controls and patients, neither regarding the carriers of mutations in exon 1 of *PTEN*. Therefore, the high levels of *PTEN* expression in these individuals did not account for *PTENP1* expression (FIGURE 16-D).

Correlation of expression levels was found for *PTEN* and *KLLN* (p=0.001), supporting a common regulation of these 2 genes, which can be explained by their shared promoter.

5.3. NGS findings

We screened 127 DNA samples for germline SNPs and indels in genes of the PI3K/AKT/mTOR pathway in an NGS panel. Genes included in the panel are shown in TABLE 3. Both *PTEN*-wt cases (n=71) and *PTEN* variant carriers (n=56) were analyzed in order to find either causal genes beyond *PTEN* or possible modifiers of the phenotype.

The variant calling of *PTEN* point mutations, both SNVs and indels, showed 100% concordance with previously detected *PTEN* variants through Sanger sequencing. Only the cases with large deletions (extending one exon or more) were not called because the bioinformatics pipeline did not allow the detection of these. Moreover, the panel also detected one case with a pathogenic mutation in *PTEN*: c.654C>A p.(C218*), a stop gain variant described in ClinVar as pathogenic, not previously detected back in 2005, which was validated then through Sanger sequencing in different DNA samples of the same patient.

By using the gene panel, we were able to check the read counts of each allele for the *PTEN* variants with very good coverage. In this way, we found 1 individual on which the allele frequency for the *PTEN* variant was 0.3 and therefore could account for a mosaicism (which is hardly discerned through Sanger sequencing). We explored if this patient had any clinical feature different from the ones present in the other *PTEN*-mut+, but apparently there was none that could suggest a mosaicism. Other possible mosaicisms occurring in genes besides *PTEN* were found (TABLE S6), but their relevance is unknown.

We found multiple patients that harbored variants in genes related to the mTOR pathway (TABLE S7), both in the group of the *PTEN*-wt patients and the *PTEN*-mut+, with no significant difference. However, all of these variants were predicted to cause a moderate effect (this refers to the missense variants, protein altering variants, inframe insertions and inframe deletions) and with no pathogenic consequence reported, and therefore we can just consider them as VUS for now.

Since the gene panel also included other cancer driver genes not closely related with PHTS (TABLE 3), we found several *PTEN*-wt patients with rare variants in genes associated to other cancer predisposition syndromes and that therefore could entail additional clinical

risks or suggest a wrong diagnosis of PHTS. One of these cases was the *FLCN* gene, found altered in some *PTEN*-wt individuals (TABLE S7) and which is associated to the Birt-Hogg-Dubé (BHD) syndrome which includes cutaneous lesions that can resemble PHTS lesions.

Moreover, we analyzed tumor DNAs paired with their correspondent blood DNA from 4 individuals in this gene panel, and we found unique somatic variants (TABLE S8). Interestingly, the only tumor which showed negative staining of PTEN in the IHC (9T40; TABLE 9) harbored a somatic splice acceptor variant in *PTEN* with an allele frequency of 0.7. This finding was expected because *PTEN* somatic mutations are frequently found in many tumors, including lung cancer (Gkountakos *et al*., 2019). Another unsurprising somatic finding was *BRAF* p.(V600E) in a papillary thyroid sample, a common event in this cancer type (Paja Fano *et al*., 2017).

5.4. WES findings

Eleven *PTEN*-wt patients were selected from our series to study their whole exome and expand the search of potential genetic alterations outside the mTOR pathway that could be driving the PHTS phenotype. The selection criteria for this study was first the absence of a *PTEN* pathogenic variant or large rearrangement together with some of the following clinical aspects: the patient met strict Pilarski's diagnostic criteria (n=6; group A), or had LDD (n=4; group B) or was a pediatric case with macrocephaly together with neurological alterations and overgrowth (n=1; group C) (FIGURE 17).

We did not find altered genes that were common between the patients (TABLE S9), apart from *RNF135* gene in 2 cases, each of them carrying a different variant. This gene is associated to overgrowth, macrocephaly and facial dysmorphism (Douglas *et al*., 2007) (OMIM 613675). However, the patients were not reported to have any of these features.

We identified several interesting variants in genes whose function is related with PTEN regulation (like *NEDD4* and *HERC1*) or with elements of the PI3K/mTOR pathway (like *PDGFRB* or *PIK3R2*). Also, other interesting variants were found in known genes associated to other cancer predisposition syndromes, like *BAP1* or *TSC1*, whose variants were also validated in the gene panel. We also found one case with a frameshift variant in *MUTYH*, a gene associated to familial adenomatous polyposis with an autosomal recessive mode of inheritance. However, this individual was found to have the *MUTYH* variant in heterozygosis, therefore it could entail a risk for the offspring, but it seems that this factor is not the driver of the patient's phenotype. Also interesting because of its frameshift consequence, was the *MLH3* variant, although we cannot exclude the possibility that the mRNA is targeted for nonsense mediated decay (NMD).

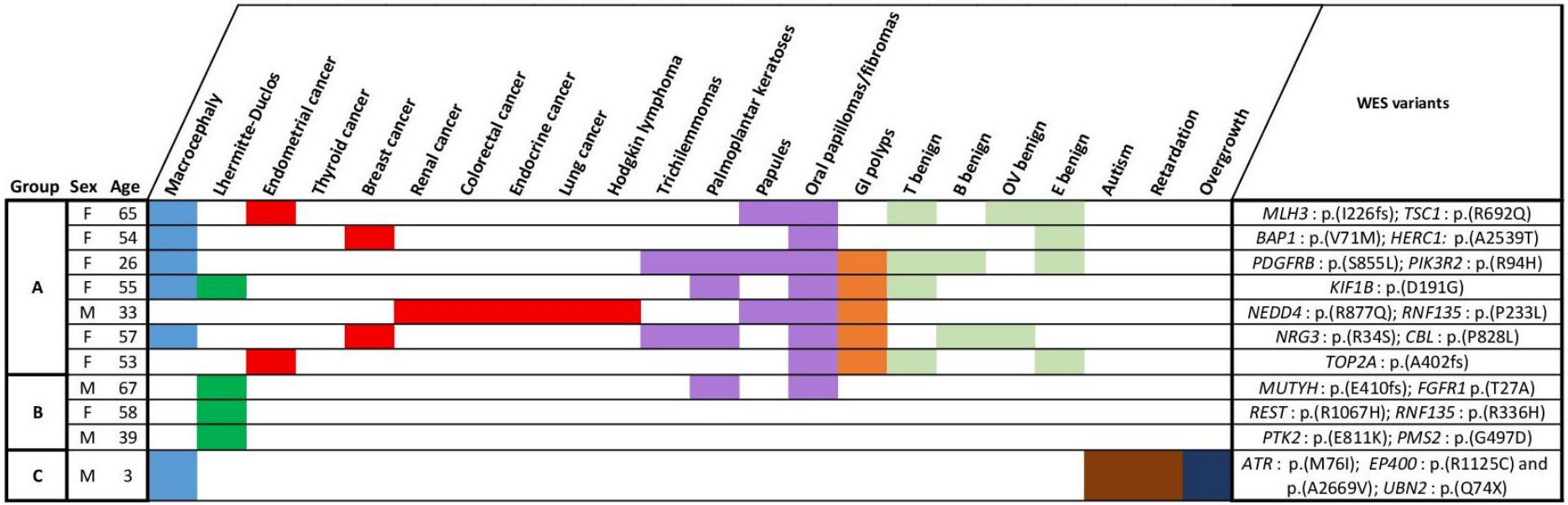

Figure 17. Clinical manifestations and selected gene variants for each *PTEN*-wt patient studied through WES. B: breast; E: endometrial; GI: gastrointestinal; OV: ovarian; T: thyroid. Groups: A) Patients who meet strict Pilarski's diagnostic criteria, B) patients who developed Lhermitte-Duclos, C) pediatric case with macrocephaly together with neurological alterations and overgrowth. Complete data on the gene variants is shown in TABLE S9.

DISCUSSION

DISCUSSION

The rareness of the PHTS, with an estimated prevalence of 1 individual with CS in 200,000 or 1 in 250,000 (Nelen *et al.*, 1999) –although this is probably an underestimation due to underdiagnosis (Eng, 2003)- together with the difficulty of establishing a diagnosis due to the outstanding variability of the associated clinical features, are two major factors that have led to a huge lack of knowledge on this disease, particularly in Spain. Only a small series of 8 Spanish families meeting criteria of CS is published in the literature (Bussaglia *et al*., 2002), together with a few smaller clinical case reports (Peiró *et al*., 2010; Martín Fernández-Mayoralas *et al*., 2007; Sabaté *et al*., 2006; Pérez-Núñez *et al*., 2004; Vega *et al*., 2003).

We decided to approach the need to improve the awareness on PHTS in the Spanish population through the work presented herein: an exhaustive clinical and molecular characterization of 145 Spanish individuals with PHTS, the first largest series on PHTS at national level (exceeding the study of Bussaglia *et al*. 2002) and the fourth at international level (after the study of Bubien *et al*. 2013 in third place, Nieuwenhuis *et al*. 2014 in second place and Tan *et al.* 2011 in first place), according to the sample size of the mentioned studies. Our findings will be useful to accelerate the diagnosis and improve the patient care in PHTS. Moreover, we have extended our research beyond *PTEN*, looking for other genetic factors that might explain the PHTS phenotype in the *PTEN*-wt patients.

1. *PTEN* molecular spectrum

In agreement with previous works in other populations (Tan *et al.* 2011; Bubien *et al.* 2013; Nieuwenhuis *et al.* 2014), the mutations found in our series were located along the *PTEN* sequence with hotspots in exons 5 and 8, but none was found in exon 9. As a difference, we found more mutations that occurred in exon 1 compared with the other studies on PHTS (FIGURE 18), suggesting this could be specific of PHTS Spanish population, although the study of new patients would be necessary.

The types of mutations found in our series were also comparable to those of the other studies (Tan *et al*. 2011; Bubien *et al*. 2013; Nieuwenhuis *et al*. 2014), as well as the recurrent mutations (c.388C>T, c.697C>T and c.1003C>T), consistent with the ones described previously (Bubien *et al.*, 2013; Tan *et al.*, 2011; Bonneau and Longy, 2000).

A high amount of the rearrangements involving *PTEN* gene in our series (5 out of 7, 71.4%), extended to the proximal gene *KLLN* and some also to other genes located more upstream, like *BMPR1A*, which is in the chr10q23.2 region and it is associated with juvenile polyposis. The deletion of *PTEN* together with *BMPR1A* occurred in 2 patients with PHTS

features but also resembling juvenile polyposis, suggesting a cooperation of these two tumor suppressor genes in promoting an overlapping phenotype. Other authors suggested that the deletion of these two genes promotes the juvenile polyposis of infancy syndrome (Delnatte *et al*., 2006) (JPI; OMIM 612242). This disease has been described only in 11 cases in the literature and it is life-threatening, mainly due to the early-onset gastrointestinal polyposis and cancer risk. Our 2 patients with the mentioned contiguous gene deletion, suffered from gastrointestinal polyposis at infancy and developed clinical features of both PHTS and JPI. Considering our finding, we believe they have overlapping characteristics of the two syndromes, rather than a different entity. Therefore, the management of these patients should combine the PHTS recommendations together with JPI recommendations, with a special follow-up of the colon.

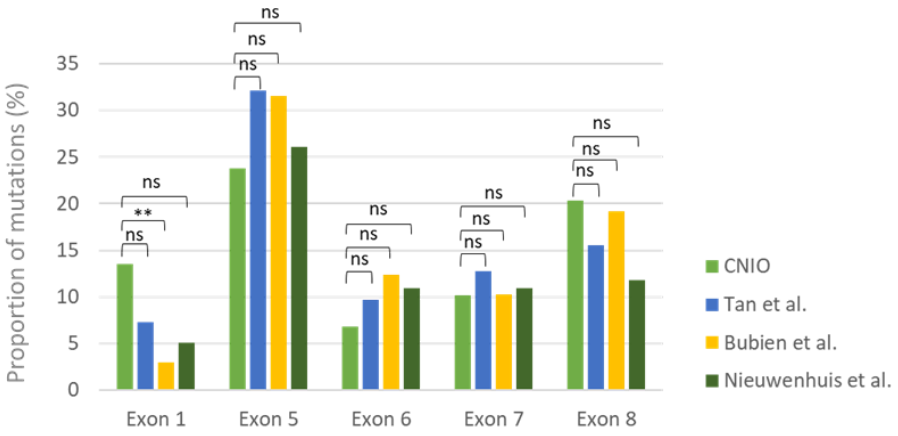

Figure 18. Proportion of mutations in the *PTEN* exons that accumulate more hotspots. Comparison between our series and the other studies published in the literature (data taken from Tan *et al.* 2011, Bubien *et al.* 2013 and Nieuwenhuis *et al.* 2014). Chi-square statistical significance (*P*<0.05) is shown.

Although the standard diagnostic *PTEN* gene testing is usually done through Sanger sequencing and MLPA in many laboratories, and recently also through gene panels which can also detect copy number variations, we have shown in our work that it is necessary to extend the study of the large deletion carriers by using other methods such as aCGH, in order to identify other genes that might be also deleted and that therefore can entail additional clinical risks for the patient.

Variants in the *PTEN* promoter, with unproven pathogenicity, have been previously proposed to account for 10% of the CS or CS-like patients without mutations in *PTEN*'s coding sequence (Teresi *et al*., 2007; Zhou, Waite, *et al*., 2003). However, we found no variants in that region in a selected subset of 31 *PTEN*-wt patients meeting strict clinical criteria of PHTS and therefore we did not extend this analysis to the remaining individuals. We only found a SNV: c.-903G>A (rs1044322) in 3 patients. However, this variant is not rare in the population (MAF=0.01 in general population and 0.04 in Spanish population,

according to 1000Genomes), it appears classified as benign in ClinVar by an expert panel and other authors have reported its presence not only in CS patients but also in control individuals (Zhou, Waite, *et al*., 2003), therefore we consider it is a polymorphism with no further relevance.

PTEN mosaicisms have rarely been described (Salo-Mullen *et al*., 2014; Gammon *et al*., 2013; Pritchard *et al*., 2013; Zhou *et al*., 2000), but we consider that this is mainly due to the method used traditionally in the *PTEN* genetic testing: Sanger sequencing, which is an unsuitable technique to discern mosaicisms. However, the use of gene panels is becoming the trend in genetic diagnosis, and this implies important increases in the sensitivity and in the detection limits. Therefore, NGS might be a better approach for the genetic diagnosis in PHTS patients. With this technology, we found that 1 case of a previous *PTEN* variant detected by Sanger and diagnosed as germline heterozygote, could be in fact a mosaicism, considering the allele frequency determined through NGS. The phenotype of this patient was not milder than that of the germline heterozygotes, in fact she suffered cancer, suggesting that she should receive similar clinical follow-up as any other PHTS patient.

Whether a VUS is pathogenic or not, is always an intriguing question, and even more when facing genetic counseling. Several approaches are needed to further characterize the consequences of the VUS and the possible clinical implications (Moghadasi *et al*., 2016) and we were able to perform some of them: literature and database search, *in silico* prediction and co-segregation analyses. However, these measures were not enough to demonstrate the deleteriousness or benignity. We found that two *PTEN* VUS segregated with the phenotype (Y177N and Q261E), but another two were *de novo* variants (T26I and T277A). The only way to demonstrate the implications of the variant at protein and cellular level is a functional study. Thus, with the hypothesis that the exonic VUS which consisted in amino acid changes and correlated with a PHTS phenotype were probably pathogenic, we reached to the groups of Dr. Pulido and Dr. Molina who have expertise in exploring the localization and protein activity of PTEN *in vitro* (Mingo *et al*., 2018, 2019; Gil *et al*., 2015; Rodríguez-Escudero *et al*., 2011). Two models were used: humanized yeasts and a mammalian cell line (COS-7), therefore faithfully capturing the lipid phosphatase activity but also other events such as post-translational modifications, protein interactions, subcellular localization or dominant-negative effects (Mighell *et al*., 2018). Thanks to their collaboration, we were able to demonstrate the pathogenic role of all the VUS that affected the N-terminal region: T26I, P95R and Y177N. These variants caused a reduced catalytic activity and an aberrant localization, supporting the relevance of the N-terminal region for PTEN's role as antagonist of the PI3K/Akt/mTOR pathway of proliferation. However,

further research is needed to functionally demonstrate the deleteriousness of the remaining VUS.

2. Clinical findings

The clinical data of our series represent minimum estimations and we probably have under-report of some clinical features in several patients. This is due to several aspects. First, we included pediatric patients, who could have developed other disease manifestations after the moment of diagnosis or consultancy visit, such as cancer, however, other features like macrocephaly or vascular lesions are thought to be evident already in the first years of life. Second, the heterogeneity of the physicians' expertise at referring the patients with a suspected diagnosis of PHTS, for example, an oncologist may focus the attention on the presence of PHTS-cancers and underlook a macrocephaly, meanwhile the pediatrician may focus in the macrocephaly and autism. Third and related with the former, the reporting of the multiple features of the PHTS patients is a hard task and the free form writing in the medical record can also lead to under-report and heterogeneity. We therefore established the collection of the clinical data with a specific checklist designed by us for this project and in order to obtain more exhaustive information in a homogeneous manner. However, this checklist was only obtained for a limited set of patients.

We are aware of the limitations of our study and the conclusions of our project should be validated in larger series. Nevertheless, we consider this work is relevant towards improving the PHTS patients' management in our country. Some of the results found in our series are concordant with those in larger studies but we also found some differences, as we will further discuss.

The most frequent manifestations in the whole series of PHTS Spanish patients included some of the classical features of PHTS: macrocephaly, mucocutaneous and thyroid lesions. Surprisingly, we found that LDD, a feature considered even as pathognomonic of CS by some authors (Colby *et al*., 2016; Zhou *et al*., 2003) and that is apparently more strongly associated with germline *PTEN* mutations when its onset is in adulthood rather than in childhood (Zhou *et al*., 2003), was not so suggestive of a *PTEN*-mut+ patient in our series, as we found it in several *PTEN*-wt individuals (n=5; FIGURE 12), all of them with adult onset.

Overall, the clinical features classically associated to a PHTS phenotype were more commonly found among the *PTEN* mutation carriers, except for breast cancer, which we found it was poorly correlated with presenting a *PTEN* mutation. However, this is due to a

bias coming from the high amount of *PTEN* studies referred due to a breast cancer diagnosis (n=31 *PTEN*-wt versus only 7 *PTEN*-mut+).

We tried to compare the clinical findings in our series with the ones in other studies, but only Tan *et al*., 2011 reported a more in-detail description of the frequencies of the different manifestations in their series. Overall, our findings were similar, with few exceptions: the proportion of patients with vascular lesions, palmoplantar keratosis or oral papillomas was significantly higher in our series, while the proportion with macrocephaly, autism disorder, mucocutaneous lesions (in general), lipomas or gynecologic lesions was significantly higher in the study of the mentioned authors (FIGURE S3). Of note, there is co-occurrence in the work of Tan *et al*. 2011 and therefore this analysis needs to be interpreted with caution. Also, the differences can be due to the different selection criteria: we used broad criteria while these authors selected patients who met relaxed ICC criteria and only the ones who were *PTEN*-mut+.

Strikingly, we found that obesity was predominant in our *PTEN*-mut+ patients (22% of this group). This feature was rarely reported in the patient series of the other studies in PHTS (Tan *et al.*, 2011; Bubien *et al.*, 2013; Nieuwenhuis *et al.*, 2014), even though the association of *PTEN* and obesity was described several years ago (Pal *et al*., 2012) and it was also already known the role of PTEN in the insulin pathway (Nakashima *et al*., 2000). Our findings support the association of *PTEN* mutations and obesity and therefore highlight another important clinical risk for the PHTS patients. Moreover, obesity is also a known risk factor of cancer (De Pergola and Silvestris, 2013), and in fact we found that half of the *PTEN*-mut+ obese patients had cancer (6 out of 13). Also related with this topic, PI3K inhibitors are being tested as a therapy for PHTS and have demonstrated efficacy at reversing skin hamartomas in mice models (Wang *et al*., 2013) and as treatment of obese mice and rhesus monkeys (Lopez-Guadamillas *et al*., 2016; Ortega-Molina *et al*., 2015). We therefore consider this is an interesting topic that requires more research.

Germline *PTEN* mutation carriers have increased risks to develop cancer. The cancer types associated to PHTS (breast, thyroid, endometrial, colorectal, renal and melanoma) were the more common among our patients, in concordance with previous findings in other studies (Tan *et al*., 2011). However, we also found other cancer types not so frequently associated to PHTS, even in the *PTEN*-mut+ patients: astrocytoma (n=1), meningioma (n=2), ovarian (n=1) and testicular cancer (n=2). Since these cancers outside the PHTS spectrum accounted only for such reduced number of cases, it is possible that they account for specific molecular factors within these families but are not related with *PTEN*.

Of note, we highlight the increased incidence of cancer with onset at young ages (18 or under) in our PHTS patient series (accounting for 14% of the *PTEN*-mut+). The most represented types were thyroid cancer and Hodgkin lymphoma. It was also interesting to find that these young patients were prone not only to cancer types within the PHTS spectrum (such as the thyroid cancer) but also to other tumors which are also infrequent to occur with such early onset, even outside a PHTS context, such as the endometrial cancer. To our knowledge, the previous and largest studies in PHTS described that the cancer usually develops during adulthood in *PTEN* mutation carriers (Tan *et al.*, 2011, 2012; Bubien *et al.*, 2013; Nieuwenhuis *et al.*, 2014), and only one study highlighted the risk of early cancer development, especially for thyroid cancer (Smpokou *et al*., 2015). The guidelines (TABLE 2) already suggest a yearly thyroid ultrasound for the patients under 18, but based on our results, we recommend the anticipation of the screenings also in the other organs, especially the ones commonly affected within the PHTS spectrum, to prevent cancer development. A larger and prospective study in young PHTS patients would be useful to identify the appropriate age at which screening should begin for each cancer type in these individuals.

3. Usefulness of the clinical features as diagnostic criteria

Since the PHTS is a rare disease, there are not many guidelines on the diagnosis or management of the patients and there are discrepancies in the diagnostic criteria proposed to date. In one hand, the International Cowden Consortium (ICC) proposed operational criteria for CS patients (Tan *et al*., 2011) based in the findings on patient series, and based on these criteria, the Cleveland Clinic (CC) established a numerical score (the CC score) (Tan *et al*., 2011) to estimate the probability of finding a *PTEN* mutation depending on the clinical manifestations. On the other hand, Pilarski and colleagues (Pilarski *et al*., 2013) proposed the revised criteria after a literature review. To date, there is no worldwide consensus in the diagnostic criteria for PHTS, although the United States' National Cancer Comprehensive Network (NCCN) prefers the ICC criteria.

We decided to evaluate how these different criteria performed in our series to identify PHTS patients based on the clinical aspects (FIGURE 19-A). The ICC criteria and CC score had a better performance to identify PHTS patients in our cohort, by identifying around 90% of our *PTEN*-mut+ patients (which we can assume are true PHTS patients), and also 40% of the *PTEN*-wt patients which we consider there was at least a suspect of presenting PHTS in spite of not harboring a *PTEN* mutation. On the contrary, when using the Pilarski criteria, we failed to identify a considerable amount of the subjects, even those who harbored *PTEN* mutations (more than 50% of this group) (FIGURE 19-A), suggesting that

these criteria are too strict, at least for its use in Spanish population. This idea is also supported when calculating the accuracy of the 3 diagnostic tools (FIGURE 19-B).

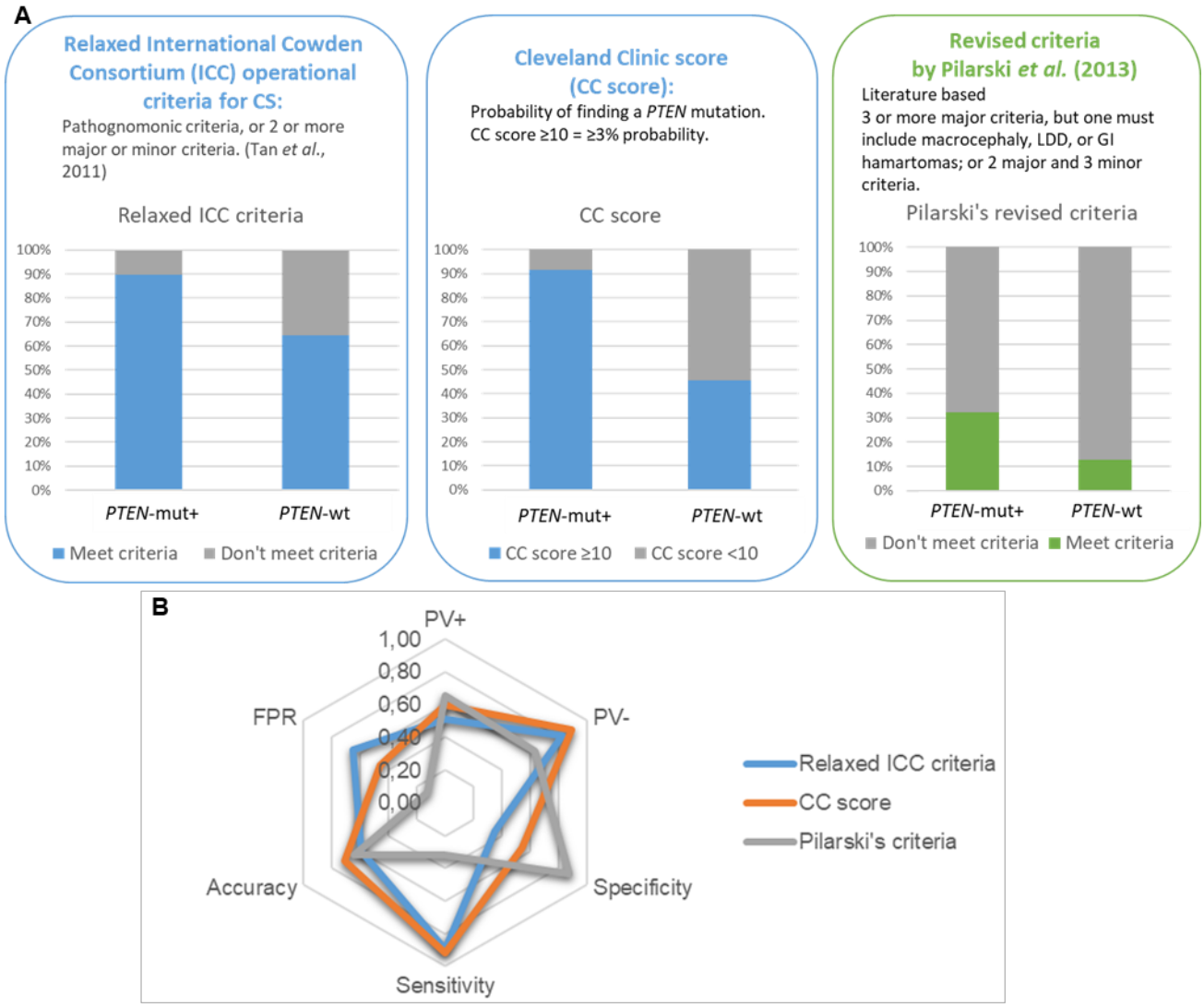

Figure 19. A) Proportion of individuals in each group of our series identified using the different diagnostic criteria. B) Rates of accuracy of the diagnostic criteria. FPR: false positive rate; PV+: positive predictive value; PV-: negative predictive value.

The 3 tools of diagnosis had similar accuracy. However, the other measures revealed considerable differences at identifying the true carriers of *PTEN* mutations and excluding the *PTEN*-wt individuals. The criteria proposed by Pilarski had high specificity (detected better the *PTEN*-wt patients) and low false positive rate (FPR; reduced identification of patients as false *PTEN* mutation carriers), due to the strictness of these criteria. However, these criteria also had very low sensitivity (correctly identifying the *PTEN* mutation carriers as so). On the other hand, the relaxed criteria of the ICC and the CC score had a slight increase in the false positive rate (FPR) while maintaining a moderately good positive predictive value (PV+; identifying the true carriers), negative predictive value (PV-; identifying the true non-carriers), specificity and accuracy, but with a very high sensitivity.

We consider that, in diagnosis, a high sensitivity would be preferable over a high specificity, in order to detect every *PTEN* mutation carrier despite studying some non-carriers. This could have negative implications at an economical level, but the authors who proposed the CC score already demonstrated that it was cost-effective (Tan *et al*., 2011).

Interestingly, considering that the relaxed ICC criteria were designed to identify CS patients, we found that 104 (72%) of the individuals included in our study met these criteria and therefore could be considered as true CS/CS-like patients, at least clinically, because only 53 (51%) of them were *PTEN*-mut+. This is in contrast with the upper rate that estimates 80 to 85% of the patients who meet CS criteria harbor a mutation in *PTEN* (Spinelli *et al*., 2015; Orloff and Eng, 2008; Zbuk and Eng, 2007; Eng, 2003). Moreover, this also implies that we had an increased detection rate of patients carrying *PTEN* mutations (51% from the 104 meeting relaxed ICC criteria), while other authors detected only a 9.5% from a very large series (Tan *et al*., 2011).

The criteria we used were not so strict as the Pilarski's ones and slightly broader than the relaxed ICC criteria mainly because we included vascular and musculoskeletal lesions, rather than focusing just in CS-associated features (as happened in Tan *et al.*, 2011). By using our own criteria, we were able to identify 6 more individuals carrying *PTEN* mutations despite studying 79 *PTEN*-wt. For these reasons, we consider our criteria could add to the previous ones described. Nevertheless, a larger study would be needed to evaluate if our criteria are more useful for Spanish patients specifically and if their application in the clinical practice to decide referral of the *PTEN* carrier candidates is cost-effective in the end.

In our series, several clinical manifestations, such as macrocephaly or oral papillomas, were more useful to identify *PTEN* mutation carriers. Meanwhile, others, such as Lhermitte-Duclos or autism, could not discriminate well between *PTEN*-wt and *PTEN*-mut+ individuals (FIGURE 19-A). These results are relevant to consider selecting the patients according to their phenotype before referral for *PTEN* genetic testing.

We noticed that many clinicians make a lot of use of the criterion of presentation of 2 or more PHTS-associated cancers in the case of study as diagnostic and enough criterion for referring the patient for *PTEN* testing. This bias is also reflected in our work as we found the more abundant cancer types were the thyroid, breast and endometrial cancers. Our study does not support the use of this criteria, at least when the individual does not have any other clinical manifestation within the spectrum of PHTS, as we found all of these individuals were *PTEN*-wt.

4. Genotype-phenotype correlations

Besides numerous efforts, the small-scale studies limit the establishment of genotype-phenotype correlations in PHTS, and to date, there are no strong ones. In our cohort, we were also not able to determine strong correlations due to the small size of our study. Only some associations were found significant but should be validated in larger cohorts. Interestingly, we observed that there were more PHTS patients harboring mutations in the exon 1 of *PTEN* than expected according to the literature (FIGURE 18) and these individuals also had an apparent increased risk of renal cancer. This finding seems interesting for further research in a larger series of *PTEN* exon 1 mutation carriers and through functional studies to unravel the implicated mechanism that contributes to renal cancer.

Only some hypotheses have been proposed but their validation in the series of patients is still difficult (Nelen *et al*. 1999; Marsh *et al*. 1998; Marsh *et al*. 1999). One extended hypothesis is the phenotype according to the lipid phosphatase activity in the mutation status: partial activity due to hypomorphic variants are apparently related with milder phenotypes, such as macrocephaly with autism, meanwhile stable but fully inactive PTEN protein might lead to more severe phenotypes with malignant lesions (Mighell *et al*., 2018; Leslie and Longy, 2016; Rodríguez-Escudero *et al*., 2011). This idea is supported by computational analyses (Smith *et al*., 2018) and experimental studies in humanized yeast models (Rodríguez-Escudero *et al*., 2011), human cell lines (Spinelli *et al*., 2015) and *in vivo* mice models (Vogt *et al*., 2015). Our results of the functional studies on the PTEN protein activity for the VUS cases could also be in agreement with the mentioned hypothesis: the PTEN VUS T26I and Y177N, which showed partial phosphatase activity in the humanized yeast model, correlated with a phenotype of macrocephaly and neurological disorders (FIGURE 10, FIGURE 11).

5. Variations of the phenotype within PHTS

On one hand, the high variability in the phenotypes among the *PTEN*-mut+ patients can be explained considering that different mutations in *PTEN* will affect differently the PTEN protein (as we have shown in the functional studies of the VUS) and have different consequences in its multiple functions, deregulating certain pathways to which specific cells or tissues will be more sensitive.

On the other hand, the fact that identical *PTEN* germline mutations can give rise to different phenotypes (even in the context of individuals from the same family), and the high heterogeneity of phenotypes among the PHTS patients suggest there are other genetic or epigenetic factors involved which favor the development of certain clinical features (Marsh

et al., 1999). In relation with this, it was suggested that *PTEN* mice models developed a certain cancer type depending less on the mutation in *PTEN* but more on the genetic background of the mice (Freeman *et al*., 2006), suggesting once more the involvement of phenotype modifiers, even in a *PTEN*-mut+ context. With this idea in mind, we sought for other elements contributing to the patients' phenotypes by performing NGS in *PTEN*-mut+ individuals, but with our results we are unable to propose strong candidates for now.

Moreover, there are genes that can contribute to an accumulative effect in the phenotype or to additional clinical risks. We found two examples in our series: *BMPR1A* gene, found entirely deleted in patients with features of PHTS and JPI (as mentioned previously in this discussion); and *APC* gene, found germline altered (c.1620dupA) together with a *de novo* *PTEN* mutation in patient 189F, who had also familial adenomatous polyposis (FAP) and whose FAP-affected family relatives also harbored the *APC* mutation (Valle *et al*., 2004).

6. Other genes in the PHTS spectrum

A considerable proportion of PHTS patients (from 20% to 90%, depending on the PHTS entity) don't harbor a *PTEN* germline mutation (Spinelli *et al*., 2015; Orloff and Eng, 2008; Zbuk and Eng, 2007; Eng, 2003) and thus, have an unknown cause of their disease. In fact, the *PTEN*-wt cases in our series who satisfy the strict diagnostic criteria (FIGURE 19-A) are suggesting the involvement of other genetic factors in the PHTS disease. Knowing the genetic cause is important to offer the best counseling, follow-up, prevention and therapeutic measures to each patient. Therefore, it is relevant to investigate deeper for yet to be discovered genetic factors involved in PHTS development.

In our patient series, we first discarded several genetic factors beyond *PTEN*, proposed in the literature as potential causes of the PHTS in the *PTEN*-wt patients: the *KLLN* promoter methylation and variants described in *SDH-B*, *SDH-D*, *PIK3CA* and *AKT1* genes (Orloff *et al*., 2013; Ni *et al*., 2008).

The promoter of *KLLN* gene has been described as hypermethylated in some PHTS cases in association with certain clinical features depending on the *PTEN* mutation status (Nizialek *et al*., 2015). Moreover, in up to 35% of the *PTEN* mutation negative individuals, the hypermethylation of *KLLN* was reported to result in downregulation of *KLLN* expression but not of *PTEN*, and associated to increased risks of breast and renal cancers (Bennett *et al*., 2010). Thus, it was suggested that *KLLN* might be not only a susceptibility gene of PHTS, but also a phenotype modifier (Nizialek *et al*., 2015). Since we were not able to reproduce the methylation experiment, we decided to explore this hypothesis through the expression analyses by qPCR of *KLLN*. However, instead of finding reduced levels of

KLLN mRNA, we found them increased in the case of patients harboring *PTEN* exon 1 mutations, together with increased expression of *PTEN* also, supporting the idea that the 2 genes are co-regulated. The association between *PTEN* exon 1 mutation carriers and increased risk of renal cancer, together with increased expression of *KLLN* and *PTEN*, is an interesting finding of our work that requires further research and validation in larger sample sizes.

In the literature, it has been previously reported a general low expression of *PTEN* at protein levels (Ngeow *et al.*, 2012), but the levels are apparently more variable when looking at the mRNA expression (He *et al.*, 2013; Bennett *et al.*, 2010), but we are unaware of previous reports of high expression levels as the ones we obtained. It is possible that some published data account not only for the *PTEN* mRNA product, but also *PTENP1*, which has a 98% of sequence similarity with *PTEN*. When we analyzed the expression of *PTEN* with primers locating to exon 1 and 2 of *PTEN* and which can also amplify *PTENP1* sequence, we still found the overexpression of the gene in the carriers of *PTEN* exon 1 mutations (FIGURE S5). The differences between our findings and the ones in the literature can also account on the sample type: we used peripheral blood leukocytes and the results on the literature come from lymphoblastoid cell lines.

PTENP1 has been described in the literature to act as a sponge of miRNAs that target *PTEN* for its inhibition. Therefore, a low expression of *PTENP1* could suggest a downregulation of *PTEN*. However, the expression levels of the pseudogene were similar in all patients and controls of our study, which discards the contribution of this pseudogene to the *PTEN* expression levels in our study.

SDH-B and *SDH-D* variants were described in the literature to be implicated in PHTS (Ni *et al*., 2008) but these are described in several databases (ExAC and gnomAD) to be common in general population (MAF around 0.6%), therefore we consider it is unlikely that these variants are involved in the PHTS etiology. Even though the *SDH* genes were not included in the gene panel of our work, nor we analyzed its sequences individually, we found one variant through WES: rs11214077 in *SDHD* gene, in 2 cases (672F and 938F), which has been described associated to CS (Yu *et al*., 2017; Ni *et al*., 2008), but again, this association casts doubts given its frequency and we did not consider it as a causal element.

PIK3CA and *AKT1* variants have been proposed to account for 9 and 2% respectively of the CS or CS-like *PTEN*-wt patients (Orloff *et al.*, 2013; Yehia *et al.*, 2019). However, the only reports of these variants at germline level come from a single study where the authors suggest their pathogenicity (Orloff *et al*., 2013), but these variants are not reported in ExAC

nor gnomAD. We suggest that for now, these variants should be considered VUS and more research is needed to confirm the association of these genes with PHTS predisposition.

In the last years, WES has become a good and affordable approach to find other unexpectedly implicated genes. This technology has been applied by other authors in samples of patients with PHTS phenotype but without mutations in *PTEN* and this is how they proposed variants in *SEC23B*, *EGFR* and *TTN* genes as potential drivers of this disease in few selected cases of PHTS in the literature (Yehia *et al*., 2015, 2017; Colby *et al*., 2016). We looked for variants in these genes in our exomes data, but we did not find any definitive one. We only found *SEC23B* rs41309927 and some variants in *TTN* gene but with an improbable clinical relevance as these did not pass our variant filtering. Therefore, we suggest these candidate genes might account only for individual cases.

From our WES data, several variants caught our attention (FIGURE 17) and suggested several hypothesis that are described in further detail hereinafter, however, we were not able to perform further studies during the time frame of this project to evaluate their implication in PHTS etiology.

All the selected variants were missense except three: two frameshift variants, one in the *MLH3* gene and the other one in the *MUTYH* gene, and one stop gain in *UBN2* gene. The *MLH3* gene is one of the DNA mismatch repair (MMR) genes, implicated in ensuring the genomic integrity during DNA replication and recombination. Defects in the MMR genes are associated to the hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome, characterized by an increased risk for colorectal and endometrial cancer (Wu *et al.*, 2001). However, it is still controversial if the *MLH3* gene is implicated in this disease (Liccardo *et al.*, 2017; Hong *et al.*, 2004; Wu *et al.*, 2001). Moreover, the only reported feature of our patient carrying the variant in *MLH3* is endometrial cancer. We were able to confirm the absence of microsatellite instability (MSI) in the paraffin embedded endometrial tumor sample, suggesting a diagnosis out of the Lynch syndrome spectrum. On the other hand, the patient 181F was found to harbor a variant in the *MUTYH* gene, which is associated to FAP syndrome in a recessive pattern. However, this variant was found in heterozygosis in this patient, meaning that he might be at risk of developing colorectal cancer, although he had no reported colonic lesions and the risk in this genetic context is still unclear (Nielsen *et al*., 2015). Nevertheless, his phenotype, including the LDD, is unlikely to be explained by this gene. *PMS2*, another known gene associated with HNPCC (Kohlmann and Gruber, 2018) was found possibly altered by a missense variant

in heterozygous state in patient 180F, who was only referred due to LDD with no known clinical history related with colon, so it is also improbable that this variant is the cause.

The genes *MLH3*, *MUTYH* and *PMS2* are associated to colorectal cancer, which is also a feature within the PHTS spectrum, and we found possible alterations on them in several PHTS *PTEN*-wt patients, raising the possibility that in these individuals, the mentioned variants entail risk to develop this cancer type but are unlikely to be responsible of the remaining characteristics of the PHTS phenotype.

Regarding genes related with the PI3K pathway, we found a variant in *HERC1*, which encodes an interactor of TSC2 and it is related with macrocephaly, dysmorphic facies and psychomotor retardation (Aggarwal *et al*., 2016). Another variant was found in *TSC1*, a known element of the PI3K pathway.

The tumor suppressor gene *BAP1* is implicated in an autosomal dominant hereditary syndrome with predisposition to mesothelioma, uveal melanoma and renal cell carcinoma (OMIM 614327), although its associated clinical spectrum is still being characterized and could also include breast cancer (Masoomian *et al.*, 2018). In our series, we found a rare missense variant in this gene in a patient who suffered breast cancer but had no other of the mentioned features.

The patient 672F was quite interesting because, besides presenting some features of PHTS, he suffered 5 cancers of different types: clear cell renal cell carcinoma, colorectal carcinoma, Hodgkin lymphoma, endocrine cancer of unspecified type and lung cancer. The WES findings in this individual were also interesting as we found *NEDD4* gene to be altered. The ubiquitin ligase NEDD4 is a known regulator of PTEN but this function might be dispensable depending on the biological context (Fouladkou *et al*., 2008; Wang *et al*., 2007), and therefore, the role of *NEDD4* as a proto-oncogenic factor still needs further research. The missense variant in this gene has a very low MAF ($<1 \times 10^{-4}$) according to ExAC, the predictors suggest deleteriousness and it is also described in Geno2MP to be present in individuals with intellectual disability, growth delay and cardiovascular lesions. The clinicians did not report any of these features for this patient. However, we consider this could be a good candidate for future research.

Another interesting case was patient 599F. This woman had one of the highest CC scores in our patient series (CC score=54). However, the interpretation of the variants we found was not so straightforward. Missense variants in *PDGFRB*, which encodes a receptor involved in the PI3K/AKT/mTOR pathway, and in *PIK3R2*, associated with megalencephaly and regulator of the PI3K, were the most interesting.

Interestingly, we found two patients (672F and 98F) carrying missense variants (each one different) in the *RNF135* gene, which caught our attention due to its association with macrocephaly, overgrowth and facial dysmorphism (Douglas *et al*., 2007). However, neither of the patients was reported to have those features, although we cannot discard that it is due to under-report.

To date, the only demonstrated susceptibility gene of LDD is *PTEN* (Zhou, Marsh, *et al*., 2003), but still many patients of this pathology have an unknown cause, and recently *EGFR* was proposed as a novel candidate of LDD susceptibility gene after its finding in a single case (Colby *et al*., 2016). We also sought for other LDD genes through WES of 4 *PTEN*-wt patients with this cerebellar tumor. We did not find any relevant alteration in *EGFR*, but of note, we found an unreported variant in *FGFR1* (TABLE S8), a gene that encodes a receptor involved in the PI3K signaling (Starska *et al*., 2018; Dey *et al*., 2010).

The autism spectrum disorder (ASD) has a complex etiology, with at least 1,000 susceptibility genes reported (Abrahams *et al*., 2013). The patient 938F had ASD together with macrocephaly, resembling a syndromic autism, and therefore, it would be expected to find a monogenic cause. In fact, *PTEN* accounts for a relevant amount of the individuals with this phenotype (Varga *et al*., 2009) and in our series, from the total of individuals with this phenotype, 7 were *PTEN*-mut+ and 5 *PTEN*-wt. This patient harbored a missense variant in *ATR* gene and the malfunction of its protein can impair fragile site stability, which can be a risk for autism. This patient also harbored a stop gain in *UBN2*, a gene putatively associated with autism (Yuen et al., 2017; Abrahams et al., 2013), and two different variants were found in *EP400* gene, described with suggestive evidence of association with autism (Abrahams *et al*., 2013).

We have proposed several variants besides the *PTEN* gene, found through NGS or WES that could account for a subset of the *PTEN*-wt patients' phenotypes, but their impact remains to be determined. A common issue in managing massive sequencing results is facing the VUS, because the interpretation of its functional impact is not straightforward. Therefore, further studies on these variants are required, mainly functional characterization of the effects at protein and cellular level, before a translation to the clinical setting. Moreover, it is possible that our results are limited by the number of patients, the heterogeneity of their clinical phenotype and not being able to recruit progenitors to study family trios.

Overall, the cause of the PHTS in a germline *PTEN*-wt context remains to be discovered. It is possible that genes not classically associated with cancer predisposition could explain these cases. Other approaches, such as RNA-seq, WGS or methylation assays, might be

also useful to unravel other genetic causes of PHTS. Moreover, processes affecting regulation regions, the nuclear functions and other roles of *PTEN* besides the PI3K pathway still need further research.

7. Usefulness of other molecular tools in PHTS diagnosis

Our results suggest that there is no correlation between the expression pattern of *PTEN* at mRNA level in peripheral blood samples with the genotype of the individual. The ascertainment of mRNA expression levels in peripheral blood is complex, and even more for mRNAs with low abundance in this tissue, and differences between expression levels in different sets of individuals would be more easily found at protein levels through western blotting.

Moreover, we have shown that the IHC of PTEN in normal and tumor paraffin-embedded tissue samples of the patient, does not contribute to make a diagnosis of PHTS. This idea is consistent with recent published results that indicated the limited usefulness of the commercially available PTEN antibodies for IHC (Mingo *et al*., 2019).

Therefore, both the *PTEN* mRNA expression in peripheral blood and the protein expression patterns in tumor tissues might not be useful to predict a diagnosis of PHTS.

Moreover, we consider that, at least for now, there are not enough evidences to set in the clinics a gene panel with other genes besides *PTEN* to diagnose the PHTS patients. The findings of variants in certain genes such as *SEC23B* or *TTN* do not explain enough patients and their implication as true causes of this disease needs further study. One criterion to decide performing genetic testing for a patient is whenever there is at least a 10% chance to find a mutation in a certain gene. This is the case for the *PTEN*-wt patients who meet the diagnostic criteria or have a CC score over 3 (Tan *et al*., 2011), which is enough to decide doing *PTEN* genetic testing, but not for studying other genes (this probably would not be cost-effective in a clinical practice). Instead, we suggest the study of other genes associated with syndromes whose manifestations overlap with PHTS in *PTEN*-wt patients, as these could have been erroneously diagnosed at clinical level. In this manner, we found patient 1174F who was reported to have several mucocutaneous lesions (trichilemmomas, acral keratosis, oral papillomas, papules and lipomas) and harbored a splicing mutation in *FLCN*, a gene associated to the BHD syndrome, characterized also by cutaneous lesions, specifically to skin fibrofolliculomas and trichodiscomas, and by renal cancer, among other features (Schmidt and Linehan, 2018; Menko *et al*., 2009), so there is a possibility that the patient's features were misdiagnosed.

Moreover, FLCN regulates the mTOR signaling (Schmidt and Linehan, 2018) and thus, an overlapping phenotype with tuberous sclerosis is also expected.

8. Recommendations on the diagnosis and management of PHTS patients

Considering our discussion, we propose the steps that should be taken to diagnose a patient with PHTS: FIGURE 20. Moreover, a multidisciplinary team including at least a geneticist, a dermatologist and an oncologist should be in charge of the follow-up of these patients. The follow-up recommendations found on the literature (TABLE 2) are appropriate, however, we suggest to anticipate the cancer screenings in young individuals at risk due to a family history of PHTS or who are demonstrated carriers of a *PTEN* mutation. Finally, prospective studies of the PHTS patients will aid in the determination of their clinical risks.

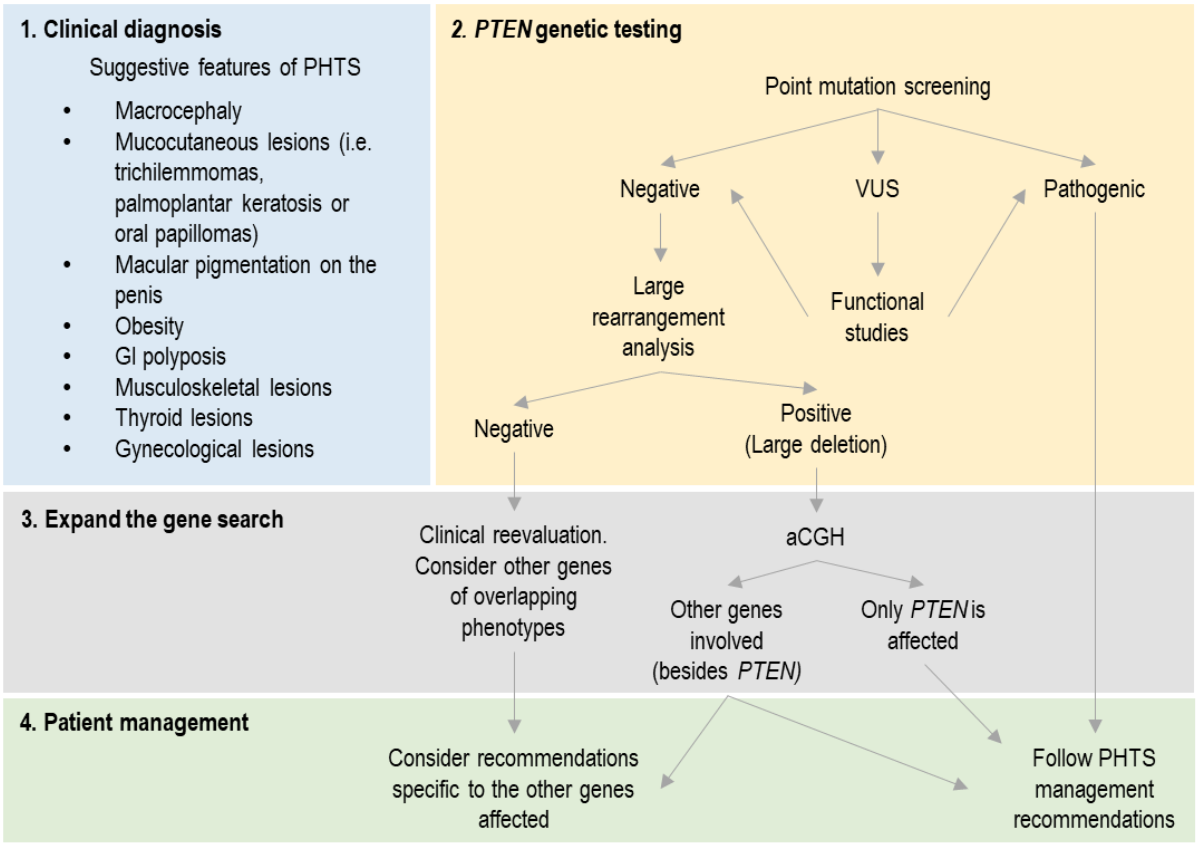

Figure 20. Recommended flowchart for the clinical diagnosis, molecular diagnosis and management of PHTS patients, based on our work.

CONCLUSIONS

CONCLUSIONS

1. Our clinical and molecular characterization of the PHTS through the study of a large series of patients diagnosed with this disease contributes to the knowledge on this rare disease and underlines the key steps to accelerate diagnosis and improve the management of these patients.
2. The systematic collection of clinical data through a specific questionnaire or checklist, together with the use of our clinical diagnostic criteria allowed in our study a good identification rate of *PTEN* mutation carriers, compared to the use of the ICC criteria or the Pilarski's.
3. Macrocephaly and mucocutaneous lesions are among the several clinical manifestations that strongly suggest the presence of a *PTEN* germline mutation, whereas referral of a patient presenting only PHTS-associated cancers for *PTEN* genetic testing is not advisable as first measure.
4. Regarding the susceptibility to cancer, we highlight: first, the occurrence of obesity in *PTEN* mutation carriers, which can increase even more the cancer risks for these patients; and second, the possible onset of cancer at very early ages (childhood or adolescence). Therefore, we suggest the inclusion of obesity check-up and anticipated cancer screenings in the PHTS patients' follow-up.
5. The genetic testing of PHTS through a multigene panel is a useful approach, as it allows further considering other genes related with cancer predisposition or overlapping phenotypes and discerning mosaicisms. In the case of finding large rearrangements involving *PTEN*, it is important to explore if other genes are also deleted, as these could entail additional clinical risks.
6. mRNA expression levels of *PTEN*, *KLLN* and *PTENP1* in samples from peripheral blood leukocytes, and the IHC of PTEN and p-AKT in tumor samples have apparently no diagnostic value, as they seem not useful to identify *PTEN* mutation carriers neither to predict the genotype or phenotype of the patient.
7. Some interesting results of our work set the basis for the continuation of the research in PHTS, with potential in a translational field, such as the association of *PTEN* exon 1 mutation carriers with high *KLLN* and *PTEN* expression levels and an apparent high risk for renal cancer, but also in basic research, such as the different consequences for PTEN protein functions depending on the genetic variants.
8. In our work, we were not able to demonstrate the implication of *TTN* and *SEC23B* genes, or the ones encoding SDH, AKT and PI3K, in the etiology of PHTS, but we found several variants in genes such as *NEDD4* that could be relevant given

its function. The cause of the disease in the *PTEN*-wt patients remains to be discovered. It is plausible that *PTEN* is the only high susceptibility gene for PHTS development and other rare factors (genetic or not) might contribute in certain individuals.

CONCLUSIONES

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1. La caracterización clínica y molecular del PHTS que hemos realizado a través del estudio de una gran serie de pacientes diagnosticados con esta enfermedad contribuye al conocimiento de esta entidad rara y destaca los pasos clave para acelerar el diagnóstico y el tratamiento de estos pacientes.
2. La recopilación sistemática de datos clínicos a través de un cuestionario específico, junto con el uso de nuestros criterios clínicos de diagnóstico, han permitido obtener en nuestro estudio una buena tasa de identificación de portadores de mutaciones en *PTEN*, en comparación con el uso de los criterios del ICC o los de Pilarski *et al*.
3. La macrocefalia y las lesiones mucocutáneas se encuentran entre varias de las manifestaciones clínicas que sugieren firmemente la presencia de una mutación germinal en *PTEN*, mientras que no es aconsejable como primera medida la derivación de un paciente que sólo presenta cánceres asociados a PHTS para estudio genético de *PTEN*.
4. Con respecto a la susceptibilidad al cáncer, destacamos: primero, la presencia de obesidad en portadores de mutaciones en *PTEN*, lo cual puede aumentar aún más los riesgos de cáncer para estos pacientes; y segundo, la posible aparición de cáncer a edades muy tempranas (infancia o adolescencia). Por lo tanto, sugerimos la inclusión de controles de peso y la anticipación de las pruebas de detección precoz de cáncer en el seguimiento de los pacientes con PHTS.
5. El diagnóstico genético de PHTS a través de un panel de genes es un enfoque útil, ya que permite considerar otros genes relacionados con la predisposición al cáncer o fenotipos superpuestos, así como detectar mosaicismos. En el caso de encontrar grandes reordenamientos que involucren a *PTEN*, es importante explorar si también se eliminan otros genes, ya que estos podrían conllevar riesgos clínicos adicionales.
6. Los niveles de expresión de ARNm de *PTEN*, *KLLN* y *PTENP1* en muestras de leucocitos de sangre periférica, y la IHC de PTEN y p-AKT en muestras tumorales carecen de valor diagnóstico, ya que parecen no ser útiles para identificar portadores de mutación de *PTEN* ni para predecir el genotipo o fenotipo del paciente.
7. Varios hallazgos interesantes de nuestro trabajo sientan las bases para continuar investigando en el PHTS, con potencial en el ámbito traslacional, como la asociación de portadores de mutación de exón 1 de *PTEN* con altos niveles de expresión de *KLLN* y *PTEN* y un riesgo aparentemente alto para cáncer renal,

pero también en investigación básica, como las diferentes consecuencias para las funciones de la proteína PTEN dependiendo de las variantes genéticas.

8. En nuestro trabajo, no hemos podido demostrar la implicación de los genes *TTN* y *SEC23B* ni los codificantes de SDH, AKT y PI3K, en la etiología del PHTS, pero hemos dado con algunas variantes en genes como *NEDD4* que por su función podrían ser relevantes. La causa de la enfermedad en los pacientes que no portan alteraciones en *PTEN* está aún por descubrir. Es posible que *PTEN* sea el único gen de alta susceptibilidad para el desarrollo de PHTS y que otros factores poco comunes (genéticos o no) estén contribuyendo en ciertos individuos.

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SUPPLEMENTARY MATERIAL

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Supplementary tables

Table S10. Medical collaborators.

Hospital/Institute/Centre	Physician/Collaborator	Autonomous community
Hospital Virgen de Valme	Cózar, María Victoria	Andalucía
Hospital Puerta del Mar	Marín, María del Rosario	Andalucía
Hospital Virgen de las Nieves	Pedrinaci, Susana	Andalucía
Hospital Lozano Blesa	Andrés, Raquel	Aragón
Hospital Central de Asturias	Plasencia, Ana María Viejo, Mónica	Asturias
Hospital Can Misses	González, Margarita	Baleares
Hospital Son Dureta	Rosell, Jordi	Baleares
Hospital Insular de Gran Canaria	Boronat, Mauro	Canarias
Hospital Nuestra Señora de la Candelaria	Martínez Bugallo, Francisco Duque, Mª del Rosario	Canarias
Materno-Infantil Gran Canaria	Santana, Alfredo	Canarias
Hospital Materno-Infantil de Canarias	Vázquez, Carlos	Canarias
Hospital General de Burgos	Lastra, Enrique	Castilla-León
Instituto Catalán de Oncología (ICO)	Blanco, Ignacio Brunet, Joan Pineda, Marta	Cataluña
Hospital Vall d'Hebrón	Balmaña, Judith Del Campo, Miguel	Cataluña
Hospital Parc Taulí	Gabau, Elisabeth Guitart, Miriam Llort, Gemma Rodà, Diana	Cataluña
Hospital Terrassa	Hernán, Imma	Cataluña
Hospital Sant Joan de Déu	Hernández, Héctor Salvador Martorell, Loreto	Cataluña
Hospital Sant Pau	Lasa, Adriana Ramón y Cajal, Teresa	Cataluña
Hospital Sant Joan de Reus	Salvat, Mónica	Cataluña
Hospital del Mar	Toll, Agustín	Cataluña
Hospital de Móstoles	Díaz Bustamante, Arantza Marrupe, David	Madrid
Hospital de Fuenlabrada	Gutiérrez Abad, David* Hernández, Almudena*	Madrid
Hospital de Alcorcón	Hernando, Susana	Madrid
Hospital Severo Ochoa	Lacambra, Carmen	Madrid
Hospital La Paz	Martínez González, Víctor Rodríguez Sala, Nuria	Madrid
Hospital Gregorio Marañón	Orera, María	Madrid
Hospital Clínico San Carlos	Pérez Segura, Pedro	Madrid
Hospital 12 de Octubre	Robles, Luis	Madrid
Hospital Puerta del Hierro	Sánchez Ruiz, Antonio	Madrid
Hospital General de Elche	Soto, José Luis	Valencia
Hospital Virgen de la Arrixaca	Carbonell, Pablo*	Murcia
Hospital Virgen del Camino	Alonso, Miguel Ángel Arias, María del Mar	Navarra

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*Direct collaborators of the FIS project.

Table S2. Nucleotide sequence of the primers and melting temperature (Tm).

Primer pair	5'-3' Forward primer	5'-3' Reverse primer	Tm (°C)
Exon 1 *PTEN*	AAGTCCAGAGCCATTTCCAT	AGTCACCCAAACTACGGACA	66-64-62
Exon 2 *PTEN*	TTCTTTTAGTTTGATTGCTGCAT	TTTTCTAAATGAAAACACAACATGAA	62-60-58
Exon 3 *PTEN*	CCATAGAAGGGGTATTTGTTGG	CAATGCTCTTGGACTTCTTGACT	64
Exon 4 *PTEN*	AAAGATTCAGGCAATGTTTGTT	TCTCACTCGATAATCTGGATGAC	60
Exon 5 *PTEN*	TGTTAAGTTTGTATGCAACATT	TCCAGGAAGAGGAAAGGAAAA	62-60
Exon 6 *PTEN*	ATGGCTACGACCCAGTTACC	TGTTCCAATACATGGAAGGATG	62-60
Exon 7 *PTEN*	TCCATATTTCGTGTATATTGCT	CACCTGCAGATCTAATAGAAAA	62-60
Exon 8 *PTEN*	TTAAATATGTCATTTCATTTCTTTTTC	CATGTTACTGCTACGTAAACACTGC	57
Exon 9 *PTEN*	TGTTCATCTGCAAAATGGAAT	AACTGGTAATCTGACACAATGT	64
PTEN promoter	GCGTGGTCACCTGGTCCTTT	GCTGCTCACAGGCGCTGA	68
3' UTR *PTEN* cDNA	GTTTACCGGCAGCATCAAAT	CCCCCACTTTAGTGCACAGT	62
KLLN cDNA	TTACCGGGTTGAGTGGAAAG	TTCCCCAACTAGGGACACAC	60
PTENP1 cDNA	TCAGAACATGGCATACACCAA	TGATGACGTCCGATTTTTCA	59
36B4 cDNA	CAGCAAGTGGGAAGGTGTAATCC	CCCATTCTATCATCAACGGGTACAA	60
1-2 *PTEN* cDNA	TGACAGCCATCATCAAAGAGA	TACGCCTTCAAGTCTTTCTGC	56
HERC1	TATGGGTGACCGCATGACT	TGTGGAATCCCAACATCAAA	64
NEDD4	AAAAACATTTCCCATTACTTTCCA	TTCTTTTAATGCCCTTACGTTCA	62
MLH3	CCTACTGGTGGGACCATTCT	TGTAAGGAGGAAATGCATGG	63
FLCN	TTGGTGTCACTAAGCGAGGA	GTGCACTGGCTGTAAGCAGA	60
STK11	TCAACCACCTTGACTGACCA	CCACTCAGTCCTCTCAATGC	64
TP53	CAGAGTGAGACCCCATCTCAA	ACACTCGTCCCTGGGTTTG	64.5

Table S3. *PTEN* pathogenic point variants (52 patients).

Sample ID	DNA variant	Protein change
186F	c.39_40del	p.(Arg14Glufs*29)
219F	c.17_18del	p.(Lys6Argfs*4)
30F	c.68_69insA	p.(Asp24fs*20)
427F	c.58_61dup	p.(Phe21Trpfs*24)
803F	c.1_2delAT	p.0?
864F	c.39_40del	p.(Arg14Glufs*29)
700F	c.49C>T	p.(Gln17*)
1274F	c.68T>G	p.(Leu23*)
1041F	c.81_82insCT	p.(Ile28Leufs*27)
1166F	c.255_256insAA	p.(Ala86Lysfs*14)
794F	c.405dup	p.(Cys136Metfs*44)
1126F	c.334C/G	p.(Leu112Val)
1157F	c.406T>C	p.(Cys136Arg)
249F	c.379G>A	p.(Gly127Arg)
532F	c.395G>T	p.(Gly132Val)
683F	c.302T>C	p.(Ile101Thr)
757F	c.389G>A	p.(Arg130Gln)
910F	c.407G>A	p.(Cys136Tyr)
1251F	c.406T>C	p.(Cys136Arg)
517F	c.332G>A	p.(Trp111*)
55F	c.388C>T	p.(Arg130*)
614F	c.388C>T	p.(Arg130*)
653F	c.388C>T	p.(Arg130*)
232F	c.622_623insT	p.(Gly208Valfs*)
777F	c.542T>C	p.(Leu181Pro)
805F	c.512A>G	p.(Gln171Arg)

Sample ID	DNA variant	Protein change
817F	c.493G>C	p.(Gly165Arg)
44F	c.493-1G>A	p.(Gly165fs*)
724F	c.493-1G>C	p.(Gly165Ilefs*9)
762F	c.723_724insTT	p.(Glu242Leufs*15)
1020F	c.686C>G	p.(Ser229*)
1085F	c.640C>T	p.(Gln214*)
499F	c.697C>T	p.(Arg233*)
518F	c.697C>T	p.(Arg233*)
788F	c.655C>T	p.(Gln219*)
1089F	c.829dup	p.(Thr277Asnfs*21)
120C	c.984_987del	p.(Asn329Lysfs*)
578F	c.817_818del	p.(Phe273*)
621F	c.1007dup	p.(Tyr336*)
659F	c.825del	p.(Val275*)
705F	c.985_986delAA	p.(Asn329*)
981F	c.955_958del	p.(Thr319*)
1105F	c.1003C>T	p.(Arg335*)
262F	c.1003C>T	p.(Arg335*)
687F	c.1003C>T	p.(Arg335*)
750F	c.1003C>T	p.(Arg335*)
795F	c.1003C>T	p.(Arg335*)
355F	c.254-1_257dup	
582F	c.492+1G>C	
189F	c.634+5G>A	
250F	c.635-1G>C	
836F	c.492+1delG	

Table S4. *PTEN* variants of unknown significance (VUS).

Patient ID	Exon or intron	DNA change	Protein change	Protein domain	Deleteriousness prediction (Condel)	Phenotype of the carrier	Variant origin
789F	E1	c.77C>T	p.(Thr26Ile)	PTP	Probably damaging	Male 11 yo. Macrocephaly, obesity, macular pigmentation penis, bilateral gynecomastia, autism, mental retardation, musculoskeletal alterations	*De novo*
1170F	E5	c.284C>G	p.(Pro95Arg)	PTP	Probably damaging	Male 39 yo. Macrocephaly, overweight, papules, palmoplantar keratoses, trichilemmomas, oral papillomatosis, lipomas, macular pigmentation penis, goiter, Hashimoto's thyroiditis, colonic and gastrointestinal polyps, cerebral hamartomas	Unknown. (Relatives were not tested)
1201F	E6	c.529T>A	p.(Tyr177Asn)	PTP	Probably damaging	Male 5 yo. Macrocephaly, motor delay	Familial. (Variant segregates in the family with the phenotype).
738F	E7	c.781C>G	p.(Gln261Glu)	C2	Probably damaging	Male 34 yo. Macrocephaly, lipomas, macular pigmentation penis, colorectal polyps, general overgrowth	Familial. (Variant segregates in the family with the phenotype).
1103F	E8	c.929A>G	p.(Asp310Gly)	C2	Probably neutral	Male 29 yo. Macrocephaly, papules, palmoplantar keratoses, thyroid adenomas, papillary-follicular thyroid cancer, colorectal polyps (inflammatory, lymphoid and hamartomatous), testicular cancer	Unknown. (Only a sister was tested resulting non-carrier).
1115F	E8	c.829A>G	p.(Thr277Ala)	C2	Probably damaging	Male 9 yo. Macrocephaly, general developmental disorder, speech delay, general overgrowth	*De novo*
707F	I5	c.254-21G>C	-	-	-	Male 10 yo. Macrocephaly, autism, hyperchromic spots	Unknown. (Relatives were not tested).

PTP: phosphatase.

Table S5. Chromosomic regions deleted as determined through MLPA and aCGH.

ID	Deleted region (MLPA)	Deleted region (aCGH)
594F	Entire *PTEN**	chr10:81,685,169-91,936,008 (10 Mb)
617F	*BMPR1A*, *KLLN* and entire *PTEN*	chr10:81,660,274-89,830,454 (8 Mb)
708F	*KLLN* and *PTEN*'s exon 1	chr10:89,625,664-89,640,157 (14 kb)
858F	*KLLN* and *PTEN*'s exon 1	chr10:89,625,664-89,640,157 (14 kb)
814F	*KLLN* and *PTEN*'s exon 1	chr10:89,653,535-89,653,594 (59 pb)
712F	*PTEN*'s exon 6	-
1054F	*PTEN*'s exon 3	-

*Study performed in 2006 with a probe kit that did not include other genes besides *PTEN* and the remaining DNA could not be used to repeat the study.

Table S6. Germline variants found through NGS that could account for mosaicisms.

Sample	PTEN germline status	ALLELE_FREQUENCIES	Variant (hg19)	Consequence	IMPACT	SYMBOL	Gene	Protein_position	Amino_acids	ClinVar	SIFT	PolyPhen	Existing_variatic
2S618	PTEN-mut+	0,15	X:76937118A>T	missense	MODERATE	ATRX	ENST00000373344	1210	D/E			benign(0)	
2S618	PTEN-mut+	0,2	9:135779797C>G	splice donor	HIGH	TSC1	ENST00000298552			pathogenic			
4S541	PTEN-wt	0,11	7:116397716C>G	missense	MODERATE	MET	ENST00000318493	664	P/A		deleterious(0)	probably_damaging(1)	
6S1181	PTEN-wt	0,11	17:29592354G>A	missense	MODERATE	NF1	ENST00000356175	1590	R/Q	uncertain_significance			rs876659197
10S808	PTEN-mut+	0,33	10:89692905G>A	missense	MODERATE	PTEN	ENST00000371953	130	R/Q	likely_pathogenic&pathogenic			rs121909229

Table S7. Germline VUS in mTOR related genes and in *FLCN*, found through NGS.

ID	Variant	Gene	AF	Consequence	IMPACT	Ref gene	Protein position	Amino acids	Existing variation	gnomAD AF	Clin_sig
499F	14:105246462G>T	*AKT1*	0,40	missense	MODERATE	ENSG00000142208	232	G/E			
1708C	14:105240256C>T	*AKT1*	0,49	missense	MODERATE	ENSG00000142208	46	D/E	rs146875699	0,0003171	uncertain_significance&not_provided
762F	19:40761140T>C	*AKT2*	0,48	missense	MODERATE	ENSG00000105221	71	N/S	rs200272953	0,0002193	
860F	22:32234798C>A	*DEPDC5*	0,49	missense	MODERATE	ENSG00000100150	819	P/T			
1105F	1:11273527G>A	*MTOR*	0,46	missense	MODERATE	ENSG00000198793	1072	P/S			
794F	1:11300579C>T	*MTOR*	0,51	missense	MODERATE	ENSG00000198793	523	D/N	rs376836258&COSM3930270	8,12E-06	
181F	17:29553639A>T	*NF1*	0,48	missense	MODERATE	ENSG00000196712	730	N/Y	rs758893131	0,000061	uncertain_significance
1173F	17:29663905T>G	*NF1*	0,50	missense	MODERATE	ENSG00000196712	2113	C/G	COSM3958176&COSM3958177		
672F	1:204438340T>A	*PIK3C2B*	0,49	missense	MODERATE	ENSG00000133056	197	Q/H	rs17847778	0,000599	
836F	1:204438908C>T	*PIK3C2B*	0,49	missense	MODERATE	ENSG00000133056	1029	F/L	rs61763420	0,002197	
1251F	1:204418411C>T	*PIK3C2B*	0,49	missense	MODERATE	ENSG00000133056	458	R/Q	rs61755372	0,000658	
1210F	1:204411723G>T	*PIK3C2B*	0,52	missense	MODERATE	ENSG00000133056	458	R/Q	rs61755372	0,000658	
1197F	1:204429727C>T	*PIK3C2B*	0,52	missense	MODERATE	ENSG00000133056	750	G/S	rs114917235	0,0005566	
1210F	1:204429727C>T	*PIK3C2B*	0,48	missense	MODERATE	ENSG00000133056	8	G/E	rs115204119	0,001045	
995F	5:67591042GGAA>G	*PIK3R1*	0,49	inframe_deletion	MODERATE	ENSG00000145675	546	E/-			
1065F	5:38962438T>C	*RICTOR*	0,50	missense	MODERATE	ENSG00000164327	565	Y/C	rs146754529	0,0007066	
1198F	5:38953625G>A	*RICTOR*	0,50	missense	MODERATE	ENSG00000164327	910	R/C	rs143469898	0,0001871	
594F	17:78857246A>G	*RPTOR*	0,49	missense	MODERATE	ENSG00000141564	407	P/L	rs922237067&COSM1579117	0,0000163	
981F	17:78935270G>A	*RPTOR*	0,49	missense	MODERATE	ENSG00000141564	538	I/V			
700F	17:78820280C>T	*RPTOR*	0,51	missense	MODERATE	ENSG00000141564	1228	V/M	rs147241989	0,0003074	
681F	19:1223163C>T	*STK11*	0,49	missense	MODERATE	ENSG00000118046	367	T/M	rs587782835&COSM21358	0,0000167	uncertain_significance
981F	19:1226587C>G	*STK11*	0,50	missense	MODERATE	ENSG00000118046	415	R/G	rs864622448		uncertain_significance
499F	9:135771994G>C	*TSC1*	0,53	missense	MODERATE	ENSG00000165699	692	R/Q	rs199755731	0,0001708	uncertain_significance&likely_benign
1172F	9:135779171C>T	*TSC1*	0,67	missense	MODERATE	ENSG00000165699	1041	S/R	rs753374839	0,0000531	uncertain_significance&likely_benign
632F	16:2110684C>T	*TSC2*	0,48	missense	MODERATE	ENSG00000103197	330	P/L	rs140910086	0,0000366	uncertain_significance
497F	16:2122869T>A	*TSC2*	0,50	missense	MODERATE	ENSG00000103197	747	L/Q	CD010683		
308F	5:67522555A>G	*PIK3R1*	0,51	missense	MODERATE	ENSG00000145675	18	R/G			
308F	16:2135023A>G	*TSC2*	0,48	missense	MODERATE	ENSG00000103197	1522	N/S	rs144062721&COSM3787107&COSM3787108	6,511E-05	uncertain_significance&not_provided, prob.benign
30F	16:2111938G>T	*TSC2*	0,5	missense	MODERATE	ENSG00000103197	396	D/Y	COSM6143684&COSM6143685		
1174F	17:17127234A>T	*FLCN*	0,49	splice_donor_variant	HIGH	ENSG00000154803			CS101080		
981F	17:17131357C>T	*FLCN*	0,49	missense	MODERATE	ENSG00000154803	32	G/E	rs587778366	0,0000122	
612F	17:17118598C>T	*FLCN*	0,5	missense	MODERATE	ENSG00000154803	445	A/T	rs41419545	0,002648	benign&likely benign&VUS

Table S8. Somatic variants found in the tumor samples through NGS.

Tumor sample	Cancer type	Paired blood DNA	Variant (hg19)2	ALLELE_FREQUENCIES	DEPTH	Consequence	IMPACT	SYMBOL	Gene	Feature	cDNA_position	Protein_position	Amino_acids	Existing_variation	VARIANT_CLASS	SIFT	PolyPhen	gnomAD_AF	CLIN_SIG
03T0179	Papillary thyroid cancer	2S232	7:140453136A>T	0,38	36	missense	MODERATE	BRAF	ENSG00000157764	ENST00000288602	1860	600	V/E	CM112509&rs113488022&COSM18443&COSM476&COSM6137	SNV	deleterious(0)	probably_damaging(0.963)		likely_pathogenic&pathogenic
09T0040	Lung adenocarcinoma	8S356	X:76855969C>G	0,46	196	missense	MODERATE	ATRX	ENSG00000085224	ENST00000373344	5846	1877	Q/H		SNV		possibly_damaging(0.68)		
09T0040	Lung adenocarcinoma	8S356	1:204438340T>A	0,57	499	missense	MODERATE	PIK3C2B	ENSG00000133056	ENST00000367187	1148	197	Q/H	rs17847778	SNV	tolerated_low_confidence(0.08)	benign(0)	6E-04	
09T0040	Lung adenocarcinoma	8S356	10:897176089G>A	0,73	197	splice acceptor	HIGH	PTEN	ENSG00000171862	ENST00000371953				CS090867&CS993675&COSM28920&COSM5971&COSM921121	SNV				
04T0158	Rectal cancer	3S26	17:7577094G>A	0,45	31	missense	MODERATE	TP53	ENSG00000141510	ENST00000269305	1034	282	R/W	rs28934574&CM056413&CM920678&TP53_g.13824C>T&COSM10704&COSM10992&COSM1636702&COSM1725698&COSM3378339&COSM3675520&COSM43813&COSM44918&COSM99925&COSM99934	SNV	deleterious(0)	probably_damaging(1)	4E-06	likely_pathogenic&pathogenic
16T0122	Thyroid cancer	11S1087	3:178952090G>C	0,39	131	missense	MODERATE	PIK3CA	ENSG00000121879	ENST00000263967	3302	1049	G/R	rs121913277&CM126700&COSM1168056&COSM12597&COSM1421012&COSM446029&COSM777	SNV	tolerated(0.09)	benign(0.096)		likely_pathogenic
16T0122	Thyroid cancer	11S1087	X:41077771TTA>T	0,53	178	frameshift	HIGH	USP9X	ENSG00000124486	ENST00000604545	6990-6991	2119-2120	LI/LX		deletion				

Table S9. Selected variants from the WES data.

Sample ID	Gene	Reference	DNA change	Protein change	Zygosity	Reason of prioritisation	Population frequency	rs	SIFT	Polyphen	CADD	REVEL	MetaLR	ClinVar	MA
98F	*REST*	NM_001193508.1	c.3200G>A	p.R1067H	Heterozygous	PTEN INTERACTOME	0		0	0,849					1,04
98F	*RNF135*	NM_032322.3	c.1007G>A	p.R336H	Heterozygous	MACROCEPHALY	0.000082	rs140592050	0	0,989	23	0,198	0,518	NA	0,928
180F	*PTK2*	NM_005607.4	c.2431G>A	p.E811K	Heterozygous	PTEN INTERACTOME	0.000082							NA	
180F	*PMS2*	NM_000535.6	c.1490G>A	p.G497D	Heterozygous	HEREDITARY CANCER	0.000100	rs199739859	0,38	0,118	11	0,251	0,458	Conflicting	0,339
181F	*MUTYH*	NM_001128425.1	c.1228_1229 insGG	p.E410fs	Heterozygous	HEREDITARY CANCER	0.000200								
181F	*FGFR1*	NM_001174067.1	c.79A>G	p.T27A	Heterozygous	HEREDITARY CANCER	0.000073	rs376018211	0,23	0,01	16	0,262	0,248	NA	
599F	*PDGFRB*	NM_002609.3	c.2564C>T	p.S855L	Heterozygous	PI3K-Akt-mTOR , PTEN INTERACTOME, OVERGROWTH	0.000004	rs1292468244	0	0,142	24	0,607	0,432	NA	0,197
599F	*PIK3R2*	NM_005027.3	c.281G>A	p.R94H	Heterozygous	PTEN INTERACTOME	0.000044	rs757629395	0	0,972	28	0,185	0,056	NA	0,488
672F	*NEDD4*	NM_198400.3	c.2630G>A	p.R877Q	Heterozygous	PTEN INTERACTOME	0.000100	rs201295772	0,01	0,893	31	0,588	0,59	NA	0,635
672F	*RNF135*	NM_032322.3	c.698C>T	p.P233L	Heterozygous	MACROCEPHALY	0.000028	rs369761172	0,61	0	4	0,027	0,041	NA	
720F	*TOP2A*	NM_001067.3	c.1206_1212 del	p.A402fs	Heterozygous	CANCER	0								
938F	*ATR*	NM_001184.3	c.228G>T	p.M76I	Heterozygous	HEREDITARY CANCER	0		0,01	0,996					1,95
938F	*EP400*	NM_015409.4	c.3373C>T	p.R1125C	Heterozygous	AUTISM	0.000032	rs773066144	0,03	0,999	32	0,904	0,924	NA	0,847
938F	*EP400*	NM_015409.4	c.8006C>T	p.A2669V	Heterozygous	AUTISM	0.000033	rs144960562	0	0,994	24	0,682	0,761	NA	0,579
938F	*UBN2*	NM_173569.3	c.220C>T	p.Q74X	Heterozygous	AUTISM	0								
779F	*KIF1B*	NM_015074.3	c.572A>G	p.D191G	Heterozygous	HEREDITARY CANCER	0								
1172F	*TSC1*	NM_000368.4	c.2075G>A	p.R692Q	Heterozygous	PI3K-Akt-mTOR	0.000200	rs199755731	0,02	0,999	29	0,567	0,805	Benign(1);Likely benign(4);Uncertain significance(2)	0,735
1172F	*MLH3*	NM_001040108.1	c.678_679del	p.I226fs	Heterozygous	HEREDITARY CANCER	0								
791F	*NRG3*	NM_001165973.1	c.102G>T	p.R34S	Heterozygous	PI3K-Akt-mTOR	0		0,01	0,01					0
791F	*CBL*	NM_005188.3	c.2483C>T	p.P828L	Heterozygous	HEREDITARY CANCER	0.000008	rs763756632	0	0,998	26	0,676	0,738	NA	0,737
1176F	*BAP1*	NM_004656.3	c.211G>A	p.V71M	Heterozygous	HEREDITARY CANCER	0.000004	rs753629908	0,04	0,982	23	0,397	0,435	NA	0,862
1176F	*HERC1*	NM_003922.3	c.7615G>A	p.A2539T	Heterozygous	MACROCEPHALY	0.000200		0,16	0,11	22	0,141	0,049	1 (VUS)	0,144

Supplementary figures

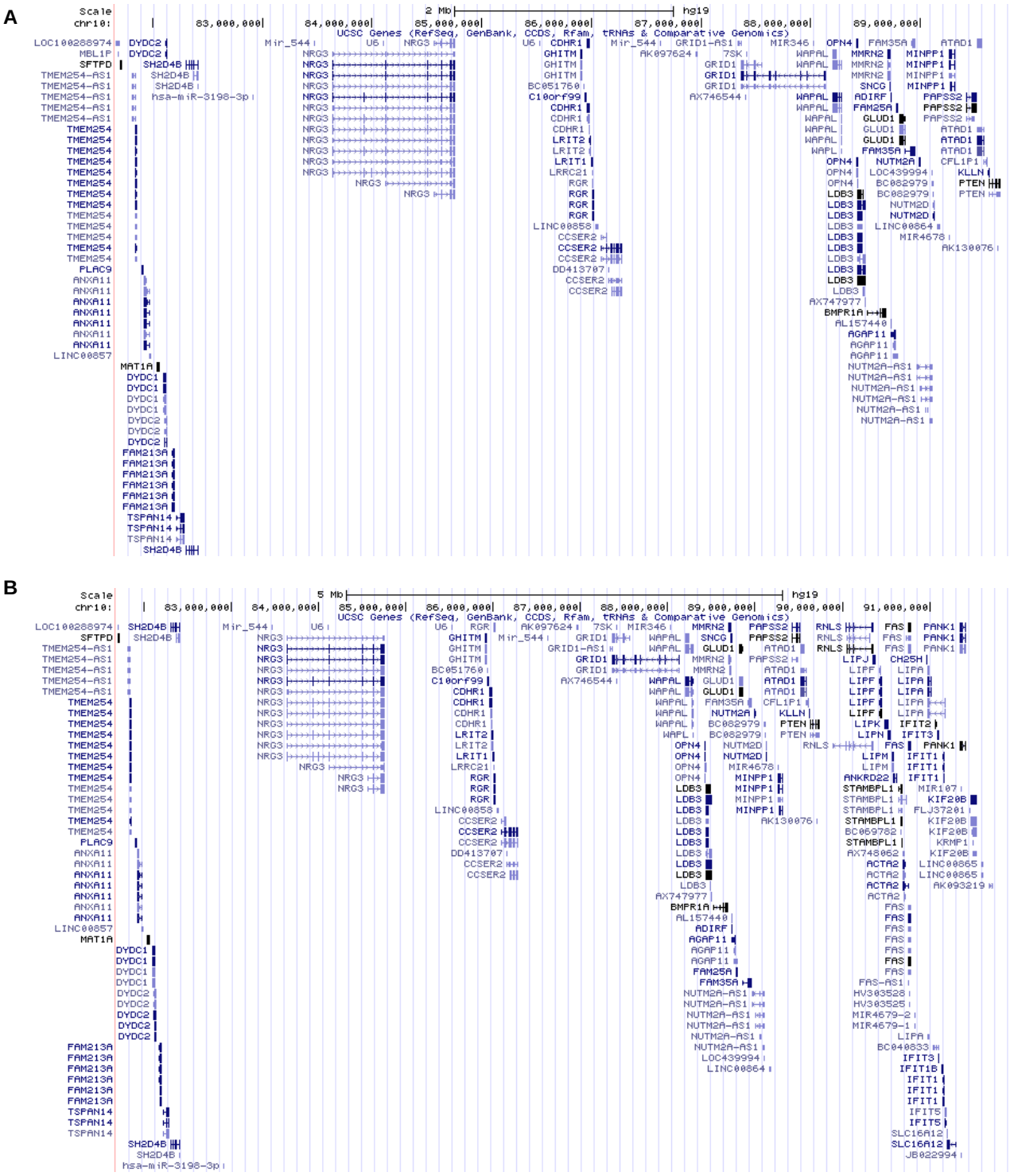


Figure S21. Genomic region comprised in the large deletions of patient 617F (A) and patient 594F (B), showing the affected genes, as determined through aCGH. (Caption obtained using the UCSC online human genome browser).

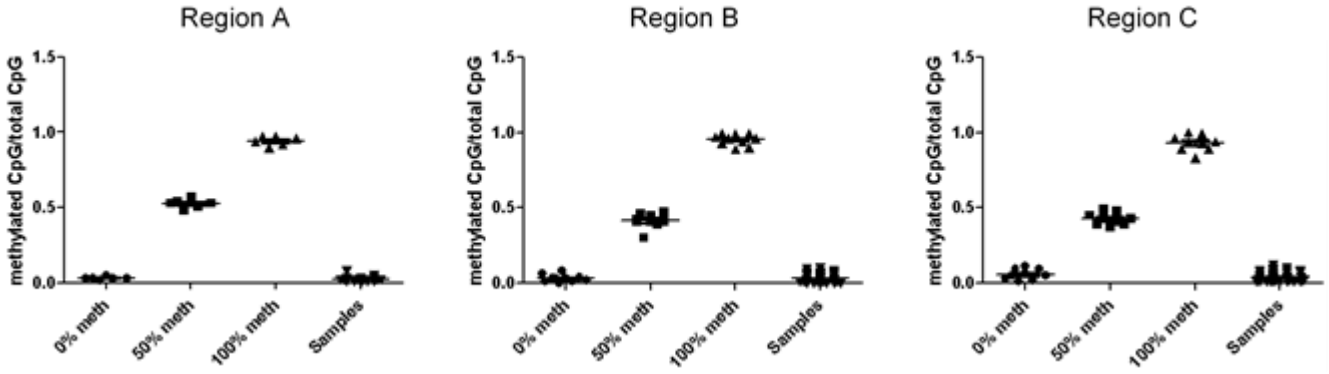


Figure S2. Methylation status of the samples compared with controls with known proportion of methylated CpG sites, assessed with MasArray Epityper at the CEGEN-USC. 3 different chromosomic regions of CpG islands were analysed: region A (chr10:89622437-89622657), region B (chr10:89621708-89622037) and region C (chr10:89622446-89622808). Each dot corresponds to a CpG site.

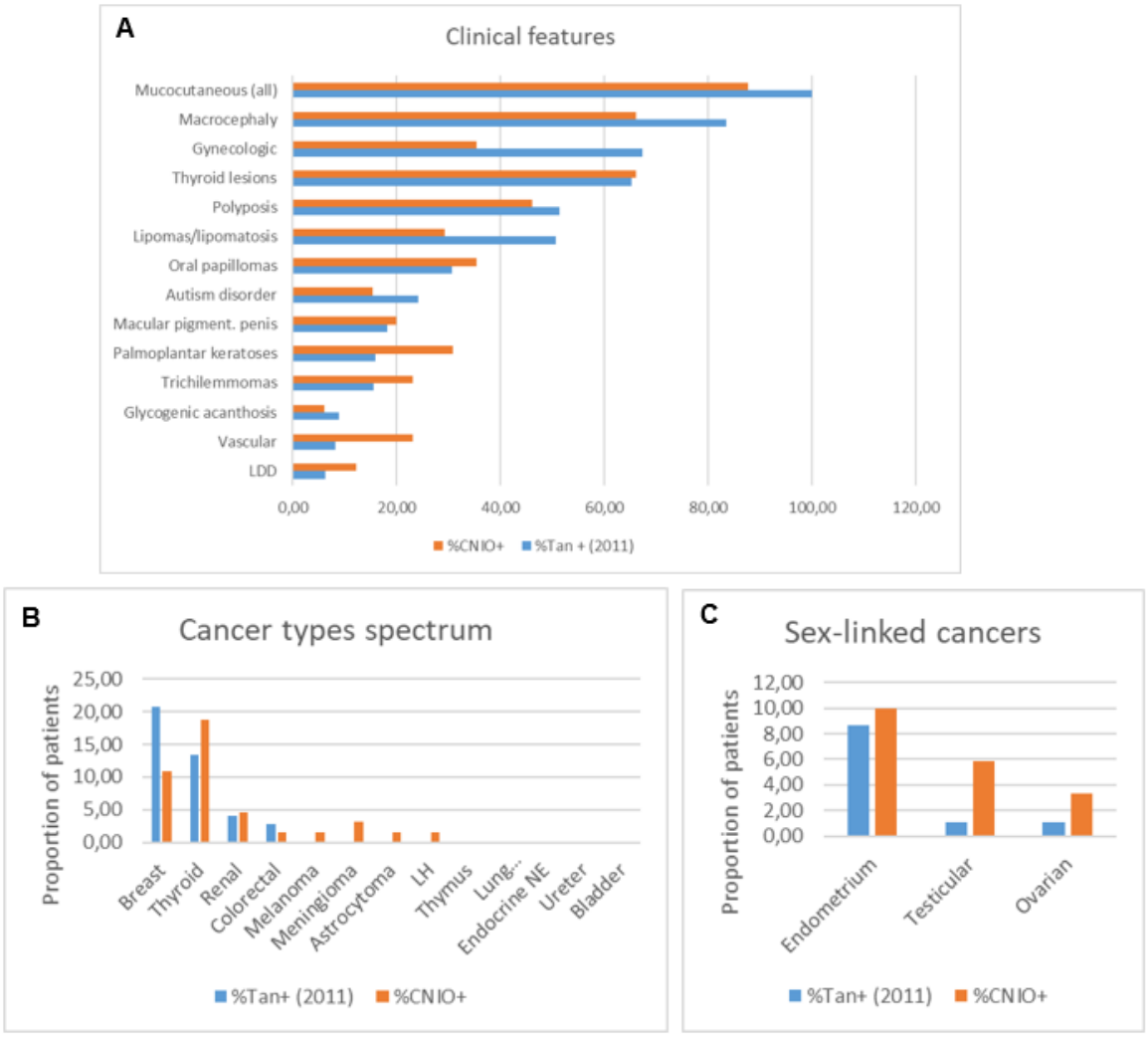


Figure S3. Comparison on the proportions of the *PTEN* mutation carriers with each clinical feature, from our work and the study of Tan *et al.* 2011. A) Non-malignant clinical manifestations. B) Cancer types not associated to one sex. C) Sex-linked cancer types.

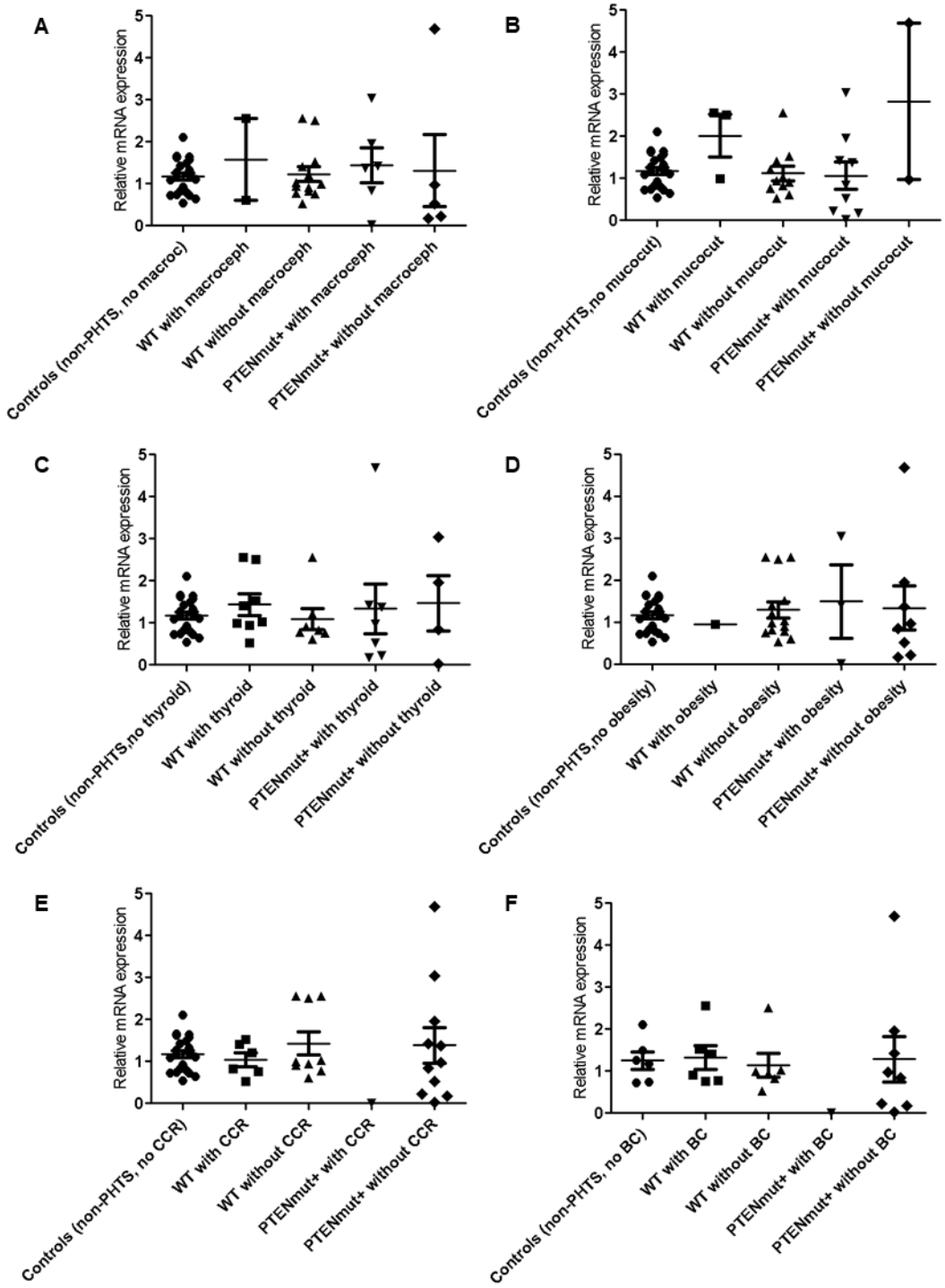


Figure S4. *PTEN* mRNA expression in controls and patients, grouped according to clinical features. Each dot corresponds to the mean value of the expression of each sample assessed in triplicate. The mean value of each group ± SEM is indicated.

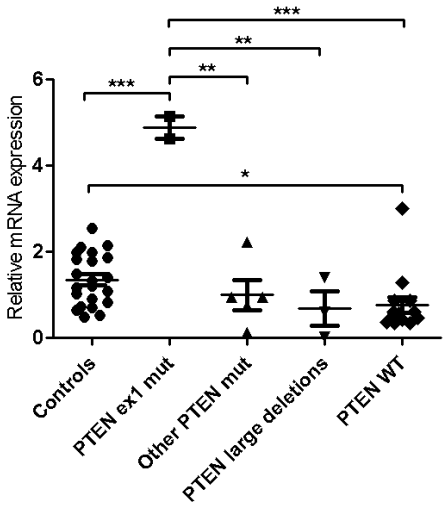
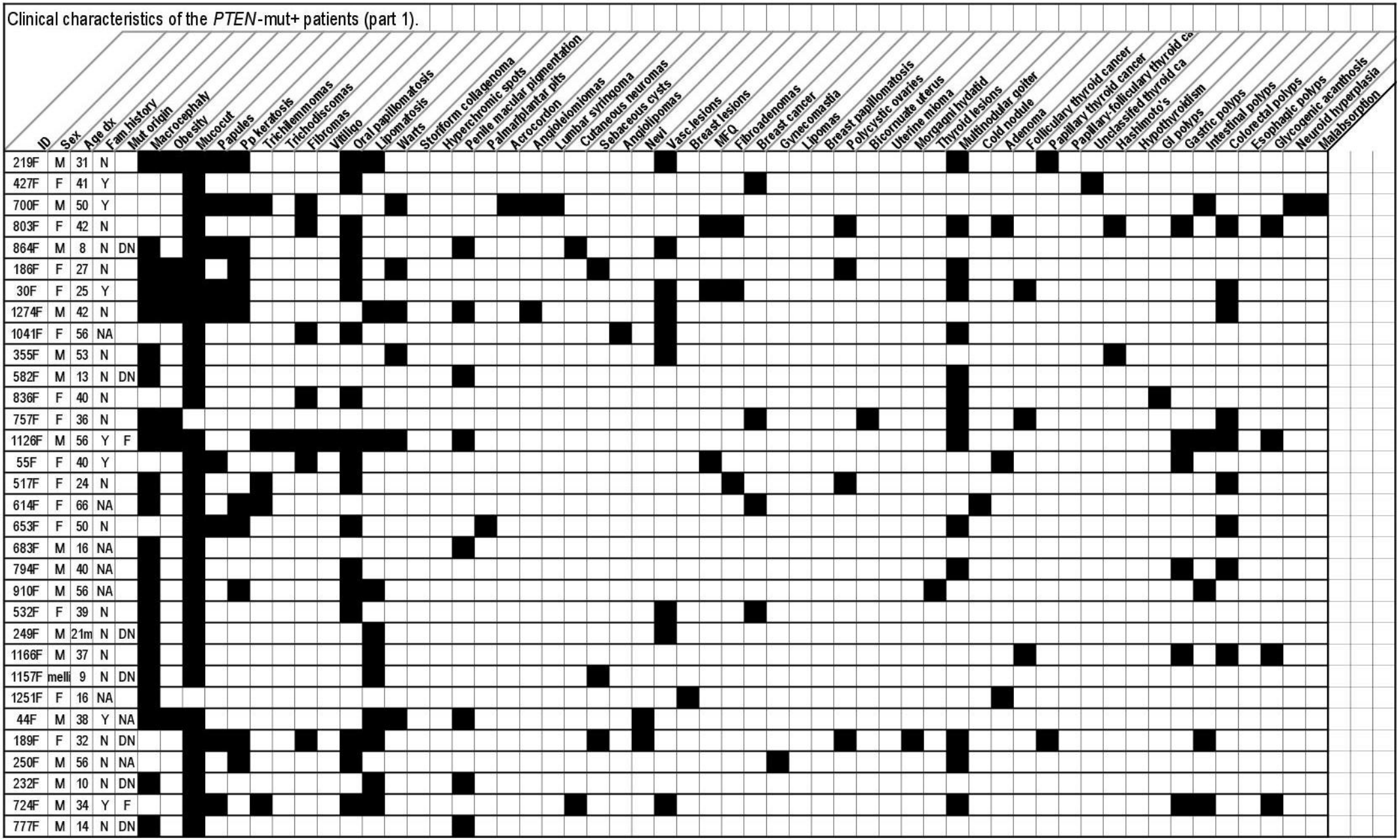
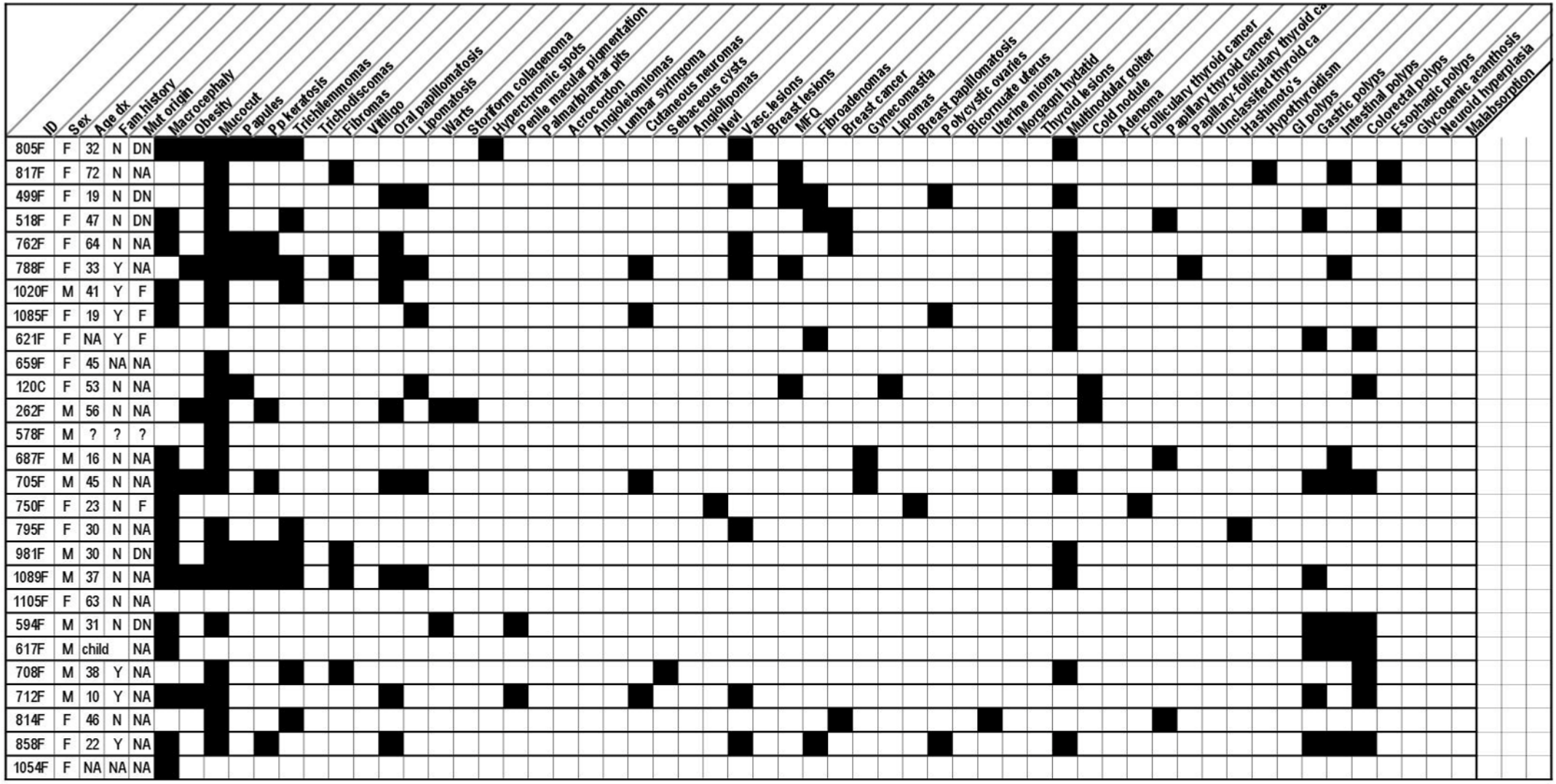
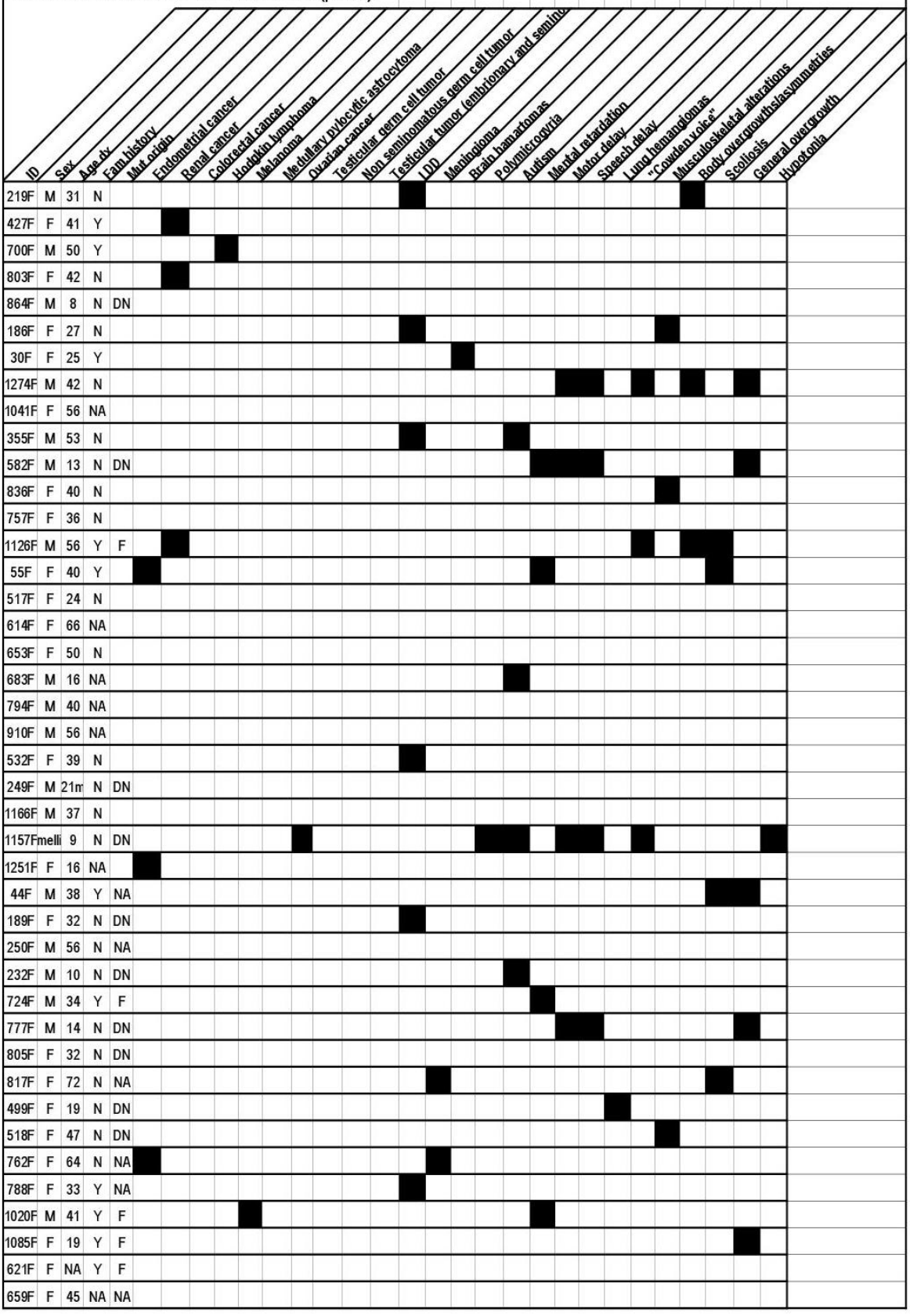


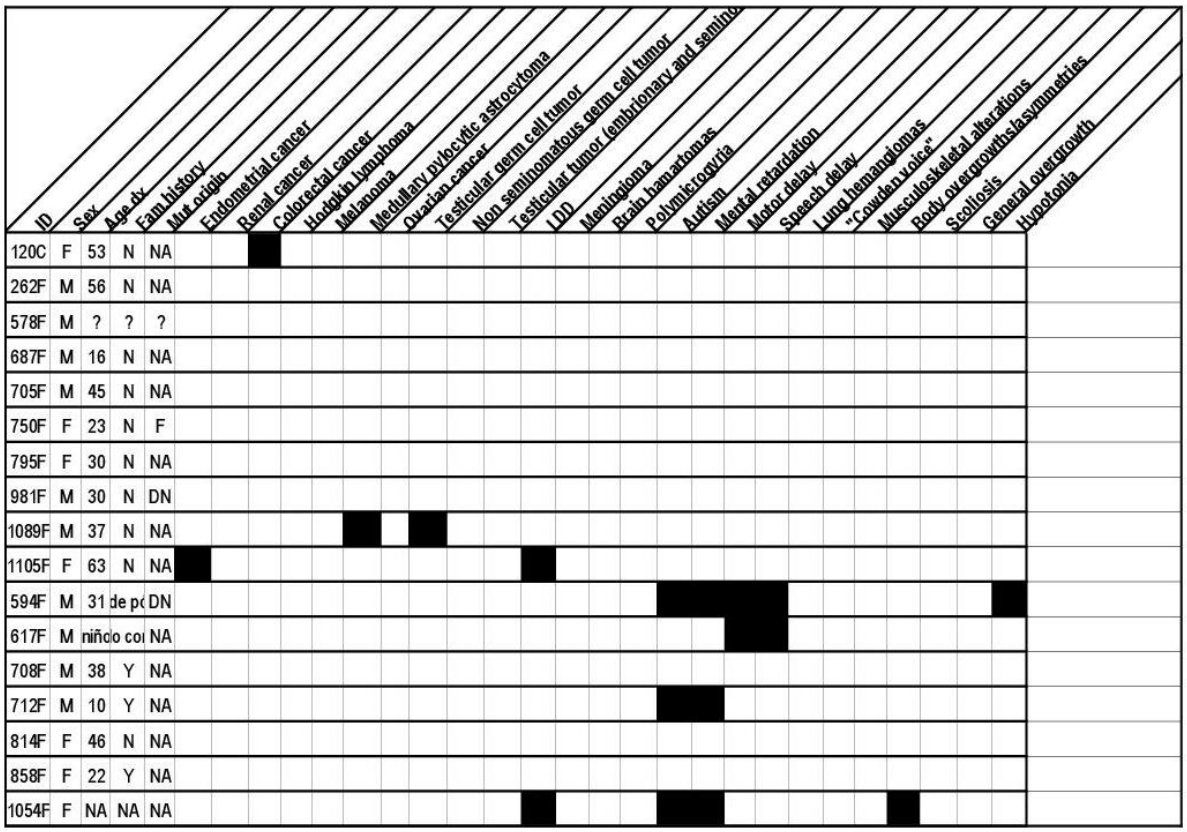
Figure S5. Relative *PTEN* mRNA expression when using primers that localize to exon 1 and exon 2 in *PTEN*. Each dot corresponds to the mean value of the expression of each sample assessed in triplicate.


Clinical characteristics of the *PTEN*-mut+ patients (part 1).
ID
Sex
Age dx
Fam history
Mut origin
Macrocephaly
Obesity
Mucocut
Papules
Pp keratosis
Trichilemmomas
Trichodiscomas
Fibromas
Vitiligo
Oral papillomatosis
Lipomatosis
Warts
Storiform collagenoma
Hyperchromic spots
Penile macular pigmentation
Palmarplantar pits
Acrocordon
Angioleiomiomas
Lumbar syringoma
Cutaneous neuromas
Sebaceous cysts
Angiolipomas
Nevi
Vasc.lesions
Breast lesions
MFQ
Fibroadenomas
Breast cancer
Gynecomastia
Lipomas
Breast papillomatosis
Polycystic ovaries
Bicornuate uterus
Uterine mioma
Morgagni hydatid
Thyroid lesions
Multinodular goiter
Cold nodule
Adenoma
Follicular thyroid cancer
Papillary thyroid cancer
Papillary-follicular thyroid ca
Unclassified thyroid ca
Hashimoto's
Hypothyroidism
GI polyps
Gastric polyps
Intestinal polyps
Colorectal polyps
Esophagic polyps
Glycogenic acanthosis
Neuroid hyperplasia
Malabsorption
219F M 31 N
427F F 41 Y
700F M 50 Y
803F F 42 N
864F M 8 N DN
186F F 27 N
30F F 25 Y
1274F M 42 N
1041F F 56 NA
355F M 53 N
582F M 13 N DN
836F F 40 N
757F F 36 N
1126F M 56 Y F
55F F 40 Y
517F F 24 N
614F F 66 NA
653F F 50 N
683F M 16 NA
794F M 40 NA
910F M 56 NA
532F F 39 N
249F M 21m N DN
1166F M 37 N
1157F melli 9 N DN
1251F F 16 NA
44F M 38 Y NA
189F F 32 N DN
250F M 56 N NA
232F M 10 N DN
724F M 34 Y F
777F M 14 N DN

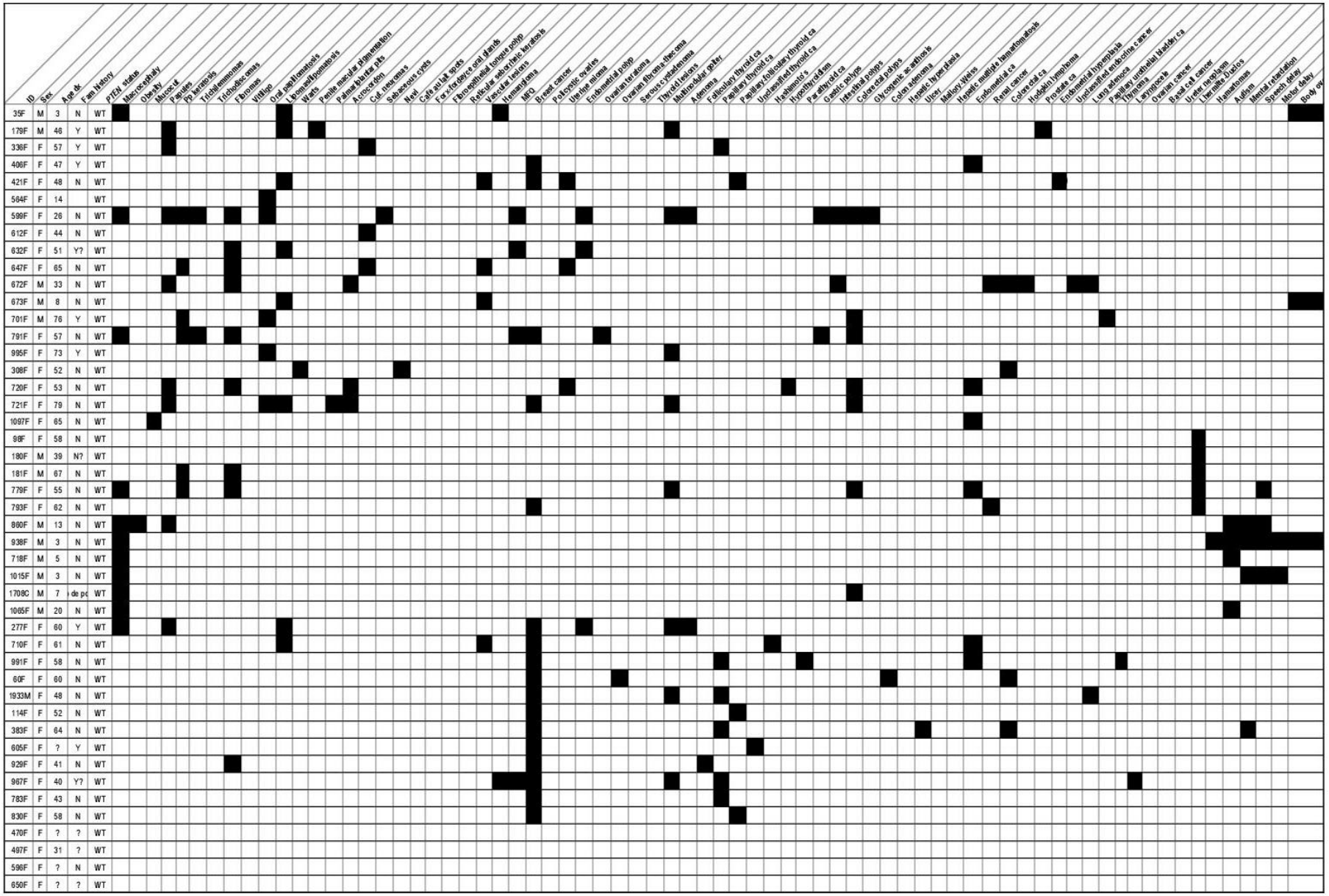

ID
Sex
Age dx
Fam.history
Mut origin
Macrocephaly
Obesity
Mucocut
Papules
Pp keratosis
Trichilemmomas
Trichodiscomas
Fibromas
Vitiligo
Oral papillomatosis
Lipomatosis
Warts
Storiform collagenoma
Hyperchromic spots
Penile macular pigmentation
Palmarplantar pits
Acrocordon
Angioleiomiomas
Lumbar syringoma
Cutaneous neuromas
Sebaceous cysts
Angiolipomas
Nevi
Vasc. lesions
Breast lesions
MFQ
Fibroadenomas
Breast cancer
Gynecomastia
Lipomas
Breast papillomatosis
Polycystic ovaries
Bicornuate uterus
Uterine mioma
Morgagni hydatid
Thyroid lesions
Multinodular goiter
Cold nodule
Adenoma
Follicularv thyroid cancer
Papillary thyroid cancer
Papillary-follicular thyroid cancer
Unclassifed thyroid ca
Hashimoto's
Hypothyroidism
GI polyps
Gastric polyps
Intestinal polyps
Colorectal polyps
Esophagic polyps
Glycogenic acanthosis
Neuroid hyperplasia
Malabsorption
805F F 32 N DN
817F F 72 N NA
499F F 19 N DN
518F F 47 N DN
762F F 64 N NA
788F F 33 Y NA
1020F M 41 Y F
1085F F 19 Y F
621F F NA Y F
659F F 45 NA NA
120C F 53 N NA
262F M 56 N NA
578F M ? ? ?
687F M 16 N NA
705F M 45 N NA
750F F 23 N F
795F F 30 N NA
981F M 30 N DN
1089F M 37 N NA
1105F F 63 N NA
594F M 31 N DN
617F M child NA
708F M 38 Y NA
712F M 10 Y NA
814F F 46 N NA
858F F 22 Y NA
1054F F NA NA NA

Clinical characteristics of the *PTEN*-mut+ (part 2).

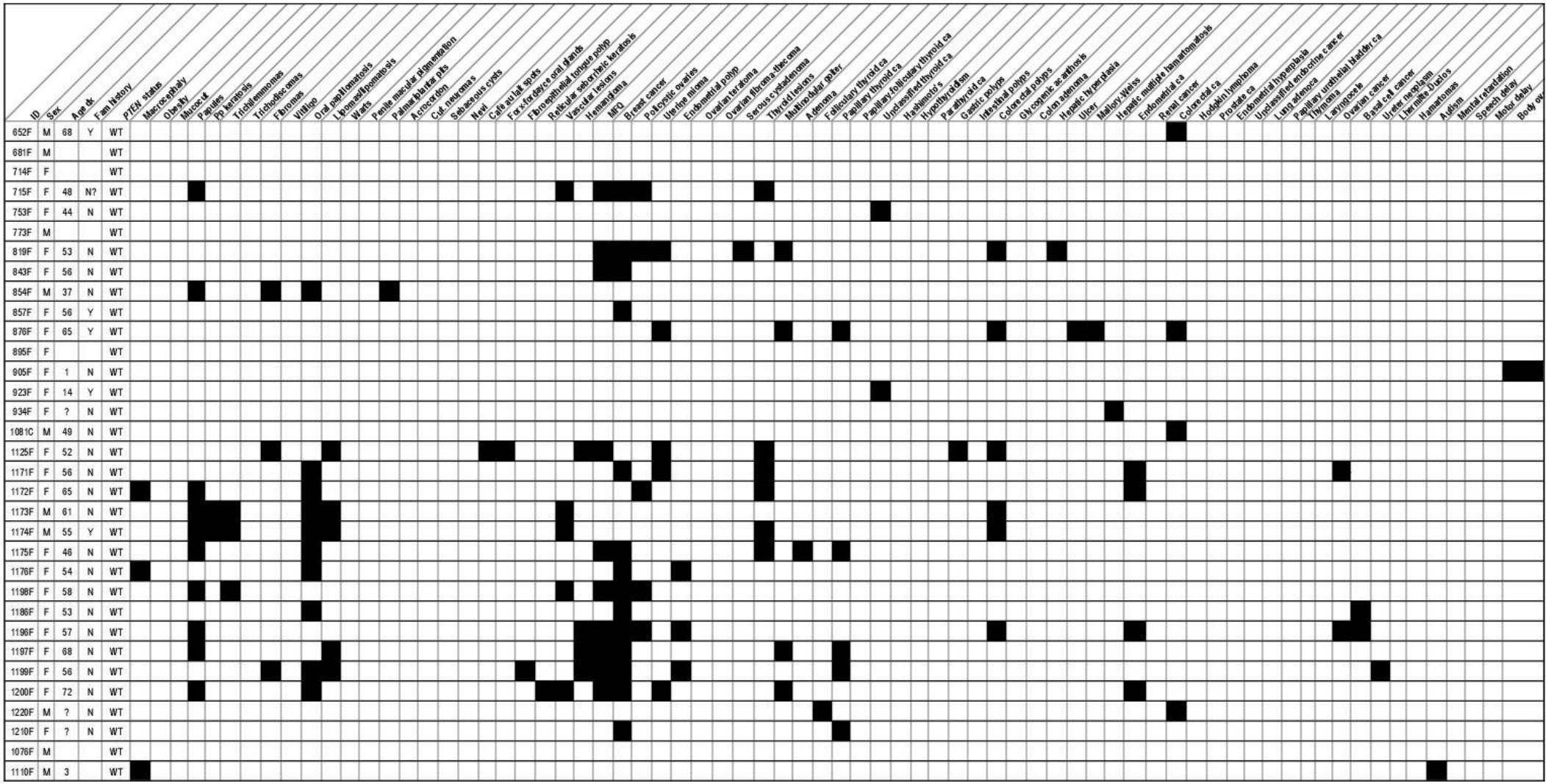



ID
Sex
Age dx
Fam history
Mut origin
Endometrial cancer
Renal cancer
Colorectal cancer
Hodgkin lymphoma
Melanoma
Medullary pylocytic astrocytoma
Ovarian cancer
Testicular germ cell tumor
Non seminomatous germ cell tumor
Testicular tumor (embrionary and semino
LDD
Meningioma
Brain hamartomas
Polymicrogyria
Autism
Mental retardation
Motor delay
Speech delay
Lung hemangiomas
"Cowden voice"
Musculoskeletal alterations
Body overgrowth
Scoliosis
General overgrowth
Hypotonia
120C F 53 N NA
262F M 56 N NA
578F M ? ? ?
687F M 16 N NA
705F M 45 N NA
750F F 23 N F
795F F 30 N NA
981F M 30 N DN
1089F M 37 N NA
1105F F 63 N NA
594F M 31 de pc DN
617F M niño co cor NA
708F M 38 Y NA
712F M 10 Y NA
814F F 46 N NA
858F F 22 Y NA
1054F F NA NA NA

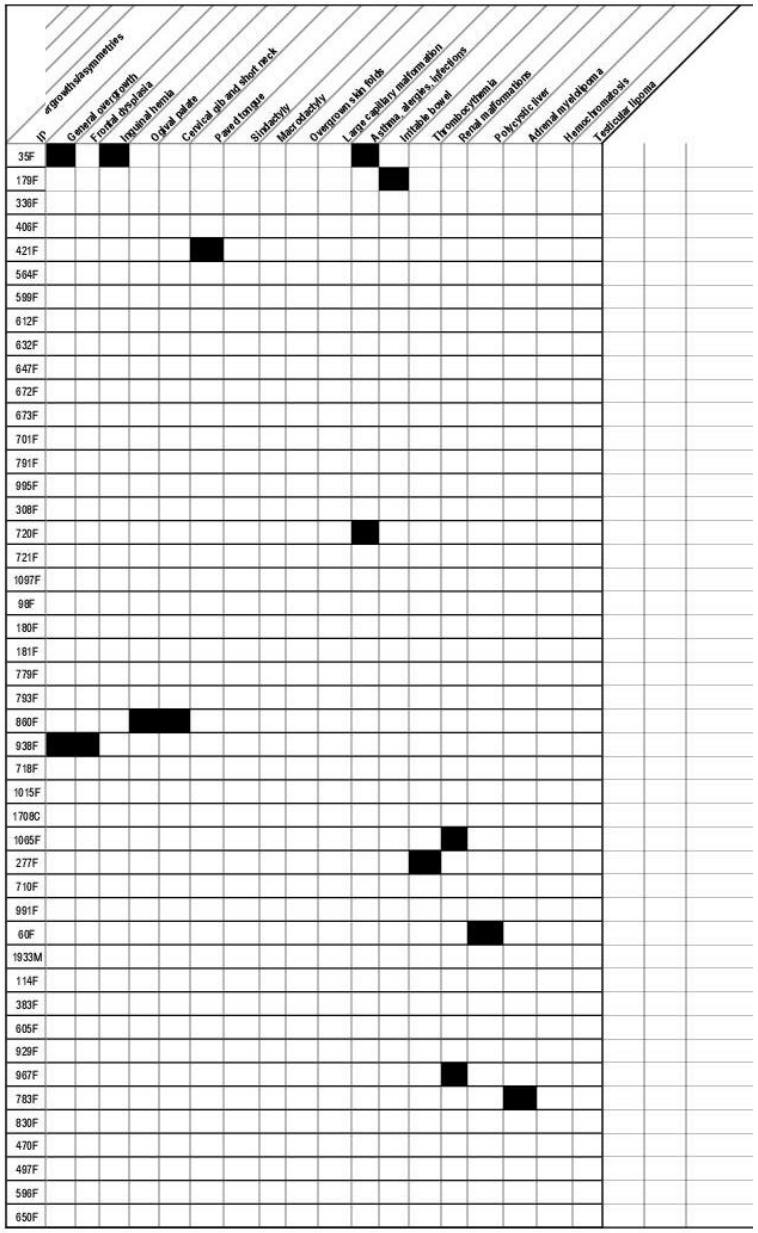
Clinical data of the *PTEN*-wt patients



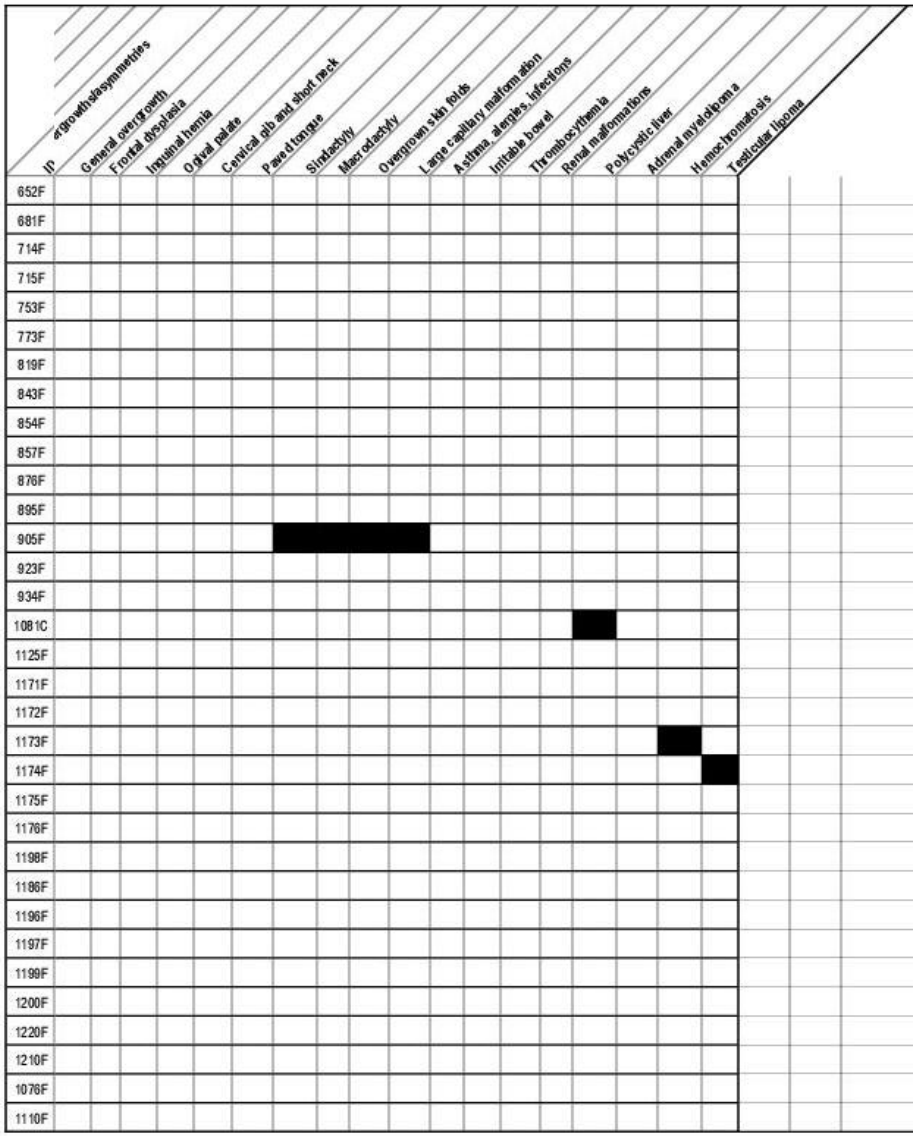
Clinical data of the *PTEN*-wt patients



Clinical data of the *PTEN*-wt patients



Clinical data of the *PTEN*-wt patients



APPENDIX I: CHECKLIST AND INFORMED CONSENT

Centro Nacional de Investigaciones Oncológicas

Checklist PHTS

Proyecto PTEN HAMARTOMA TUMOR SYNDROME

Nombre/iniciales paciente: Fecha nacimiento:
Fecha exploración:
Dr/Dra:
Centro hospitalario:

Piel y mucosas	NO	SÍ	Número, localización, fecha/edad diagnóstico,...
Pápulas faciales			
Papilomas orales			
Queratosis acral			
Tricolemomas			
Máculas pigmentarias en el pene			
Lipomas cutáneos			
Fibromas: esclerótico, oral			
Neuromas mucocutáneos			
Digestivo	NO	SÍ	Número, tipo histológico, fecha/edad diagnóstico,...
Pólipos colónicos			
Pólipos gástricos			
Acantosis glucogénica en esófago			
Otros			
Lesiones lipovasculares	NO	SÍ	Número, localización, extensión, fecha/edad diagnóstico,...
Lipomatosis			
Lesiones o malformaciones vasculares			
Otras			

Remitir a:
Laura Pena
Unidad Clínica de Cáncer Familiar
Centro Nacional de Investigaciones Oncológicas (CNIO)
C/ Melchor Fernández Almagro, 3. 28029 Madrid
Email: lpenac@cnio.es

Centro Nacional de Investigaciones Oncológicas

Checklist PHTS

Proyecto PTEN HAMARTOMA TUMOR SYNDROME

Alteraciones neurológicas	NO	SÍ	Número, localización, fecha/edad diagnóstico,...	
Lhermitte-Duclos				
Retraso mental				
Retraso psicomotor				
Autismo				
Epilepsia				
Otras				
Genitourinario	NO	SÍ	Número, localización, fecha/edad diagnóstico,...	
Quistes ováricos				
Fibromas o miomas uterinos				
Lipomas testiculares				
Otros				
Tiroides	NO	SÍ	Número, localización, fecha/edad diagnóstico,...	
Nódulos				
Bocio multinodular				
Adenomas				
Tiroiditis de Hashimoto				
Otras				
Mamas	NO	SÍ	Número, localización, fecha/edad diagnóstico,...	
Mamas fibroquísticas				
Otros				
Otras	NO	SÍ		
Macrocefalia			PC nacimiento:	PC actual:
Sobrecrecimiento			Talla nacimiento: Peso nacimiento:	Talla actual: Peso actual:
Otras				

Remitir a:
Laura Pena
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Email: lpenac@cnio.es

Centro Nacional de Investigaciones Oncológicas

Checklist PHTS

Proyecto PTEN HAMARTOMA TUMOR SYNDROME

CÁNCER			
Cáncer de mama	NO	SÍ	Características histológicas, estado receptores hormonales, Her2, fechas/edad diagnóstico,...
Cáncer de endometrio	NO	SÍ	Fechas/edad diagnóstico, características histológicas,...
Cáncer de ovario	NO	SÍ	Fechas/edad diagnóstico, características histológicas,...
Cáncer de tiroides	NO	SÍ	Fechas/edad diagnóstico, características histológicas,...
Cáncer colorrectal	NO	SÍ	Fechas/edad diagnóstico, características histológicas,...
Cáncer renal	NO	SÍ	Fechas/edad diagnóstico, características histológicas,...
Melanoma	NO	SÍ	Fechas/edad diagnóstico, características histológicas,...
Otros			Fechas/edad diagnóstico, características histológicas,...

Remitir a:
Laura Pena
Unidad Clínica de Cáncer Familiar
Centro Nacional de Investigaciones Oncológicas (CNIO)
C/ Melchor Fernández Almagro, 3. 28029 Madrid
Email: lpenac@cnio.es

Centro Nacional de Investigaciones Oncológicas

Hoja de información y consentimiento informado.

Introducción

Le solicitamos su participación en un estudio destinado a la **caracterización clínica y búsqueda de nuevos genes implicados en el síndrome de PTEN-tumores hamartomatosos (PHTS).**

El estudio se está llevando a cabo en la Unidad Clínica de Cáncer Familiar del Centro Nacional de Investigaciones Oncológicas en Madrid. Su participación en el estudio es voluntaria, puede rechazar participar en él y puede retirarse del estudio en cualquier momento sin que esto afecte su atención médica posterior.

Cuando ya no se prevea la utilización de la muestra en los correspondientes estudios esta será eliminada, anonimizada o cedida con sus datos a una colección o a un biobanco según lo que Ud. nos indique en la hoja de consentimiento informado.

A continuación describiremos los datos y las pruebas que son necesarias y para las cuales solicitamos su colaboración:

1. Se le pedirá actualizar la información clínica sobre sus antecedentes personales y familiares mediante una entrevista telefónica o personal.
2. Se le pedirá una muestra de sangre.
3. En caso de haber sido diagnosticada de cáncer es posible que se le pida su aprobación para obtener tejido parafinado del tumor y acceso a su historial clínico.

Extracción de muestras

La recogida de muestras para el estudio aprovechará las intervenciones médicas que se le realicen en relación al diagnóstico y tratamiento de su enfermedad para evitarle molestias y riesgos. En caso de que esto no sea posible, la extracción de sangre se llevará a cabo mediante la venopunción (extracción de sangre por aguja), cuyos riesgos son mínimos y se incluyen en el Anexo 1.

En caso de que se produzca un efecto adverso derivado de su participación en el estudio, usted debe de ponerlo en conocimiento del Investigador Principal a través de los datos de contacto que se facilitan en la parte final del documento, debiendo acudir, además, a su médico lo antes posible.

Uso de muestras y datos

Todas estas muestras serán utilizadas exclusivamente con fines científicos relacionados con los objetivos del estudio.

El material biológico que nos cede formará parte del bio-depósito de este centro y será registrado hasta su uso en el proyecto de investigación. Una vez finalizado el estudio, la muestra será almacenada en una colección (C.0001446) registrada en el Instituto de Salud Carlos III, de la que es titular el Dr. Miguel Urioste Azcorra, Jefe de la Unidad Clínica de Cáncer Familiar del CNIO; su cesión a investigadores de otros centros participantes estará condicionada a la aprobación del Comité Científico del estudio.

El material se conservará durante un máximo de 50 años para realizar, posiblemente, posteriores determinaciones como parte de este proyecto o de futuros estudios relacionados. En este último caso su muestra solo será utilizada si el proyecto es autorizado por un Comité de Ética de la Investigación (CEI).

Confidencialidad

Los datos personales que se recojan sobre usted, serán confidenciales y procesados de acuerdo con la Ley Orgánica 15/1999 de Protección de Datos de Carácter Personal, así como de acuerdo a la legislación sanitaria y a la relativa a la investigación biomédica vigente 14/2007, tratándose los mismos únicamente de acuerdo con los objetivos descritos en el presente proyecto de investigación u otros relacionados sobre el síndrome de PHTS. Asimismo, se informa de que los resultados obtenidos de los diferentes estudios llevados a cabo con las muestras, pueden ser publicados en revistas científicas, sin embargo, nunca será facilitada su identidad o datos que puedan llegar a identificarle.

En el momento que usted consienta el uso de la muestra para los fines de investigación aplicada descritos, dicha muestra será sometida a un proceso de disociación. Es decir, sólo será identificado por un código, constando todos sus datos debidamente codificados, sin embargo, y dado que se trata de un análisis cuyo resultado podría redundar en beneficio del paciente y/o de sus familiares, únicamente el investigador principal tendrá acceso a la información necesaria para asociar el código con sus datos personales.

De igual modo, sus datos personales serán incorporados a un fichero automatizado de carácter confidencial, cuya titularidad corresponde al CNIO, debidamente inscrito en la Agencia Española de Protección de Datos con número 2042540353, conforme a los términos establecidos en la Ley Orgánica 15/1999, pudiendo ejercer en cualquier momento, los derechos de acceso, rectificación, cancelación u oposición, reconocidos por la citada normativa en materia de protección de datos de carácter personal, poniéndose para ello en contacto con el Responsable del Estudio en su Hospital. En caso de revocación, su muestra dejará de formar parte de la investigación y se destruirá aunque los datos obtenidos hasta ese momento sí formarán parte de la misma.

La información obtenida en el estudio será confidencial, de acuerdo con lo que establece la Ley tratándose únicamente de acuerdo con los objetivos descritos en el presente documento. Nadie, excepto los miembros del estudio, LAS AUTORIDADES SANITARIAS, ASÍ COMO COMITÉS DE ÉTICA DE INVESTIGACIÓN CLÍNICA, tendrá acceso a su información.

Consejo Genético

Los datos que se obtengan de los análisis de las muestras serán archivados, y formarán parte del proyecto de investigación, manteniéndose durante un máximo de 50 años para realizar, posiblemente, posteriores análisis. Los métodos utilizados en investigación biomédica suelen ser diferentes a los aprobados para la práctica clínica, por lo que puede no tener valor clínico para usted.

No obstante, le corresponde a usted decidir si quiere recibir o no dicha información, tanto en relación con su persona como con su familia, para lo que deberá cumplimentar el apartado correspondiente de la hoja de consentimiento.

Si se adquiere un compromiso de facilitarle esta información, y de conformidad con la normativa vigente, una vez obtenidos y evaluados los resultados de los análisis realizados y en caso de que éstos tengan un valor clínico comprobado, nos comprometemos a suministrarle consejo genético, es decir, informarle sobre las posibles consecuencias tanto para usted como para sus descendientes de los resultados de los referidos resultados, así como de sus ventajas y riesgos.

Seguro

De acuerdo con lo dispuesto en el Art. 18 de la Ley 14/2007 de Investigación Biomédica, se ha suscrito una póliza de seguro para cubrir los daños que pudiera ocasionársele por la extracción de la muestra de sangre descrita anteriormente para poder llevar a cabo este proyecto de investigación (Póliza nº 0971570018377 del 15/03/15 de MAPFRE)

Beneficios potenciales, riesgos y compensación

La donación tiene, por disposición legal, carácter altruista, por lo que usted no obtendrá ni ahora ni en el futuro ningún beneficio económico por la misma. No está previsto compensarle por los productos desarrollados a partir de esta investigación. Sin embargo, su participación, junto con la de otras muchas personas, es muy importante para ayudar a entender mejor cómo se desarrolla y evoluciona el PHTS y para que, con ello, se beneficien otros pacientes futuros. Los resultados de la investigación corresponderán a los investigadores participantes en el proyecto.

Asimismo, y para poder completar el presente proyecto de investigación, cabe la posibilidad de que sea necesario requerir nuevamente su participación para realizarle una nueva extracción de sangre u obtener otra muestra biológica, siempre que la viabilidad de la presente investigación científica pueda requerirlo.

A continuación se adjunta:
- **Hoja de consentimiento**
- **Anexo 1: Riesgos de la venopunción**
- **Anexo 2: Resumen del proyecto**

Consentimiento Informado

	Sí	No
1. Confirmo que he leído y entendido la hoja de información, y que he tenido la oportunidad de hacer todas las preguntas que creía necesarias.	☐	☐
2. Estoy de acuerdo en participar en las siguientes partes del estudio:		
Entrevista personal	☐	☐
Recogida de sangre	☐	☐
Recogida de tejido parafinado	☐	☐
Acceso al historial clínico	☐	☐
Contacto personal en el futuro	☐	☐
Contacto familiar en el futuro	☐	☐
3. En el caso de producirse resultados que pudieran ser clínicamente relevantes, **relacionados con la susceptiblidad a padecer cáncer**, para mí o mi familia deseo que se me comuniquen estos datos.	☐	☐
4. En el caso de producirse resultados que pudieran ser clínicamente relevantes, **no relacionados con la susceptiblidad a padecer cáncer**, para mí o mi familia deseo que se me comuniquen estos datos.	☐	☐
5. Una vez finalizado el proyecto, doy permiso a que el sobrante de mis muestras biológicas se almacenen para su uso en otros estudios no relacionados*:	☐	☐
- Tras su anonimización (proceso por el cual no es posible establecer relación entre un dato o muestra y el sujeto al que se refiere)	☐	☐
- De forma no anónima (aunque con procedimientos de disociación para preservar la confidencialidad de los datos de carácter personal).	☐	☐

* La no aceptación de este punto implica que la muestra será destruida una vez finalizado el proyecto.

Otorgo mi consentimiento de manera voluntaria y entiendo que soy libre de retirarme en cualquier momento, sin dar explicaciones, sin consecuencias legales y sin que tenga ningún efecto sobre mi tratamiento médico futuro.

Nombre y Apellidos del participante	Fecha	Firma
Nombre del representante legal	Fecha	Firma
Nombre del médico/investigador	Fecha	Firma

Le agradecemos su cooperación en este proyecto de investigación. Si tiene alguna pregunta sobre el estudio, puede llamar al investigador principal del estudio, el Dr. Miguel Urioste Azcorra, jefe de la Unidad Clínica de Cáncer Familiar del CNIO, Madrid, Tfno: 912246900, ext 3315. Este protocolo ha sido revisado y aprobado por el Comité Ético del Instituto de Salud Carlos III.

ANEXO I
Riesgos de la venopunción

Riesgos generales de la venopunción:

· Sangrado excesivo (ej: casos de uso de medicamentos anticoagulantes como Simtron)
· Desmayo o sensación de mareo
· Hematoma (acumulación de sangre debajo de la piel)
· Infección (un riesgo leve en cualquier momento que se presente ruptura de la piel)
· Punciones múltiples para localizar las venas

De estos son habituales o frecuentes el hematoma y las punciones múltiples. El tamaño de las venas y las arterias varía de un paciente a otro y de una parte del cuerpo a otra, por tal razón obtener muestras de sangre en algunas personas puede ser más difícil que en otras.

Consideraciones especiales

· Puede existir una mayor dificultar de acceso venoso en caso de afectación cutánea en la región del antebrazo.
· Existe un mayor riesgo de sangrado excesivo por déficit de plaquetas y factores de la coagulación.
· Existe un mayor riesgo de trombosis e inflamación venosa en algunas personas.

En el caso de que se materialice algún efecto adverso de los citados anteriormente, durante su participación en este Proyecto, debe ponerlo en nuestro conocimiento, debiendo acudir, además, a su médico lo antes posible.

ANEXO II
Resumen del proyecto

El término PTEN-tumores hamartomatosos (PHTS) engloba un conjunto de síndromes caracterizados por una gran variabilidad clínica y heterogeneidad genética, que condicionan una demora en el diagnóstico y en el establecimiento de las medidas de prevención y detección precoz, circunstancias especialmente inadmisibles en pacientes con un riesgo de cáncer del 85%. Tanto el abanico de signos clínicos, como los criterios diagnósticos, así como los riesgos de cáncer y las bases genéticas del síndrome, distan mucho de ser capítulos cerrados y están en permanente revisión.

Hemos recogido información y muestras de 120 probandos remitidos a nuestro laboratorio desde todas las CCAA para estudio del gen PTEN por sospecha de PHTS. En el 36,6% de los casos se identificó una mutación patogénica en PTEN.

Buscamos definir las características clínicas y conocer las bases genéticas del síndrome en esta importante serie de pacientes españoles, mediante:

1.- Recogida sistemática de la información clínica y la historia familiar.
2.- Investigación en profundidad del papel del gen PTEN.
3.- Análisis de otros genes previamente implicados en el desarrollo del síndrome o en la modificación del fenotipo.
4.- Búsqueda de nuevos genes mediante el análisis del exomas en casos seleccionados que se ajusten a los criterios clínicos más exigentes.
5.- Búsqueda de correlaciones genotipo-fenotipo y definición de criterios diagnósticos en nuestra población.

Es un objetivo complementario del proyecto la promoción de una red que favorezca la atención e investigación del síndrome en la que participen tanto pacientes como profesionales o población general con interés en esta entidad.

APPENDIX II: Publications

PUBLICATIONS

First-authored publications:

Pena-Couso, L., Perea J., Melo, S., Mercadillo, F., Figueiredo, J., Sanches, J. M., Sánchez-Ruiz, A., Robles, L., Seruca, R., Urioste, M. 'Clinical and functional characterization of the *CDH1* germline variant c.1679C>G in three unrelated families with hereditary diffuse gastric cancer.' (2018). *European Journal of Human Genetics*. vol. 26, no. 9, pp. 1348-1353.

Pena-Couso, L., Ercibengoa, M., Hernández, A., Gutiérrez-Abad, D., Mercadillo, F., Carbonell, P., PHTS Working Group, Perea, J., Urioste, M. 'Phenotype and genotype findings in a series of Spanish patients with PTEN hamartoma tumor syndrome.' (Manuscript in preparation).

Co-authored publications:

Tapial, S., Olmedillas-López, S., Rueda, D., Arriba, M., García, J. L., Vivas, A., Pérez, J., Pena-Couso, L., Olivera, R., Rodríguez, Y., García-Arranz, M., García-Olmo, D., González-Sarmiento, R., Urioste, M., Goel, A., Perea, J. 'Cimp-Positive Status is More Representative in Multiple Colorectal Cancers than in Unique Primary Colorectal Cancers.' (2019). *Scientific Reports*. vol. 9, no. 1, pp. 10516.

Brandariz, L., Arriba, M., García, J. L., Cano, J. M., Rueda, D., Rubio, E., Rodríguez, Y., Pérez, J., Vivas, A., Sánchez, C., Tapial, S., Pena, L., García-Arranz, M., García-Olmo, D., Urioste, M., González-Sarmiento, R., Perea, J. 'Differential clinicopathological and molecular features within late-onset colorectal cancer according to tumor location.' (2018). *Oncotarget*. vol. 9, no. 20, pp. 15302-15311.

Alonso, N., Cañueto, J., Ciria, S., Bueno, E., Palacios-Alvarez, I., Alegre, M., Badenas, C., Barreiro, A., Pena, L., Maldonado, C., Nespeira-Jato, M. V., Peña-Penabad, C., Azon, A., Gavrilova, M., Ferrer, I., Sanmartin, O., Robles, L., Hernandez-Martin, A., Urioste, M., Puig, S., Puig, L., Gonzalez-Sarmiento, R. 'Novel clinical and molecular findings in Spanish patients with naevoid basal cell carcinoma syndrome.' (2018). *British Journal of Dermatology*. vol. 178, no. 1, pp. 198-206.