When, where and which *PIK3CA* mutations are pathogenic in congenital disorders

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Abstract

PIK3CA encodes for the class I PI3Kα isoform and is frequently mutated in cancer. Activating mutations in *PIK3CA* also cause a range of congenital disorders featuring asymmetric tissue overgrowth, known as the *PIK3CA*-related overgrowth spectrum (PROS), with the vasculature frequently involved. In PROS, *PIK3CA* mutations arise postzygotically during embryonic development leading to a mosaic distribution resulting in a variety of phenotypic features. A clear skewed pattern of overgrowth favouring some mesoderm and ectoderm-derived tissues is observed but is not understood. Here, we summarize current knowledge on the determinants of *PIK3CA*-related pathogenesis in PROS, including intrinsic factors such as cell lineage susceptibility and *PIK3CA* variant bias and extrinsic factors which refers to the environmental modifiers. Gaining biological understanding of *PIK3CA* mutations in PROS will contribute to unravel the onset and progression of these conditions, and ultimately impact on their treatment. Given that *PIK3CA* mutations are similar in PROS and cancer, deeper insight into one will also inform about the other.

PIK3CA encodes for p110α, one of the four class I phosphatidylinositol 3-kinase (PI3K) catalytic subunits. p110α is an obligate heterodimer with a p85-type regulatory subunit, with no evidence of the existence of p85-free p110α[1–4]. For simplicity, we will use below PI3K when referring to the p110s isoforms. PI3Kα is ubiquitously expressed and is activated by tyrosine kinases. *PIK3CA* is the most frequently mutated oncogene across all human cancers with the high prevalence in breast and endometrial cancers[5,6]. Activating mutations in *PIK3CA* span almost the entire *PIK3CA* gene product, with most frequently mutated hotspots found in the helical (E542K and E545K) and kinase (H1047R) domains. *PIK3CA* mutations are acquired in a somatic fashion and are largely present in heterozygosity. Nevertheless, there is evidence of the presence of double *PIK3CA* mutations in cis which further increase its PI3K activity[7].

Our Review stems from the remarkable discovery of oncogenic mutations in *PIK3CA* being causative of sporadic mosaic congenital disorders characterised by tissue overgrowth, with the vascular compartment as the most frequently affected. These conditions have become widely known as the *PIK3CA*-related overgrowth spectrum (PROS) and they can range from isolated (e.g. skin-related lesions, vascular malformations, brain or muscle overgrowth) to complex and syndromic phenotypes, where several tissues are affected (Fig. 1). Enigmatically, a clear biased pattern of disease manifestation, favouring some mesoderm-derived tissues, is observed but is not understood[8]. PROS are considered monogenic diseases; albeit emerging evidence indicate that co-occurrence of several genetic events, at least in the vasculature, is more frequent than previously anticipated[9–12]. This Review focuses on the pathogenic effects of somatic activating *PIK3CA* mutations when are acquired at different developmental stages. We will discuss how the interplay between genetics, cell identity and the environment explain the onset, progression, and severity of these disorders. Also, we will provide an overview about the impact of distinct *PIK3CA* variants in these congenital conditions. Finally, we include a dedicated section on vascular malformations given that the vascular compartment appears most affected in PROS. For congenital disorders caused by other PI3K signaling components we refer the reader to Box 1. Of note, mirroring the similarities between RAS and PI3K congenital manifestations, the term PIK3Copathies has been proposed when referring to all PI3K-related conditions[13].

Class I PI3Ks

PI3Ks are a large family of lipid kinases that catalyse the phosphorylation of the 3-hydroxyl group of the inositol ring of different phosphatidylinositol (PtdIns) lipid substrates present at the cellular membranes. In vertebrates, PI3Ks are divided into three classes (class I, class II, and class III) based on their structure, substrate preference, distribution, mechanism of activation and function (Box 2 includes extended information on class II and class III)[3,14,15].

Basic concepts on class I isoforms. Class I PI3Ks are heterodimers composed of a catalytic and a regulatory subunit[4]. The p110 (here refer to as PI3K) subunit confers the lipid kinase activity while the regulatory subunit modulates the activity, stability, and subcellular localization of the complex. Class I PI3Ks are subdivided into class IA and class IB depending on their ability to bind to different regulatory subunits[14–16]. Class IA catalytic subunits PI3Kα, PI3Kβ, and PI3Kδ (encoded by ***PIK3CA***, *PIK3CB* and *PIK3CD* respectively) interact with one of five p85-type regulatory subunits p85α (or its splice variants p55α and p50α), p85β, and p55γ (encoded by *PIK3R1*, *PIK3R2* and *PIK3R3* respectively). PI3Kα and PI3Kβ evenly interact with p85α and p85β. Instead, PI3Kδ preferentially binds to p85α[17]. p85 stabilizes but inhibits PI3K kinase activity in the basal state. Upon stimulation, p85 allows PI3K activation by promoting their recruitment to pTyr residues in receptor tyrosine kinases (RTK) and adaptor molecules[1]. Both, p85-mediated inhibition and recruitment to pTyr residues occur via the same Src homology 2 (SH2) domains (in p85)[1,15]. Of note, the (basal) inhibition of PI3Kα involves the nSH2 and iSH2 domains of p85, whereas PI3Kβ and PI3Kδ also require inhibition by the sSH2 domain[18–20]. This may explain why mutations in PI3Kα easier result in PI3K activation (loss of p85-dependent inhibitory effect) compared to other catalytic subunits[16,21]. Class IB is solely composed by the PI3Kγ catalytic subunit (encoded by *PIK3CG* gene) which may interact with one of two regulatory proteins, p101 or p84/p87 (encoded by *PIK3R5* and *PIK3R6* genes respectively)[22]. Class I PI3Ks catalytic subunits show specific expression patterns, being PI3Kα and PI3Kβ ubiquitously expressed and PI3Kδ and PI3Kγ enriched in some cell lineages such immune cells, neurons, and heart[3,14,15].

All class I PI3K are activated by extracellular signals at the plasma membrane. PI3Kα and PI3Kδ are recruited to the plasma membrane via binding of the SH2 domains of p85 to tyrosine-phosphorylated proteins. Instead, the PI3Kγ heterodimer is activated by the Gβγ subunits released by activated G protein-coupled receptor (GPCRs)[22–24]. PI3Kβ is unique in that multiple active membrane receptors, including both RTKs and GPCRs, may potentially recruit it and activate it[25–27]. This has led to the interpretation that full activation of this isoform

likely involves cooperation of several inputs, albeit further evidence is required to fully demonstrate this. All class I PI3K catalytic isoforms contain a RAS-binding domain (RBD) which allows them to interact with membrane-bound small GTPases and provide an extra input of activation. This includes RAS for PI3Kα, PI3Kδ and PI3Kγ[28–30] or RAC1 and CDC42 for PI3Kβ[30].

Canonical class I PI3K signalling. Activated class I PI3Ks phosphorylate phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) at the plasma membrane and generate the second messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3; also known as PIP_3)[31]. A transient rise in PIP_3 levels engages a signalling cascade that is required for the regulation of the broad range of cellular functions including growth, proliferation, metabolism, migration, and survival[3,14]. How and when PIP_3 favours one or another cellular function is not well understood but it quite likely involves (1) the activation of PI3K by different extracellular inputs, (2) the intensity and duration of PI3K activation, (3) the specific localization and amount of PIP_3 produced, and (4) the rate of phosphate hydrolysis; all ultimately leading to the activation of distinct downstream effectors[32]. PIP_3 serves as ligand and functional regulator of a group of proteins which contain a pleckstrin homology (PH) domain such as phosphoinositide-dependent kinase 1 (PDK1) and the serine-threonine kinase AKT (also known as protein kinase B (PKB))[2,33,34]. Other PI3K effectors with PH domains are tyrosine kinases (e.g., BTK in B-lympocytes), several GEFs and GAPs that regulates small-GTPases of the RAC, RAS, RHO and ARF families (e.g., GRP1, ARAP3) and protein adaptors (e.g., GAB1, GAB2, TAPP1 or DAPP)[35]. Their recruitment and activation are isoform-selective and cell-type dependent comparing to a more universal activation of AKT. Phosphatase and tensin homolog (PTEN) and Src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1 and 2 (SHIP1 and SHIP2) counterbalance the transient increase in PIP_3. PTEN converts PIP_3 back to PI(4,5)P_2 while SHIP dephosphorylates PIP_3 into PI(3,4)P_2, which is then further dephosphorylated by the INPP4B phosphatase[36,37].

AKT is the most widely studied effector of PI3K and comprises three isoforms (AKT1, AKT2 and AKT3) which have different patterns of expression and localization[38,39]. Upon binding to PIP_3, AKT is recruited to the plasma membrane through its PH domain. There, AKT is phosphorylated on Thr308 by PDK1, which is also recruited to the membrane through its PH domain. Nevertheless, full activation of AKT requires an additional phosphorylation on Ser473 by mammalian target of rapamycin complex 2 (mTORC2). Activated AKT can

exert its function in the cytoplasm and nucleus, where it phosphorylates and consequently activates or inhibits different downstream substrates. More than 100 non-redundant substrates of AKT have been identified[39]. Among them, we highlight tuberous sclerosis complex 2 (TSC2) and forkhead box protein O1 (FOXO1) for their importance in cancer biology. Activated AKT phosphorylates, and in turn inhibits both TSC2 and FOXO1. Upon phosphorylation, TSC2 loses its ability to inhibit mTOR complex 1 (mTORC1). Of relevance, activation of mTORC1 occurs at multiple levels, thus, it is incorrect to assume that PI3K signalling encompasses full activation of mTORC1[40]. FOXO1 is phosphorylated by AKT at 3 serine/threonine residues, which results in its nuclear exclusion and in turn in the inactivation of its transcriptional activity[41].

Oncogenic *PIK3CA* mutations beyond cancer

In 2012, activating *PIK3CA* mutations were linked for the first time to mosaic, congenital, and progressive overgrowth disorders for which the name PROS was coined[42–45]. PROS features anatomically variable admixture of overgrown tissues, with vasculature and adipose tissue most severely affected macroscopically. Previously described disorders that now are grouped under the umbrella of PROS are: Congenital Lipomatous Overgrowth, Vascular malformations, Epidermal nevi, Scoliosis/ skeletal and spinal (CLOVES) syndrome[44]; Capillary malformation of the lower lip, Lymphatic malformation of the face and neck, Asymmetry of the face and limbs, and Partial or generalized Overgrowth (CLAPO) syndrome[46]; Klippel-Trenaunay Syndrome (KTS)[47]; Dysplastic MegalEncephaly (DMEG)/HemiMegalEncephaly(HME)/Focal cortical dysplasia (FCD)[48]; FibroAdipose hyperplasia or Overgrowth (FH/FAO)[43]; Fibroadipose Infiltrating Lipomatosis/facial infiltrative lipomatosis (FIL)[49]; HemiHyperplasia Multiple Lipomatosis (HHML)[50]; Macrodactyly[51]; Muscular HemiHyperplasia (MHH)[52]; Diffuse Capillary Malformation with Overgrowth (DCMO)[53]; Lipomatosis Of Nerve (LON)[54]; Megalencephaly-Capillary malformation syndrome/macrocephaly-capillary malformation (MCAP/M-CM)[42]; and FibroAdipose Vascular Anomaly (FAVA)[55] (Fig. 1).

A few years later somatic activating *PIK3CA* mutations were discovered as a cause of congenital sporadic venous malformations (VMs) and lymphatic malformations (LMs)[55–58].This exposed that, beyond being associated with complex phenotypes, *PIK3CA*-related vascular malformations may also occur in isolation. Since then, different subtypes of new *PIK3CA*-related disorders have been described in the literature. This has confused the field as it is not clear whether all or only some pertain to the so-called PROS. Strictly speaking,

all conditions involve tissue overgrowth (beyond other phenotypes); thereby suggesting that all should be grouped under the umbrella of PROS. Nevertheless, we favour the subclassification proposed by Mirzaa and colleagues which distinguishes between isolated or syndromic PROS[59]. The former includes any clinical manifestations which occurs as a focal lesion affecting only one tissue or body part. Instead, syndromic PROS are those conditions in which tissue overgrowth is not focal, affects several tissues and is presented with others features (Fig. 1). The terms isolated or syndromic PROS will be used across this Review. Of note, Victor Martinez-Glez et all., have recently described *PIK3CA* mutations in patients that present **segmental undergrowth** (in length or volume) of musculoskeletal tissues together with vascular malformations and with or without associated overgrowth[60]. This reflects that the understanding of *PIK3CA*-related congenital disorders is still in its infancy and indicates that current classification may need to be revisited in the future.

Determinants of clinical phenotypes in PROS. Overgrowth in PROS is characteristically present at birth, progressing during childhood and sometimes adulthood. Activating *PIK3CA* mutations in **PROS** arise postzygotically and stochastically during embryonic development leading to a mosaic distribution where only a subset of cells carries the mutation resulting in a variety of phenotypic features.

Germline vs. Mosaicism. Within PROS, most activating *PIK3CA* mutations have been detected in a mosaic fashion with very low allelic frequency in the affected tissues, and absent in blood cells. Likely, activating mutations in *PIK3CA* in the human zygote cause early embryonic death[61]. This has been shown in mice where expression of the *Pik3ca*^{H1047R} variant in the germline leads to embryonic lethally[62,63]. This is not unique of *PIK3CA* mutations, as many oncogenes have a dominant lethal activity that can only survive through mosaicism[13,64]. Of note, germline mutations in *PIK3CA* have been reported in 13 cases of PROS with macrocephaly. Ten of these cases carried missense mutations of uncertain significance, and none were identified in the cancer hotspot sites[42,65–67]. There is a recent case of a child showing a mild PROS phenotype which presented a *PIK3CA* G364R germline mutation. This variant is annotated as functional activating mutation in cancer[5] and was detected in 50% variant allelic frequency (VAF) in peripheral blood cells, buccal smears, and skin fibroblasts. Patient-derived fibroblasts carrying this mutation showed increase PI3K signalling[67]; albeit it is not clear whether the increase in PI3K activity occurs at the same level as cancer hotspots. Together, these data suggest that there is a threshold of PI3Kα

activity that a living organism can tolerate, with quite likely only weak *PIK3CA* variants surviving in the germline. While *PIK3CA* mutations are primary presented in a mosaic fashion in PROS, it is still not clear whether overgrown lesions are exclusively composed of mutant cells, or they are also mosaic.

Does the time of mutation acquisition define PROS clinical severity? Clinical severity is defined by simultaneous presence of pain and disability. Without exceeding the threshold of PI3Kα activity that it is compatible with life, it is expected that postzygotic activating *PIK3CA* mutations which arise at early developmental stages affect a higher number of cell lineages leading to a more widespread, pleiotropic, and severe condition. On the other hand, if *PIK3CA* mutations were acquired later in development or after birth it would result in a lineage-specific pathogenesis, such as that found in isolated vascular malformations. This has led to the assumption that the latter is a less clinically severe PROS. The implementation of next generation sequence (NGS) into the clinical practice to diagnose PROS has allowed to study and follow large cohorts of patients. This has provided substantial evidence that there is not always a clear correlation between the type of cell lineages which carry the mutation, the VAF of the mutation in the affected tissue and the severity of the clinical outcome[68]. In fact, there are patients who develop an isolated, but very severe lesion and other patients with a widespread overgrowth, but with lower severity. This indicates that severity primary relies on the anatomic location and extension of the overgrown tissue rather than the degree of widespread. This also suggests that severity and phenotypes (number of tissues affected) are not synonymous in PROS. In addition, it poses the notion that it is not accurate to assume that the earlier a mutation appears the more severe the pathogenic outcome is. Instead, we believe that intrinsic (cell-autonomous) and extrinsic factors to which mutated clones are exposed to, are also key determinants to PROS severity*;* including cell lineage that acquired the mutation (e.g., mesoderm vs endoderm precursor, progenitor vs. differentiated cell), the degree and mechanism of PI3Kα activation (*PIK3CA* variant) and the spatiotemporal environmental modifiers of PI3Kα signalling (availability of growth factors, paracrine activation of wild-type cells surrounding mutant cells, cell-cell and cell-extracellular matrix (ECM) interactions and mechanical signals among others). We propose that the final phenotypic outcome of PROS would be the consequence of a unique combination of all these parameters (which, when and where). Below we further develop each of these aspects (Fig. 2).

Tissue patterns of PIK3CA pathogenesis. A remarkable observation within the variable spectrum of PROS is the clear biased pattern of tissues that present phenotypic traits, including adipose, muscle, bone, nervous, vascular and, skin (epidermis and dermis)[44]. Yet, the exact cell type or types that carry the mutation in each case is not always clear. Most of genetic testing has been done using biopsies of affected tissues in which many different cell types are found, including non-mutated cells. In addition, the VAF within these biopsies range from 0.5% to 50% indicating for instance that in cases where 1% VAF is detected only 2 out of 100 cells carry the mutation. However, there is an intrinsic variability based on sample handling; often patients need to be biopsied several times before the presence of a *PIK3CA* mutation is detected[69]. Cell type specific isolation and *in vitro* culture of patient derived cells have clarified that *PIK3CA* mutations are present in keratinocytes[70], blood endothelial cells (BECs) and lymphatic endothelial cells (LECs)[69,71–73], fibroblasts[43,74–76] and adipose-derived stem cells and adipocytes[77,78]. Most of the tissues carrying a *PIK3CA* mutation are mesodermal derivates (vasculature, adipose, muscle, and bone) and/or ectodermal derivates (nervous tissue, epidermis, and connective tissues of the head)[48,54,79]. Within the nervous tissue, it is not clear which specific cell linages carry *PIK3CA* mutations as biopsies have not discriminated between neurons, macroglia and microglia (being neurons and macroglia of neuroectodermal origin and microglia derived from the mesoderm line)[48,78,80,81]. Also, it is incorrect to consider that all PROS-related neuropathies involve overgrowth of neuroectodermal derivates. For example, LON (lipomatosis of nerve) is a subtype of isolated PROS in which patients suffer from enlargement of nerve bundles primarily caused by overgrown adipose and fibrous tissue[54]. In line with this, a recent case report has confirmed that *PIK3CA* mutations in LON are prominently found in mesoderm-derivate lineages[81].

No endodermal-derived tissues (e.g., epithelial lining of the gastrointestinal and respiratory tract, the parenchyma of tonsils, liver, thymus, thyroid, parathyroid and pancreas) are usually found phenotypically affected in PROS. This is not unique of congenital disorders, as *PIK3CA* mutations also have a dominant role in ectodermal and mesoderm-derived cancers such as breast and endometrial [82,83]. An enigmatic aspect about the lineage skewing pattern in PROS is whether *PIK3CA* mutations are present, but silent, in non-pathogenic tissue, or instead they are not present in those tissues. One possibility is that mesoderm and ectoderm-derived tissues are more sensitive to PI3K overactivation while in endoderm-derived tissues *PIK3CA* mutations are not enough to cause pathogenesis. It is also possible that mutation acquisition favours differentiation into specific lineages, as

shown in breast cancer[84]. In fact, homozygous *PIK3CA* mutations induce self-sustained stemness and resistance to spontaneous differentiation in human induced pluripotent stem cells (iPSC)[85,86]. This suggests that *PIK3CA* mutations persist better in less differentiated cell states. Nevertheless, we cannot rule out that *PIK3CA* mutant clones undergo negative selection (either by out competition or by cell death) in specific cell lineages. Of note, *PIK3CA* mutations have been found in healthy adult tissues from endoderm-derived tissue such as oesophagus[87,88]. While *PIK3CA* mutant clones outcompete their wild-type neighbours in that context, they do not lead to abnormal tissue growth[87]. This would fit with *PIK3CA* mutations being silent in endoderm-derived tissues.

The bias of PIK3CA variants in PROS. Missense activating mutations in *PIK3CA* span almost the entire gene in cancer and PROS. In cancer, more than 80% of somatic mutations are found in three hotspots located in the helical (E545K, E542K) and kinase (H1047R) domains[5]. While the mutational profile of *PIK3CA* in PROS is similar than in cancer, the occurrence of mutations other than these three hotspots is much higher[68] (Fig. 3). Mutations with lesser gain-of-function activity are quite likely no that frequent in cancer because of their lower oncogenic potential. Instead, it seems that mild and weak activating *PIK3CA* mutations are enough to generate a pathogenic response when acquired at embryonic stages. Indeed, G914R and E726K, which are likely non-strong activating *PIK3CA* mutations, are also hotspots in PROS[7,68]. Intriguing, sporadic cases of isolated PROS with two mutations have been identified, where a hotspot mutation is combined with a non-hotspot mutation[48,89]. Yet, it is not clear whether these mutations are presented in the same clone in cis or trans or in different clones. It is important to bear in mind that cancer hotspot mutations have been preferentially mapped for genetic testing in PROS which has quite likely underestimated the occurrence of mutations beyond the hotspots mentioned above. In line with this, there is also a bias in the clinical visibility of severe cases which tend to overrepresented for genetic testing[90].

The emerge of numerous genetic studies in the context of PROS is allowing for the first time to conceptualize phenotypes from genotypes. For example, the majority of MCAP (megalencephaly-capillary malformation syndrome) patients with a reported genetic diagnosis carry a non-hotspot mutation in *PIK3CA* with G914R and E726K being the most common variants[42,68,76,91–95]. Also, MCAP is a subtype of PROS disorder in which affected tissues derive from two different developmental layers, ectoderm (neurons and macroglia) and mesoderm (vasculature and microglia); thereby suggesting that weakly activating

mutations in *PIK3CA* are compatible with their existence before the divergence of the germline layers. Another study with a large cohort of patients with lymphatic malformations (LMs) has revealed genotype-phenotype associations; with cancer hotspots (E545K, E542K, H1047R) being overrepresented in localized LMs and KTS. [69]. On the other hand, non-hotspot mutations were found significantly more frequently and at higher VAFs in LMs presented in CLOVES and unclassified PROS. Many clinical units worldwide are currently implementing in their routine pipeline genetic studies for PROS patients. We anticipate that this will shed light into new genotype-phenotype correlations.

Output of PIK3CA variants. Based on the impact of each mutation on the protein conformation of PI3Kα [96,97], *PIK3CA* mutations display quantitative differences in the intrinsic PI3Kα lipid kinase activity. For example, the H1047R variant (1) leads to increase interaction of PI3Kα with lipid membranes, (2) enhances PI3Kα kinase activity under growth factors stimulation and (3) becomes insensitive to RAS binding[98–102]. On the other hand, the E545K/E542K helical variants require RAS-GTP binding to be fully activated but are no longer inhibited by p85 (the regulatory subunit). This propels PI3Kα in a basal active state that mimics the activation induced by RTK[98,102,103] and explains why growth factors' stimulation does not add greater activity to PI3Kα compared to basal state[100]. Instead, mutants in the C2 domain, such as the C420R variant, result in increased positive surface charge; thereby leading to an enhanced recruitment of PI3Kα to cellular membranes[96]. Indeed, C420R shows a greater increase in PI3K signalling than E545K/E542K upon growth factor stimulation[96,104]. Of relevance, there is a large amount of less frequent mutations in *PIK3CA* for which there is very little knowledge. Several groups have shown that most of non-hotspot mutations are also gain-of-function mutations and signal constitutively through AKT[104,105]. Yet, structural insights and biochemical insights of the impact of these less frequent variants are lacking which hampers the understanding of the mechanisms by which they promote high PI3Kα activity.

New evidence has emerged that distinct *PIK3CA* variants induce different molecular programs in glioblastoma[106]. In breast cancer, instead, the expression of the same variant in different mammary gland populations results in different molecular programs which cause different tumour types and clinical outcomes[84]. While these differences are yet to be described in PROS, the variety of clinical manifestations in these conditions suggests that *PIK3CA* variants exhibit qualitative differences by means of variant-specific molecular

signals[106]. Another important unresolved question is whether *PIK3CA* variant-related different pathogenesis confer differential susceptibility to classical PI3K inhibitors.

Extrinsic factors in PROS pathogenesis. The comparison between *PIK3CA*-related phenotypes in PROS and cancer suggests that the timing when the mutation is acquired (development vs. adult) confers different susceptibilities to *PIK3CA* mutations. Tissue growth is chiefly taking place during embryogenesis and early postnatal periods, during which most cells in the organism divide and growth extensively. It is during these growing phases when activating mutations in *PIK3CA* favour PROS onset. In line with this, tissues with high plasticity which are in constant adaptation to the microenvironmental needs, such as the vasculature and adipose tissues, are highly affected in *PIK3CA*-related congenital disorders. Recent data have shown that onset and growth of *Pik3ca*-related vascular malformations relies on the synergy between *Pik3ca* mutations and growth factors[71,107]. This explains why in the adulthood when the growth factor signals are residual, these lesions do not form the novo or existing ones progress very slowly. This also fits with the observation that many lesions regrowth after incomplete surgical removal, when the body reacts locally busting the production of growth factors to promote wound closure and explains why some asymptomatic lesions ignite its growing during injury, adolescence and pregnancy (hormonal changes). Thus, patients may benefit from therapies in which, at specific timing windows, for example after a resection, microenvironment-derived paracrine specific signals are inhibited[107]. The FAVA disorder is an example of a PROS condition in which patients are asymptomatic at birth with lesions developing in the extremities during late childhood and adolescence. In fact, some patients have reported that FAVA lesions appear after an accidental event causing physical injury (Eulàlia Baselga's personal communication) which is coherent with the push-growth notion, as damage often results in hypoxia and acute production of growth factors. The notion that growth factors are critical for PROS pathogenesis opens the discussion if the degree of widespread is dependent on *in situ* production of growth factors at the time that a *PIK3CA* mutation is acquired. This would imply that the penetrance (proportion of cells carrying a particular variant) of PROS would be primary linked to the local production of lineage-specific growth factors when the mutation occurs.

It is still not fully understood to at what extend tissue overgrowth in PROS relates to cell growth and/or cell proliferation. While PI3K signalling mediates both cellular functions, cell growth and cell cycle can be also independently regulated. Modelling *Pik3ca*-related

single and complex PROS in mice has shown that overactivated PI3Kα results in an obvert hyperplasia with an increase in the projected surface area; thereby indicating that *PIK3CA* mutations alter both proliferation and growth[108,109]. However, data on PROS patients treated with alpelisib (an allosteric PI3Kα specific inhibitor) have showed that, upon treatment, overgrowth lesions essentially reduced their volume without cell death which suggests that treatment primary interferes with cell size [108]. Albeit it is also possible that such prominent effect on lesion size relates to reduce swelling. Given that PROS hyperplastic phenotypes largely depend on the presence of growth factors, it is tempting to speculate that onset of PROS relies on cell proliferation and that the slow progression during the lifetime of the patient is more dependent on the intrinsic cell growth. This would fit with the observation that PROS lesions are considered as non-proliferative lesions at the time of diagnose[110].

Cells are in constant exposure to biomechanical cues (shear, tensile and compressive stresses)[111], being at foremost play during developmental stages[112,113]. Indeed, mechanotransduction (the cellular response induced by biomechanical cues) is believed to contribute to cell fate decisions[114–116]. This is particularly relevant for those cells/lineages which co-exist in cell-cell or cell-extracellular matrix contact. For example, endothelial cells (ECs) which establish adherent junctions to one another and are in constant interaction with both their luminal and abluminal extracellular space are extremely dependable of mechanobiology signalling[117]. Intriguing, the anatomical location of a mutant clone has been recently identified as critical factor for tumorigenesis[118]. Hence, it is tempting to speculate that tissue architecture and mechanical forces contribute to define such anatomical-related pathogenesis. Several evidence suggest that oncogenic signalling synergises with mechanotransduction to promote pathogenesis[119–121]. Specifically, PI3K signalling cooperates with several components of the mechanobiology machinery such as YAP/TAZ, cadherins and actin remodelling proteins[122–124]. Based on these novel concepts, it is tempting to speculate that aberrant crosstalk between biomechanical cues and *PIK3CA* contribute to the clinical manifestation of PROS.

Other reports have identified that *PIK3CA*-related pathogenesis is supported by non-cell autonomous mechanisms. For example, Martin-Corral et al. have showed that the presence of *Pik3ca* mutant clones in lymphatic vessels results in the accumulation of immune cells, including macrophages, which then become a major source of VEGF-C than can further promote pathological lymphangiogenesis[107]. Other such studies have provided evidence that *PIK3CA* mutant clones in tumours, through paracrine communication, interfere with wild-type or *HER2* mutant clones in close proximity and thus catalyse their aberrant

behaviour[125,126]. While this has not been reported in the context of PROS yet, it is possible that mosaic lesions also require to attract non-*PIK3CA* mutant cells for pathogenesis. Indeed, this has been reported in some vascular malformations[127,128]. We anticipate that understanding how mutant clones hijack these other clones may open new therapeutic opportunities.

***PIK3CA* mutations in vascular malformations.**

Vascular malformations occur in both isolated and syndromic PROS. Even within the syndromic PROS, which tend to feature a variable admixture of overgrown tissues, the vascular compartment/tissue is most commonly affected. This indicates that vascular malformations are a hallmark of PROS. This is coherent with PI3Kα being a master regulator of endothelial cell biology (Box 3). Vascular malformations are abnormal vessels that grow aberrantly during embryonic development and are manifested at birth (congenital) or throughout the life of affected individuals. They slowly grow and do not regress spontaneously over time. Depending on the type(s) and localization of the affected vessels, patients' symptoms can range from mild to severe, even life-threatening. These vascular lesions often cause pain, swelling or bleeding, together with cosmetic deformities that can interfere with the normal function of the affected areas. Recent evidence shows that occasionally vascular malformations are not clinically manifested until a pathophysiological condition such as adolescence, pregnancy, or injury triggers their growth[129,130]. Due to the variability in the clinical manifestations, the diagnosis and treatment are not easy and require a multidisciplinary team of specialists.

Subtypes of *PIK3CA*-related vascular malformations. Vascular malformations are divided in low-flow (venous, lymphatic and capillary) and fast-flow (arteriovenous) lesions. They can also be classified as **simple**, when only a specific vascular bed is affected such as capillary, venous, lymphatic, or arteriovenous anomalies, or **combined**, when a lesion presents two or more vascular malformations or mixed vascular beds characteristics such as capillary-venous malformations (CVMs), lymphatic-venous malformations (LVMs) or capillary-lymphatic venous malformations (CLVMs), among others. Intriguing, *PIK3CA*-related vascular malformations are restricted to low-flow lesions, including isolated venous malformations (VMs), capillary malformations (CMs), and lymphatic malformations (LMs) or in combination. It is not clear why the presence of *PIK3CA* mutations has not been reported in arteriovenous malformations (AVM). One possibility is that *PIK3CA* mutations behave as silent mutations in arteries. Indeed, arterial ECs exhibit molecular refractoriness to other

vascular-related mutations[131]. However, targeted sequencing for the *PIK3CA* gene in more than 100 surgically resected human brain AVMs did not identify any positive case[9,10]. Another possibility is that arterial mutant clones are negatively selected during vascular development or homeostasis. Studies using inducible genetic models which allow to express *PIK3CA* mutations in different endothelial cell populations would help to clarify this conundrum. Below we summarize the most recent relevant aspects of *PIK3CA*-related specific vascular malformations subtypes. For more general aspects, we refer the reader to specialized reviews on vascular malformations[132–135].

Lymphatic malformations (LMs): a prototypical example of *PIK3CA* dominance*.* LMs are debilitating vascular anomalies classified as cystic LMs (micro- or macro-cystic) and complex lymphatic anomalies. While the former appears in isolation, complex lymphatic anomalies show diffuse and multifocal pattern and may cause defects in the central collecting lymphatic channels such as generalized lymphatic anomalies (GLA), Gorham-Stout disease (GSD), kaposiform lymphangiomatosis (KLA), and central conducting lymphatic anomalies (CCLA). Activating mutations in *PIK3CA* have been detected in the majority of cystic LMs and GLA [55,136]*.* Of significance*,* there is a clear genotype to phenotype association in *PIK3CA*-related LMs, with the so-called cancer hotspots (H1047R, E545K and E542K) being dominant. While this could be explained by the notion that variants with lesser gain-of-function activity are not sufficient to induce pathogenesis in the lymphatic endothelium, it is also possible that this vascular compartment is more tolerant than other linages to high PI3K signalling. Of note, mutations in the helical domain (E545K and E542K) are more common than H1047R in LMs [69], albeit the significance of this remains to be determined.

The generation of mouse models of *PIK3CA*-related LMs has provided significant insights on the molecular and cellular factors that determine the onset and the subtype of LMs[107,108,136]. For example, the expression of *Pik3ca*^{H1047R} mutation in VEGFR3-positive cells during early embryonic development recapitulates traits of macro-cystic LMs. Instead, if the same mutation is expressed in VEGFR3-positive cells during late embryonic or early postnatal stages, mice develop micro-cystic LMs[107]. Another study showed that the expression of P110* (a dominant active *Pik3ca* transgene with 20 times higher kinase activity than H1047R) in VEGFR3 positive cells in adult mice causes multifocal LMs[108]. Similarly, the expression of the *Pik3ca*^{H1047R} mutation in PROX1-positive lymphatic cells after weaning resulted in GLA[136]. These data suggest that the type of cell/precursor, the time of activation and the mouse modelling genetic approach used to activate of PI3Kα signalling

are key factors in the onset of different subtypes of LMs. Another important lesson learnt from these mouse models is that, rapamycin, an allosteric inhibitor of mTOR, alone is not sufficient to revert LMs[107,108]. Martin-Corral et al. showed that VEGFC-VEGFR3 dependent activation of mutated PI3Kα promotes the growth of microcystic LMs in mouse. This explains why combined inhibition of mTOR and VEGFC leads to the regression of microcystic LMs [107]. Recently, it has been shown that alpelisib ameliorates LM symptoms in mouse models and in patients with cystic LMs that previously do not respond to rapamycin[108]. Currently, it is not clear why rapamycin and alpelisib induced different responses in LMs. However, it is tempting to speculate that the latter provides an overall better response due to a direct inhibition of the mutant protein. Also, given that rapamycin only targets a branch of the PI3K pathway, it is possible that *PIK3CA*-related pathogenesis occurs through mTOR dependent and independent mechanisms. Collectively, this emphasises the importance of generating faithful preclinical models for each subtype of LMs towards the so-called personalized medicine.

PI3K overactivation in venous malformations (VMs): a matter of *PIK3CA* and *TEK*. VMs are bluish lesions caused by aberrant EC proliferation that show enlargement, tortuosity, reduced mural coverage and impaired functionality. VMs are classified into Common VMs, Familial VM cutaneo-mucosal (VMCM), Blue rubber bleb nevus syndrome (BRBNS), Glomuvenous malformations (GVM), Cerebral cavernous malformation (CCM), Familial intraosseous vascular malformation (VMOS), and verrucous venous malformation (VVM). Common VMs are the most frequent VMs (90% of all VMs). They are caused by activating mutations in *TEK* (60%)[137,138] or *PIK3CA* (20-25%)[56–58] with both mutations being largely mutually exclusive. While *PIK3CA* mutations only occur in a somatic fashion, both somatic and germline mutations in *TEK* can cause VMs. *PIK3CA* and *TEK* mutations lead to increased PI3Kα signalling, albeit *TEK* mutations activate the pathway to a lower extent [56,71,139]. This quite likely explains why some *TEK* mutations are compatible with their survival in the germline. Of note, there is a clear tissue-genotype association between *TEK* and *PIK3CA*, with *TEK*-related VMs being mostly found in the skin surface while *PIK3CA*-related VMs are preferentially located in intramuscular areas[56,57]. An important aspect to consider is that *TEK*-related lesions tend to be purer VMs than *PIK3CA*-related lesions which often also express some lymphatic markers. Data on the co-occurrence of *PIK3CA* and *TEK* mutations in the same lesion are also emerging[57,139]. Yet, it is too early to say whether

second events play a wider role in the severity of these diseases and whether multi-genetic events co-occur in the same cells.

Mouse models of *Pik3ca*-related common VMs have also flourished. For example, Castillo et al. reproduced the aetiology of VMs by widespread mosaic induction of *Pik3ca*^{H1047R} under the endogenous promoter in the mouse lateral plate mesoderm during embryonic development. At present, it is still enigmatic why this mouse model solely develops vascular malformations, predominantly VMs, while avoiding major alterations in other tissues. Based on these data, it is tempting to speculate that the type of PROS also relates to the subtype of (mesoderm) precursor where the mutation occurs. Another study has shown that ubiquitous, but mosaic, expression of *Pik3ca*^{H1047R} in adult mice also leads to a rapid and exclusive development of VMs[57]*.* Collectively, these mouse models have shown that *Pik3ca*-related VM form through proliferation and are deprived of mural cells[57,58]. Xenographs models using human - ECs derived from patients have also emerged[139]. While these models become a relevant tool for preclinical testing, they have limitations for the understanding of the onset and biology of these diseases. Taken together, data from modelling PROS in mice support the notion that BECs are particularly sensitive to *PIK3CA* pathogenesis.

Cerebral cavernous malformations and *PIK3CA*: the advent of multigenic events in vascular malformations. Activating *PIK3CA* mutations have been also found in cerebral cavernous malformations (CCMs), that are capillary-venous malformations specifically located in the brain and spinal cord. There are two types of CCM diseases, familiar CCMs (20% of CCMs) and sporadic CCMs (80% of CCMs). Until recently it was believed that CCM were monogenic diseases, largely caused by inherited or somatic loss-of-function mutations in one of the three genes (*KRIT1 (also known as CCM1), CCM2 or PDCD10 (also known as CCM3)*) that encode for the heterotrimetic CCM protein complex[140,141]. In 2021, somatic gain-of-function mutations in *MAP3K3* (encoding for MEKK3*)* were also found in sporadic orphan CCMs[142–144]. In CCM, *MAP3K3* mutations largely lay in the I441M spot, and lead to enhanced activation of MEKK3 signalling. In addition, several studies have showed that CCMs with a prominent and rapid growth and associated with strokes and seizures, carry an additional somatic genetic hit in *PIK3CA,* chiefly on a cancer hotspot[10,142,143]. While mutations in *MAP3K3* and *CCMs* genes are mutually exclusive, *PIK3CA* mutations may co-occur with any (*MAP3K3, KRIT1, CCM2* and *PDCD10*). This is coherent with the observation

that *KRIT1, CCM2*, *PDCD10* and *MAP3K3* mutations all lead to activation of MEKK3 signalling.

Modelling CCMs in mice using endothelial-specific CreERT2 mouse models has confirmed that the synergy between loss of *Krit1* and expression of *Pik3ca* occurs by interaction within or between ECs[10]. In line with this, isolation of ECs from human CCM has confirmed that these cells carry *PIK3CA* mutation[142]. However, another such study in mice has proposed that *Pik3ca* mutations in CCMs occur in pericytes, a subtype of mural cells which adhere to and support capillary endothelial function[9,145]. Yet, these data remain controversial as the Cre mouse line employed to activate the expression of *Pik3ca*^{H1047R} is neither inducible nor pericyte-specific. In addition, no proof that ECs in the mouse model do not carry *Pik3ca* mutations is provided[146]. In support of a possible involvement of mural cells in the CCM disease, another report showed that specific deletion of *Pdcd10* in mural cells, including both pericytes and smooth muscle cells, resulted in CCM. While proof that human brain pericytes carry *PIK3CA* mutations would be required to validate these findings, it is possible that *PIK3CA* mutations in endothelial cells and mural cells account for different subtypes of CCMs. Of note, pericytes do not rely on PI3Kα, but PI3Kβ to activate PI3K signalling which would be coherent with no involvement of *PIK3CA* mutations in pericytes in the progression of the CCM[147].

Intriguingly, sporadic CCMs are frequently found in close proximity to developmental venous anomalies (DVAs)[148,149]. DVAs are the most common vascular malformations (present in about 10% of the adult population) and are largely develop before the age of 20. Emerging data suggest that sporadic CCMs may, at least some, derive from DVAs. While comparing the mutational status of DVA and its paired CCM (present in the same patient), it was identified that DVA and CCM carried both a somatic activating *PIK3CA* mutation, while CCM lesions harboured mutations only in *MAP3K3*. Based on these finding, it has been proposed that individuals who have a *PIK3CA*-related DVAs are predisposed to develop sporadic CCM in close proximity to the DVA[144]. Collectively, these studies have catapulted *PIK3CA* as a critical genetic hit for the CCM disease. Yet, it is not clear whether *PIK3CA* mutations are required for the formation of CCMs, or they serve as endothelial clonal amplifiers.

Capillary malformations (CMs): the least pathogenic vascular malformations within PROS. Low-flow CMs are anomalies composed of dilated capillaries near the surface of the skin which normally present a macular, pink to red stain. *PIK3CA*-related CMs have been

largely described as part of some PROS, such as MCAP and DCMO, where they are largely caused by activating non-hotspot mutations in *PIK3CA*[53,93]. Also, a recent case with an acquired capillary malformation (after birth) associated with V344M *PIK3CA* variant has been reported[150]. Overall, these lesions tend to be largely cosmetic; thereby indicating that they are less severe than LMs or VMs. Mutations in *GNAQ* and *GNA11* (encoding for $G\alpha_q$ and $G\alpha_{11}$ respectively) are overrepresented in cutaneous CMs. Intriguing, co-occurrence of *PIK3CA* and *GNAQ* has also been reported in combined vascular malformations[11]. Whether this co-existence relates to higher severity is not clear yet.

Treatment options for PROS: the era of repurposing drugs used in oncology.

Yet, there is no approved molecular treatment for PROS patients. The broad clinical manifestations in PROS together with the fact that they pertain to the category of rare diseases have compromised the implementation of effective and safety targeted therapies for their treatment. Until very recently, the standard care was surgical debulking (including amputation, lesion debulking/resection among others) and/or scleroembolization of vascular malformations. However, these treatments are not curative and have high risk of recurrence (hypertrophy). In line with this, treatments to diminish symptoms such as pain (steroids or antihistamines) or seizures (epilepsy medication) have been prescribed in some cases. With the discovery of *PIK3CA* mutations being causative of PROS, treatment possibilities emerged. Initial efforts have been centered on the repurpose of PI3K inhibitors used in oncology. Below we summarized most promising attempts (Box1). Of note, with the emerge of pharmacotherapy for PROS, the efficacy and the best regimen is also being established. We refer the reader to Table 1 for specific details on completed and ongoing clinical trials for PROS.

Sirolimus: the first targeted therapy to treat PROS. Sirolimus (also known as rapamycin) is an allosteric mTOR inhibitor approved by both the food and drug administration (FDA) and European medicine agency (EMA). Hence, it is no surprising that siroliums was first proposed for the treatment of PROS. The very first clinical trial was planned in patients with vascular anomalies, even prior to the implementation of the genetic diagnosis as a precondition for trial inclusion (NCT00975819). Together with other follow up clinical trials, it showed that sirolimus reduced the overall volume of vascular malformations with LMs exhibiting the highest susceptibility to the drug[151–154]. However, several adverse events (AEs) and lesion regrowth upon treatment withdrawal were also identified on those studies

[153]. The PROMISE study, which tested a lower dose of sirolimus, arose as the first clinical trial specifically for syndromic PROS patients [45]. In that context, sirolimus showed a modest reduction in the volume of the overgrown tissues which was also accompanied with frequent AEs. Although these results were not as good as the initial expectations, it is fair to acknowledge that many patients have benefited from sirolimus. This explains why current standard of care includes sirolimus (with and without surgery) for PROS patients. However, should an individual risk-benefit evaluation by the physician be considered[45]. Currently, there are several ongoing clinical trials which are assessing the potential benefits of sirolimus for PROS (NCT04598204, NCT03987152, NCT04128722, NCT03972592, NCT02638389, NCT03767660). Topic administration on superficial lesions is being also considered (NCT03972592), given that reduced side-target effects are expected. However, it is important to consider that topic administration is not an option for syndromic PROS with internal lesions.

Miransertib (currently known as MK-7075): an AKT inhibitor for the treatment of PROS. Miransertib is an allosteric highly selective AKT inhibitor which was initially developed for oncology [155]. Given that AKT is the main effector of PI3Kα signaling pathway, repurposing of miransertib was propose not only for Proteus Syndrome (PS, caused by gain of function mutations in AKT1, Box 1) but also for PROS. This was first used in a compassionate basis with partial therapeutic efficacy and no major toxicities[156,157]. An important aspect to bear in mind about miransertib is that it seems most efficient in severe PROS. Preclinical studies in isolated vascular malformations have also showed promising data, even when using half of the dose used in oncology[71]. Clinical trials testing the safety and effectiveness in PS and PROS patients with a confirmed genetic diagnosis will remain open until June 2022 (NCT04316546, NCT03094832, NCT03317366).

The use of PI3K inhibitors for PROS: pan vs. PI3Kα selective targeting. PROS are caused by activating *PIK3CA* mutations, hence selective inhibition of PI3Kα shall be the most accurate targeted treatment. Nevertheless, a clinical trial using taselisib, a selective pan class I PI3K (PI3Kα, β δ, γ) inhibitor was first approached (NCT03290092)[158]. PROS patients treated with taselisib showed clinical improvement with reduced pain, chronic bleeding resolution and functional improvement. However, presentation of severe drug-related AEs in some patients led to the early termination of the trial. These adverse effects were thought to be caused by the impact of inhibition of PI3Kδ/γ on the immune system;

thereby suggesting that selective inhibition of the PI3Kα isoform would be a better choice in that context. Since then, expectations have been put in alpelisib, a PI3Kα selective inhibitor which has been recently approved by the FDA for metastatic breast cancer[159]. Alpelisib was first tested in mouse models of CLOVES-like syndrome showing promising results with higher efficacy than sirolimus[94]. Similar results have been recently obtained in a mouse model of LMs[108]. However, withdrawal of the treatment led to the recurrence of the lesions, indicating that a chronic treatment should be considered for these patients[94]. The exciting observations that topic administration of alpelisib in xerograph mouse models induced a prominent regression in vascular skin lesions[57], enhances the prospects for topical administration of this inhibitor in humans.

An unregistered case series of patients with PROS with confirmed *PIK3CA* mutations treated with alpelisib on a compassionate basis, reported evidence of efficacy with minor side effects[94]. However, neither safety nor efficacy endpoints were pre-specified in that study[94]. Later, other studies also confirmed promising results while using of alpelisb in a compassionate basis[160–162]. Nevertheless, the lack of evidence of benefit-risk assessments in a long-term basis pushed a retrospective and non-interventional study of 32 patients treated with this inhibitor (EPIK-P1). Current reported analysis on the first endpoint (after 24 weeks of treatment) exposed that 37.5% of alpelisib-treated patients exhibit clinical benefits and 38.6% of the patients suffer of hyperglycemia, aphthous ulcer and stomatitis[163]. EPIK-P3 is now continuing EPIK-P1 to evaluate the long-term safety and efficiency (NCT04980833; 2020-005896-12). Alpelisib treatment has also been tested in patients with LMs who showed a general improvement with decrease volume of LMs and reduced tiredness and pain while reporting moderate AEs such as aphthous and diarrhea[108]. The promising data on alpelisib for compassionate has finally catalyzed an ongoing prospective, multicenter, randomized, double-blind and, with placebo-controlled period clinical trial (EPIK-P2) to demonstrate the efficacy, tolerability, and safety of alpelisib (NCT04589650).

While we are still learning from all the pharmacotherapy studies described above, it is now clear that these drugs have become essential tools to treat PROS. Collectively, they have shown a clear impact on the quality of life of these patients while different penetrance in the reduction of volume lesion and symptoms were observed. It is important to consider that PROS patients exhibit high heterogeneity in their clinical manifestations (from isolated vascular malformations like VM or LMs to complex and syndromic cases such as CLOVES or KTS among others) which quite likely explains the heterotypic responses to the different treatments. Thus, once safety of these treatments is well established, physicians will have

to evaluate the risk-benefits of each patient based on their specific needs and responses. We believe preclinical studies are being instrumental to define dosing regiments (e.g. high *vs* low dose; intermittent *vs* continuous) as well as to test combination therapies.

Conclusions and perspectives

With the advent of NGS *PIK3CA* has emerged as frequently mutated oncogene in congenital disorders with asymmetric overgrowth. This has accelerated the generation of mouse models of these diseases towards the understanding of the mechanisms which lead to their onset and progression. Lessons learnt suggest that the pathogenic score of activating *PIK3CA* mutations in congenital disorders is a combination of the developmental time (when) that these mutations appear together with intrinsic factors (cell-autonomous), such as the mechanisms and degree of activation of PI3Kα (*which*) and the cell lineage specificity of the mutated cell (*where*) and extrinsic factors (non-cell autonomous), which refers to environmental modifiers of the PI3Kα outcome. Unraveling the specific contribution of each of these elements will be required to define better treatments for these patients. In line with this, mapping the entire *PIK3CA* gene should be considered when genotyping patients with a suspicion of PROS.

With regard to the clear skewing tissue pattern in PROS, it has become clear that some lineages are more sensitive to PI3K overactivation. Yet, it has to be learned the basis of such context-dependent pathogenesis. We propose that scRNAseq approaches in combination with genetic mouse models which allow to express *Pik3ca* mutations in specific subpopulations will be critical to identify cell lineage histories of mutant clones and to understand how PI3Kα activation subverts cellular processes in a context-dependent manner. Second or even triple genetic hits in vascular malformations are emerging in aggressive clinical cases. This overrules the original idea that congenital vascular malformations are mostly monogenic disorders that behave differently from cancer. This highlights the urge to look for multiple genetic hits in other *PIK3CA*-related disorders that may explain the severity or tissue-specific pathogenesis. Finally, it is of vital importance to consider non-cell autonomous mechanisms for future treatments. Response to co-inhibition of specific upstream or downstream signalling pathways may be also different among the *PIK3CA* variants. Targeted studies of molecular programs underlying distinct types of *PIK3CA*-related overgrowth are needed to inform precision medicine strategies.

BOX1. Non-*PIK3CA*-related congenital disorders caused by aberrant PI3K signalling.

Mutations in several components of the PI3K pathway other than *PIK3CA* have been identified in congenital disorders characterized by aberrant activation of PI3K signalling and tissue overgrowth[164]. This includes loss of *PTEN*[165] and *TSC1* and *TSC2*[166,167] and germline or somatic activating mutations in *AKT1*[168], *AKT2*[169], *AKT3*[42,170,171], *PIK3R1*[172], *PIK3R2*[42] *and mTOR*[79,173–176]. Heterozygous loss of *PTEN and TSC1/TSC2* and activating mutations in *AKT3*, *PIK3R2* and *mTOR* may occur in the germline or in a somatic fashion. The germline (heterozygous) loss of *PTEN* is known as *PTEN* hamartoma tumour syndrome (PHTS) and includes Bannayan-Riley-Ruvalcaba syndrome (BRRS), Cowden syndrome (CS) *PTEN*-related Proteus syndrome (PS), and *PTEN*-related Proteus-like syndrome[165]. While PHTS patients are prone to develop benign and malignant tumours, evidence has emerged that 50% of them also develop vascular malformations. Autosomal dominant loss of *TSC1/TSC2* leads to Tuberous sclerosis complex (TSC), a neurocutaneous disorder frequently associated with abnormalities in the brain[177]. Somatic activating mutations in *AKT1* lead to the so-called Porteous syndrome (PS)[168] and in *PIK3R1* have been associated with vascular malformations and overgrowth similar to PROS/PS[42,170,172]. Mutations in *AKT3* (highly expressed in brain and heart) and in *PIK3R2* have been associated with brain overgrowth. Only very few cases with *AKT2* mutations have been found and they have been associated with severe fasting hypoglycemia and asymmetrical growth[169]. Although there are overlapping characteristics between these genetic conditions, the specific expression and regulation of each of the member define the ultimate clinical outcome[178]. The correct genetic diagnosis is relevant to find proper treatments as well as to better understand the gene-specific functions during development. It is important to bear in mind that overactivation of the PI3K signalling pathway may not be only promoted by the PI3Kα isoform. Figure BOX1 integrated in this box.

BOX2. Class II and Class III PI3Ks

Class II and class III PI3K still remain quite enigmatic. Class II PI3K is composed of three catalytic isoforms PI3KC2α, PI3KC2β and PI3KC2γ (encoded by *PIK3C2A*, *PIK3C2B and PIK3C2G* respectively*)* that lack dedicated regulatory subunits. These enzymes can generate PI(3)P and $PI(3,4)P_2$ and regulate vesicular trafficking, including receptor endocytosis, endosomal trafficking, neurosecretory granule release and insulin secretion. PI3KC2α and PI3KC2β are broadly expressed while PI3K-C2γ expression is limited to some tissues such as liver, pancreas, prostate, and breast. Due to the lack of a specific regulatory

subunit, the mechanisms through which class II PI3Ks are recruited to specific sites and activated are still unclear[14,179,180]. Class III PI3K is composed by a solely catalytic subunit Vps34 (encoded by *PIK3C3*) associated with the regulatory protein Vps15 (encoded by *PIK3R4*) which controls Vps34 localization and activation. Vps34 catalyzes the phosphorylation of phosphatidylinositol (PtdIns) to phosphatidylinositol-3-phosphate (PtdIns3P) and, principally, it is involved in vesicle trafficking and autophagy controlling nutrient acquisition pathways[14,181].

BOX3. PI3Kα in the endothelium

PI3Kα has emerged as master regulator of vascular morphogenesis in blood and lymphatic vessels[182]. Genetically engineering mouse models have shown that both inactivation and overactivation of PI3Kα activity in the germline or specifically in BECs cause profound defects in the blood vasculature ultimately resulting in embryonic lethality[57,62,63,183–185]. This is explained by PI3Kα being the main producer of PIP3 upon RTK stimulation in ECs. In line with this, inactivation or deletion of other class I PI3K isoforms does not interfere with vascular development nor with embryonic development[183]. Lymphatic endothelial-specific depletion of PI3Kα results in perinatal lethally due to selective alterations in mesenteric and intestinal lymphatic vessels[186]. Stanczuk et al. also showed that LECs differently rely on VEGFR3-PI3Kα signaling pathway depending on their origin and tissue location. An intriguing aspect of PI3Kα is that it is activated through distinct mechanism in the BECs and LECs. BECs utilise the regulatory subunits to recruit PI3Kα to the plasma membrane upon RTK stimulation. Instead, the LECs also require the RBD domain of PI3Kα intact. Hence, mice carrying mutations in *Pik3ca* that block PI3Kα binding to RAS exhibit selective defects lymphatic vessel development[187]. PI3Kα regulates vessel growth through both AKT dependent and independent mechanisms[188,189], including (1) control of cell cycle progression through the inactivation of FOXO1[188,190]; (2) stimulation of vein specification by increasing COUP-TFII levels upon TIE2 activation[191]; (3) regulation of junctional remodelling and cell rearrangements through the control of NUAK1/MYPT1/MLCP[122]. Endothelial-specific loss of *Pten* in mice also result in aberrant vascular development and early embryonic failure. Mechanistically, PTEN fine tunes endothelial cell proliferation during the early steps of the angiogenic process[192,193]. Of note, loss of *Pten* metabolically rewires endothelial cells towards lipid consumption[194].

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Number	Title	Phase	Nº Patients/Pathology	Age	Type of administration and dose	Results	Ref
Sirolimus/Rapamycin							
NCT00975819	Clinical Trial Assessing Efficacy and Safety of the mTOR Inhibitor Sirolimus in the Treatment of Complicated VA.	Phase 2; Interventional; Single Group Assignment	60; Complicated Vascular Anomalies without genetic testing	up to 31Y	Oral; Liquid dosing based on trough levels	-Efficacious and well tolerated. -Effective at stabilizing or reducing signs/symptoms in GLA and GSD patients.	151,154
NCT02509468	Treatment of Superficial Voluminous Complicated Slow-flow Vascular Malformations With Sirolimus.	Phase 2; Interventional; Crossover Assignment	59; Slow-flow vascular malformations including syndromic forms*	6Y to 18Y	Oral solution or tablets, starting with 0.08mg/kg/twice/d, with dose adjustments (6mg/d maximum)	- Decrease LM volume together with oozing, and bleeding. - Less efficient for VMs.	152
NCT02443818 2014-000484-41 NCT02428296	PROMISE: Sirolimus Effect on Hypertrophic Syndromes Related Gene PIK3CA.	Phase 2; Interventional; Single Group Assignment	16;11;32; PROS with **confirmed *PIK3CA* mutations**	3Y to 65Y	Adult: tablet 1mg/d; Children: oral solution 0.5mg/twice/d tablet/solution. Max 1.5mg	- Beneficial especially for PROS with progressive adipose tissue overgrowth. - High rate of discontinuations -No clear decrease in AE rates compared with higher-dose therapy.	45
NCT01811667; 2012–001262-15	Clinical Study on Efficacy and Safety of the mTOR Rapamycin Inhibitor Found in the Complex Vascular Malformations.	Phase 3; Interventional; Single Group Assignment	19; microcystic LM, GLA or complex vascular malformations*	3Y to 64Y (media n: 15)	Children under 12: Oral solution 0.8 mg/m2 /twice/d; Adult: tablet, 2mg/d	- Partial response in all patients, reducing symptoms and increasing QoL.	153
NCT04598204	A Phase II Clinical Study to Evaluate the Efficacy and Safety of Rapamycin in Complex Vascular Anomalies in Pediatric Patients	Phase 2/3; Interventional; Single Group Assignment	30; patients with KHE and TA and complicated vascular malformations	1M to 14Y	Oral. Children under 3: 0.8mg/m2/daily; Above 3: twice/d for a dosage of 10-15ng/ml in plasma	- Ongoing trial.	195
NCT03987152	Treatment of Congenital Vascular Malformations Using Sirolimus: Improving Quality of Life.	Phase 3; Interventional; Challenge-Dechallenge-Rechallenge	75; Congenital VM, or LM or combined.	≥1Y	Daily intake	- Ongoing trial.	-
NCT04128722	TOPGUN: TOPical Sirolimus in linGUal Microcystic Lymphatic Malformation.	Phase 2; Interventional; Crossover Assignment	12; LMLMs not associated to CLAPO	≥5Y	Oral solution; 1mg/mL-0.5 mL to 1 mL/d on LMLM according to the size of the lesion	- Ongoing trial.	-
NCT03972592	0.1% Topical Sirolimus in the Treatment of CMLM in Children and Adults.	Phase 2; Interventional; Parallel Assignment	55; Primary CMLM	≥ 6Y	Topical 0.1% sirolimus daily on the randomly allocated area	- Ongoing trial.	-
NCT02638389	Efficacy and Safety of Sirolimus in VA That Are Refractory to Standard Care.	Phase 3; Interventional; Single Group Assignment	250; VM, LM or complex vascular malformations (KTS, PTEN, etc.)	3M to 70Y	Not specified	- Ongoing trial.	-
NCT03767660	Efficacy of Rapamycin (Sirolimus) in the Treatment of BRBNS, Hereditary or Sporadic VM.	Phase 4; Interventional; Single Group Assignment	20; BRBNS and VMs	all	Oral; Children: 1 mg/m2 body surface area/d; Adults: 2 mg/m2/d	- Ongoing trial.	-
Miransertib							
NCT04316546	A Multi-Cohort Phase 2 Dose-Escalation Study of MK-7075 (Miransertib) in Proteus Syndrome.	Phase 2; Interventional; Single Group Assignment	45; Proteus syndrome with confirmed somatic *AKT1* mutation	≥3Y	Not specified	- Ongoing trial.	-
NCT03094832	MOSAIC: Study of Miransertib (MK-7075) in Participants With PIK3CA-related Overgrowth Spectrum and Proteus Syndrome.	Phase1/2; Interventional; Parallel Assignment	85; PIK3CA-Related Overgrowth Spectrum (PROS)/Proteus Syndrome with **confirmed *PI3KCA* or *AKT1* mutation.**	≥2Y	Oral capsules; 15 mg/m2; up to 25 mg/m2	- Ongoing trial.	-
NCT03317366	Expanded Access of MOSAIC.	-	Overgrowth diseases and/or vascular anomalies with **confirmed *PI3KCA* or *AKT1* mutation.**	≥2Y	Capsules	- Ongoing trial.	-
Taselisib							
NCT03290092	TOTEM: Trial of Taselisib in Overgrowth.	Phase 1/2; Interventional; Single Group Assignment	19: PROS with **confirmed *PI3KCA* mutations**	16Y to 65Y	1 or 2 mg/d	- 76.4% reported clinical improvement. - No reduction of lesion volume. - Unfavorable safety profile in KTS and CLOVES,	158
Alpelisib							
NCT04285723	EPIK-P1: Retrospective Chart Review Study of Patients With PROS Who Have Received Alpelisib.	Observational : case only	57; PROS with **confirmed *PIK3CA* mutation**	≥2Y	Adult:250 mg/d; Children: 50 mg/d	- Reduction in lesion volume - Improve QoL due to reduction of major symptoms/sings in the majority of cases. - 39% alpelisib-related AE: hyperglycaemia, aphthous ulcer and stomatitis.	163
NCT04980833/ 2020-005896-12	EPIK-P3: Study Assessing Long-term Safety and Efficacy of Alpelisib in Patients With PROS Who Previously Participated in Study EPIK-P1.	Phase 2; Interventional; Single Group Assignment	50; PROS with **confirmed *PI3KCA* mutation**	≥2Y	Doses permitted are 50, 125, 200 and 250 mg.	- Ongoing trial.	-
NCT04589650	EPIK-P2: Study Assessing the Efficacy, Safety and PK of Alpelisib (BYL719) in Pediatric and Adult Patients With PROS.	Phase 2 multi-center; Interventional; Parallel Assignment	150; PROS with **confirmed *PIK3CA* mutation**	≥2Y	Oral tablet; Children 2-17: 50 mg/d; Adults: 125 mg/d	- Ongoing trial.	196

Table 1: Summary of previous and ongoing PROS clinical trials with PI3K-related inhibitors. (Y) Years; (M) months; (d) daily; (AE) Adverse events; (PROS) *PIK3CA*-related overgrowth spectrum; (CLAPO) Capillary malformation of the lower lip, lymphatic malformation of the face and neck, asymmetry and partial/generalized overgrowth; (LMLM) Lingual Microcystic Lymphatic Malformation; (CMCM) Cutaneous microcystic lymphatic malformations; (LM) lymphatic malformations; (VM) venous malformations; (GLA) Generalized lymphatic anomaly; (GSD) Gorham-Stout disease;(BRBNS) Blue rubber bleb nevus syndrome. *Some patients included in the clinical trial have a genetic diagnosis.

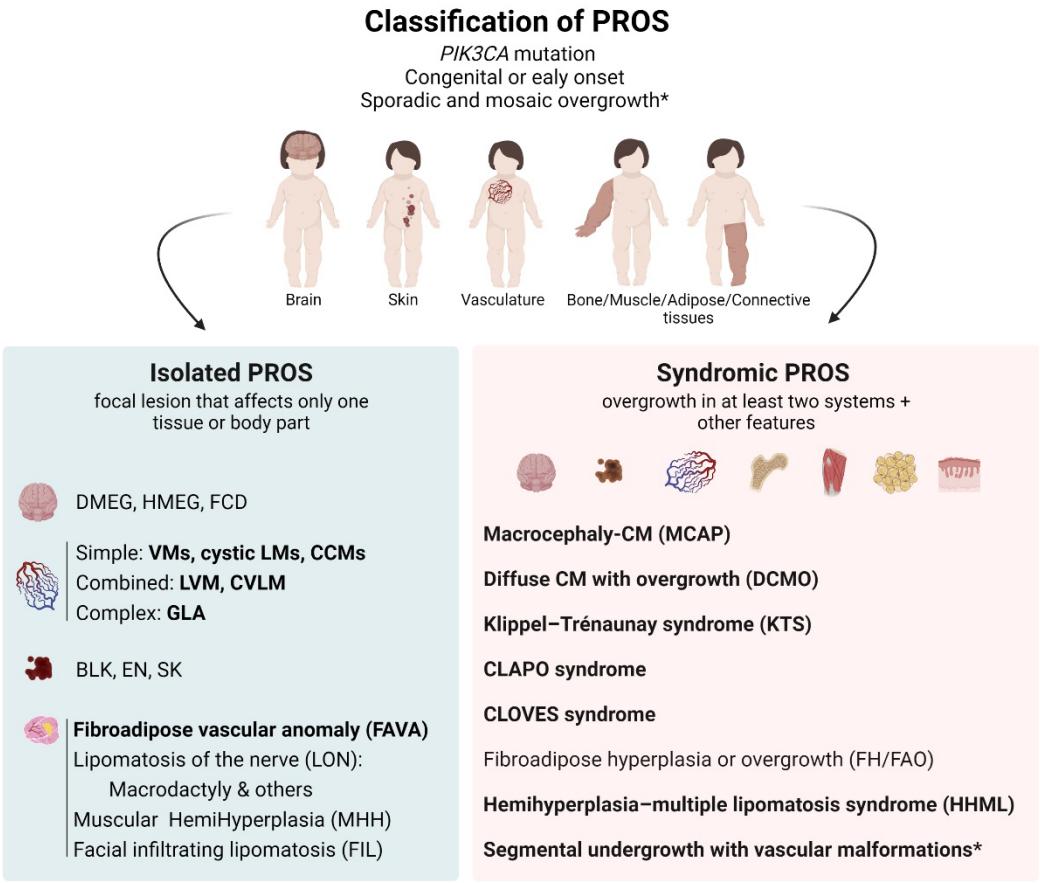

Figure1. Classification of *PIK3CA*-related overgrowth spectrum (PROS) disorders. PROS patients share a variety of clinical manifestations, with overgrowth being found in different tissues such as brain, skin, vasculature, bone, muscle, adipose and connective. PROS disorders are divided in: isolated PROS, when overgrowth is locally found affecting only one tissue or body part, and syndromic PROS, when overgrowth is not focal, and it is presented in at least two different systems together with other features[59]. Belong to isolated PROS: (1) Brain overgrowth: DMEG- Dysplastic Megalencephaly; HMEG- HemiMegalencephaly; FCD- Focal Cortical Dysplasia; (2) Vascular overgrowth/malformations: VMs-Venous Malformations; LMs-Lymphatic Malformations; CCMs-Cerebral Cavernous Malformations; LVMs- Lymphatic-Venous Malformations; CVLMs- Capillary-Venous-Lymphatic Malformations; GLA- Generalized Lymphatic Anomaly; (3) Skin lesions: BLK-Benign Lichenoid Keratosis; EN- Epidermal Nevi; SK- Seborrhoeic Keratosis; (4) Combined lesions (2 or more tissues locally affected): FAVA- Fibro Adipose Vascular Anomaly, LON- Lipomatosis of Nerve (Macrodactyly and others), MHH- Muscular HemiHyperplasia and FIL- Facial Infiltrating Lipomatosis. Belong to syndromic PROS: MCAP- Megalencephaly-Capillary malformation (CM) syndrome; DCMO- Diffuse Capillary Malformation (CM) with Overgrowth; KTS- Klippel-Trenaunay syndrome; CLAPO syndrome- Capillary vascular malformation of the lower lip, Lymphatic malformations of the head and neck, Asymmetry and Partial/generalized Overgrowth; CLOVES syndrome- Congenital Lipomatous Overgrowth, Vascular malformations, Epidermal nevi, and Skeletal/Spinal abnormalities; FH/FAO- FibroAdipose Overgrowth; HHML- HemiHyperplasia-Multiple Lipomatosis; Segmental undergrowth with vascular malformations. *Recently, some patients with activating *PIK3CA* mutations presented tissue undergrowth together with vascular malformations with or without the presence of tissue overgrowth[197].

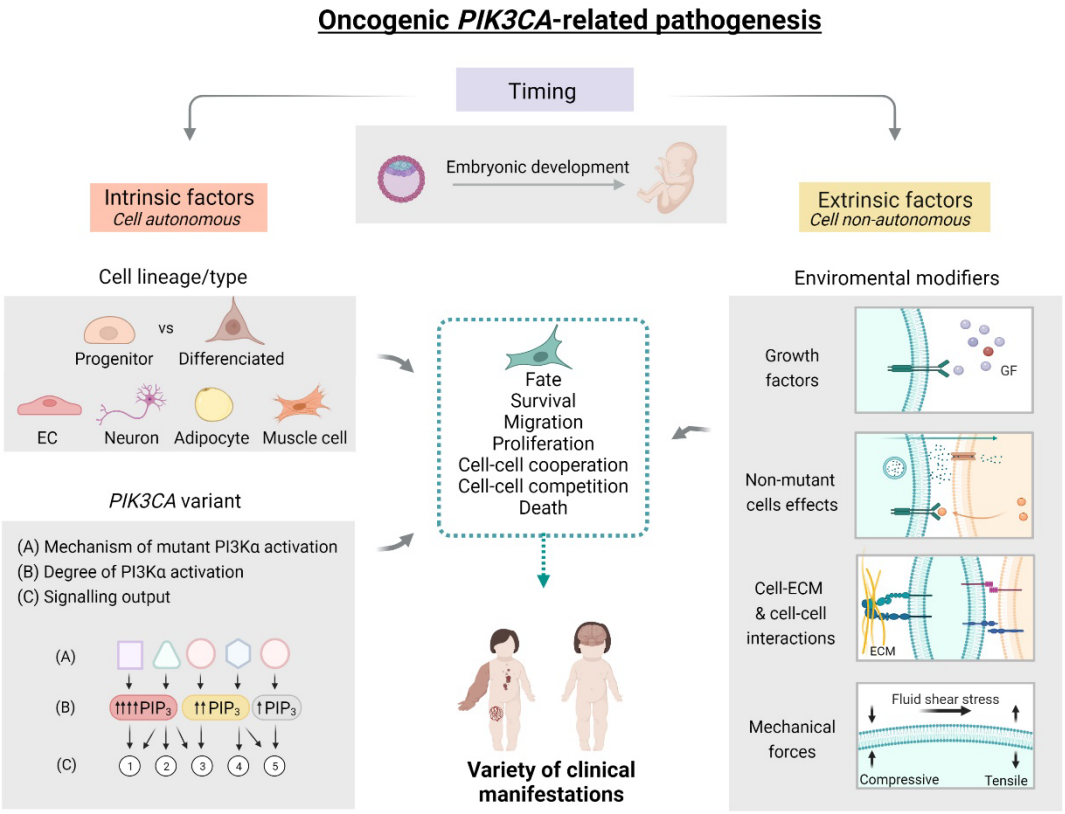

Figure 2: Intrinsic and extrinsic factors that define *PIK3CA*-related pathogenesis in PROS. Somatic activating *PIK3CA* mutations are acquired during embryonic development. However, a variety of elements are at play when defining the ultimate clinical outcome: the developmental time when the genetic error occurs, the type of cell lineage that acquire a *PIK3CA* mutation, the degree and mechanism of PI3Kα activation (*PIK3CA* variant) and the spatiotemporal environmental modifiers of PI3Kα signalling.

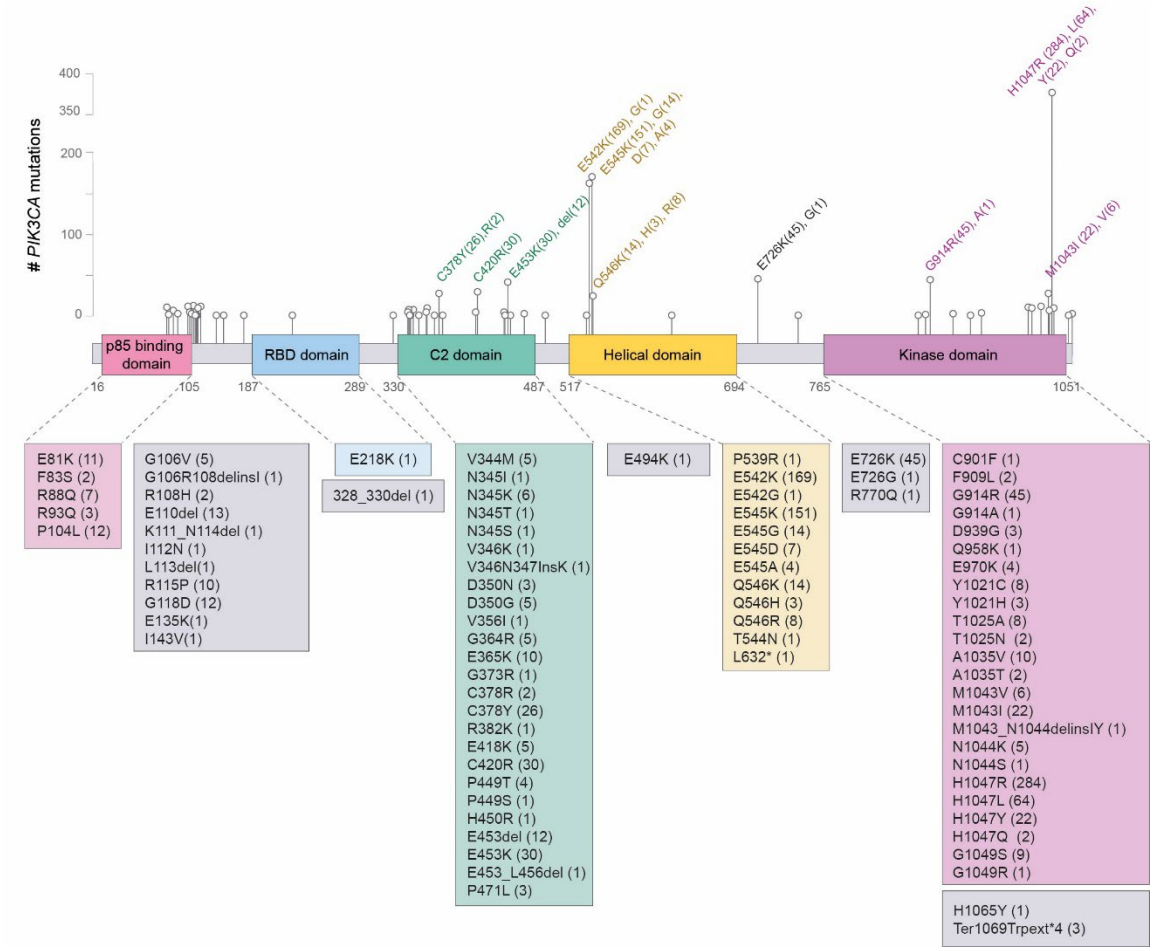

Figure 3. *PIK3CA* variants in PROS. Summary of the documented mutations in *PIK3CA* that have been found in 1173 PROS patients[12,42–58,61,65–69,72–74,76,78–80,89–91,93–95,108,136,150,160,161,197–229,229–266]. Two patients showed two different mutations in *PIK3CA*.

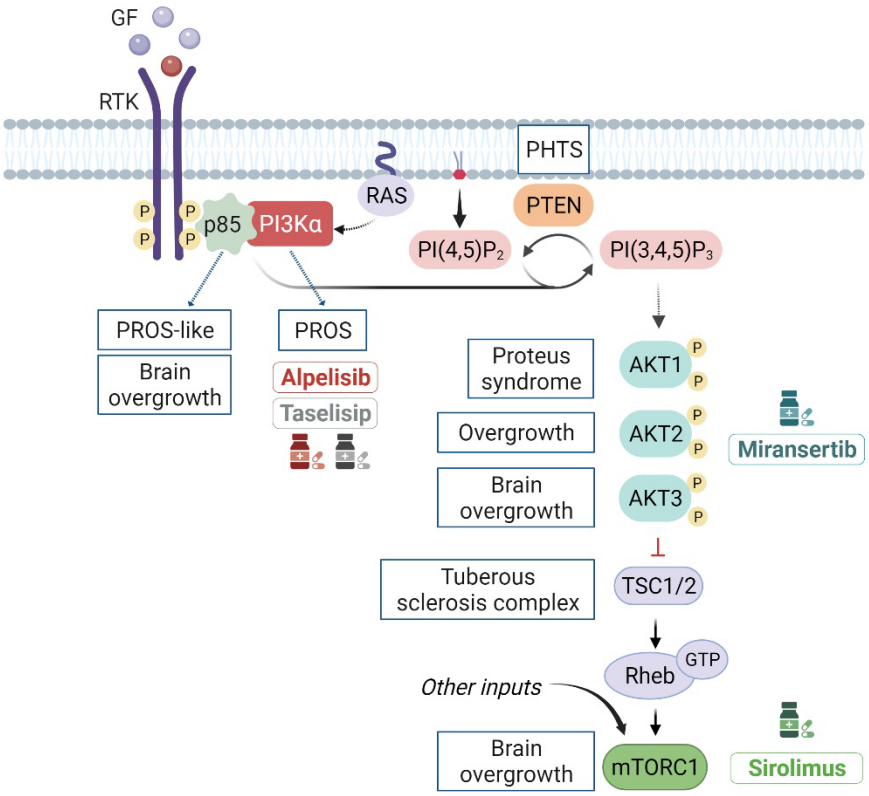

Figure BOX1. PI3K-related congenital disorders and tested inhibitors. Tested or current inhibitors in clinical trials for PROS are alpelisib (PI3Kα isoform specific inhibitor), taselisip (pan-PI3K inhibitor), miransertib (pan-AKT inhibitor) and sirolimus (mTOR inhibitor). GF- Growth factor; RTK- Receptor tyrosine kinase; PROS- *PIK3CA*-related overgrowth spectrum; PHTS- PTEN hamartoma tumor syndrome.