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Abstract

Background
Postzygotic activating *PIK3CA* variants cause several phenotypes within the *PIK3CA*-related overgrowth spectrum (PROS). Variant strength, mosaicism level, specific tissue involvement and overlapping disorders are responsible for disease heterogeneity. We explored these factors in 150 novel patients and in an expanded cohort of 1007 *PIK3CA*-mutated patients, analysing our new data with previous literature to give a comprehensive picture.

Methods
We performed ultradeep targeted next-generation sequencing (NGS) on DNA from skin biopsy, buccal swab or blood using a panel including phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway genes and *GNAQ*, *GNA11*, *RASA1* and *TEK*. Additionally, 914 patients previously reported were systematically reviewed.

Results
93 of our 150 patients had *PIK3CA* pathogenetic variants. The merged PROS cohort showed that *PIK3CA* variants span thorough all gene domains, some were exclusively associated with specific PROS phenotypes: weakly activating variants were associated with central nervous system (CNS) involvement, and strongly activating variants with extra-CNS phenotypes. Among the 57 with a wild-type *PIK3CA* allele, 11 patients with overgrowth and vascular malformations overlapping PROS had variants in *GNAQ*, *GNA11*, *RASA1* or *TEK*.

Conclusion
We confirm that (1) molecular diagnostic yield increases when multiple tissues are tested and by enriching NGS panels with genes of overlapping 'vascular' phenotypes; (2) strongly activating *PIK3CA* variants are found in affected tissue, rarely in blood: conversely, weakly activating mutations more common in blood; (3) weakly activating variants correlate with CNS involvement, strong variants are more common in cases without; (4) patients with vascular malformations overlapping those of PROS can harbour variants in genes other than *PIK3CA*.

Key messages

What is already known on this topic?

- The clinical manifestations of overgrowth caused by activating somatic mutations of PIK3CA are numerous and clinically heterogeneous and are collectively included in the umbrella acronym of PROS (PI3KCA-related overgrowth spectrum).

What this study adds?

- This comprehensive systematic review of >1000 cases with PROS and PROS-like conditions shows that defining clear-cut genotype/phenotype correlations for all PROS entities is not possible.
- Just a few genotype/phenotype correlations are possible, including by variants/domains interested, variant activating strength and variant allele frequency.
- Angiogenic signalling pathways genes can be responsible for PROS-resembling phenotypes.

How this study might affect research, practice or policy?

- A 10 hotspots tailored first-tier approach will diagnose 70% of cases, reducing analytic costs, the subsequent molecular approach should include angiogenic signalling pathways genes to increase the diagnostic yield.
- There is an urgent need to investigate phenotypes overlapping PROS without a known genetic alteration.

Introduction

Gain-of-function (GoF) somatic variants in the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*; MIM #171834) oncogene are widely observed in human cancers.[1] Activating *PIK3CA* variants have an impact on kinase activity and downstream signalling through the AKT/mammalian target of rapamycin (mTOR) pathway, resulting in a variable degree of hyperactivation of the phosphatidylinositol 3-kinase (PI3K) network, leading to the concept of 'variant strength', where strong variants are more highly activating and weak variants are less activating.[2] Among the numerous *PIK3CA*-activating variants identified in cancer, three mutational hotspots are characterised by strong oncogenic activity (p.His1047Arg, p.Glu542Lys and p.Glu545Lys) cumulatively accounting for 80% of cases.[3 4] Somatic activating variants in *PIK3CA* have more recently been linked also with the heterogeneous spectrum of rare congenital segmental overgrowth phenotypes grouped under the term PROS (*PIK3CA*-related overgrowth spectrum, an acronym adopted in 2015).[5 6] In the PROS, excessive PI3K signalling leads to excessive proliferation of mesodermic and/or ectodermal tissues from embryogenesis onwards: the variable combination of different kind of overgrown tissues and diverse body regions affected results in a wide phenotypic spectrum.[5] The latter include:

- Fibroadipose (and bone) hyperplasia or overgrowth without (FAO) or with vascular anomalies;
- Hemihyperplasia multiple lipomatosis (HHML);
- Type I macrodactyly;
- Muscle or fibrous hyperplasia (MH, FH);
- Facial infiltrating lipomatosis (FIL);
- Congenital lipomatous overgrowth, vascular malformations, epidermal nevi, scoliosis/skeletal and spinal syndrome (CLOVES, OMIM #612918);
- Klippel-Trenaunay syndrome (KTS, OMIM #149000);
- Diffuse capillary malformation with overgrowth (DCMO);
- Megalencephaly-capillary malformation polymicrogyria syndrome (MCAP, OMIM #602501; previously/also known as macrocephaly-capillary malformation (M-CM) or macrocephaly-cutis marmorata telangiectasia congenita);
- Hemimegalencephaly (H-MEG), dysplastic megalencephaly (D-MEG) and focal cortical dysplasia (FCD);
- Capillary malformation of the lower lip, lymphatic malformation of the face and neck, asymmetry and partial/generalised overgrowth (CLAPO, OMIM #613089).

Epidermal nevi, seborrheic keratoses, benign lichenoid keratosis and isolated vascular malformation (IVM, including lymphatic, capillary and venous malformations) are considered part of the PROS spectrum as variants in *PIK3CA* have sometimes been found, although these are conditions well known to have genetic heterogeneity.

As has been speculated by previous authors, an individual PROS disease phenotype is likely determined in embryonal life based on three factors: the functional consequences of the mutation (strength, ie, degree of GoF), the timing of onset during fetus development and, consequently, the degree of mosaicism and the tissues involved.[5 7] Tissue overgrowth severity and evolution over time are connected with the degree of hyperactivation in PI3K signalling induced by the several classes of *PIK3CA* variants, including strong, intermediate and weak mutants.[8] Therefore, variant strength also likely affects the phenotype in both embryonal and later life.

Ultradeep sequencing or alternative highly sensitive molecular techniques have allowed for the identification in PROS of a plethora of *PIK3CA* pathogenetic variants spanning the entire coding sequence and with different levels of mosaicism. Even these advanced and highly sensitive genetic techniques identify causal mutations in only a fraction of individuals with clear-cut PROS or overlapping phenotypes: no pathogenic variants are found in 15%–60% in different case series of *PIK3CA*.[9–13] Variants in genes of angiogenic signalling pathways, including *GNAQ*, *GNA11*, *RASA1* and *TEK* have been shown to contribute to some of these clinically typical but *PIK3CA* mutation negative cases.[14]

Prior studies have explored the wide spectrum of *PIK3CA* variants and the heterogeneity of related phenotypes in PROS.[9–32] The aim of this study was to describe genotypes and phenotypes of a previously unreported cohort of 150 patients with overgrowth syndromes with clinical features in the PROS spectrum, which taken together provide the first comprehensive systematic review of >1000 *PIK3CA*-mutated cases.

Materials and methods

Patient population

The cohort was set up including cases from 14 referral centres from Italy that submitted samples for genetic testing to a central laboratory (Department of Biomedical Sciences and Human Oncology of the Medical Genetics of the University of Bari 'Aldo Moro', NR). A total sample of 200 consecutive cases was collected between January 2010 and February 2021. According to accepted diagnostic criteria, 150 individuals (76 females and 74 males) met the clinical criteria for PROS.[5 32–39]

Phenotype classification

According to literature, patients were classified based on clinical phenotype as following: MCAP/M-CM,[33] D-MEG/H-MEG/FCD,[34] CLOVES,[35 36] FAO, FIL,[5 37] HHML,[37] KTS, DCMO,[40] IVM, CLAPO,[38] FH/MH,[32] isolated macrodactyly[39] and epidermal naevus syndrome (ENS) (see online supplemental file 1 for definitions). In the statistical analysis, we grouped

together entities with overlapping clinical features in which a clear-cut differentiation was not always easy or possible: for example, KTS+DCMO, MCAP+M-CM, FAO+FIL+HHML, IVM+CLAPO, FH+MH.
Based on the involvement of the central nervous system (CNS), the cohort was further divided into two subgroups: (a) CNS phenotypes, when MRI study detected CNS abnormalities such as MCAP/M-CM, H-MEG, D-MEG or FCD and (b) non-CNS phenotypes, if no CNS involvement was present. MRI study was performed in cases with macrocephaly, neurological symptoms (neurodevelopmental disorders, epilepsy) and cases with skin lesions or tissue overgrowth localised to the head or face (n=41).
A comprehensive literature review (updated in April 2021) was performed, searching for papers in PubMed using the following keywords: PROS, *PIK3CA*-related overgrowth spectrum disorders, MCAP, M-CM syndrome, KTS, CLOVES syndrome, CLAPO syndrome, fibrous hyperplasia, fibro adipose overgrowth, HHML, megalencephaly, dysplastic megalencephaly, cortical dysplasia and ENS, and including papers in the references and with the 'similarity' attribute from the PubMed search engine. The final revision included only papers providing sufficient clinical description for a diagnosis of PROS and reporting the related variants in *PIK3CA*. Detailed clinical data of the final cohort were collected using a standard spreadsheet completed by referring clinicians who were asked to update the clinical information of patients with the last follow-up visit (online supplemental file 2). Photographic documentation was required by the laboratory in specific cases (n=26), where the classification was not clear from the referring clinician description. All material was blindly reviewed by the two expert clinicians (CL, AM). They independently provided a diagnosis and subsequently discussed and agreed on the phenotypic allocation of these examined cases.
Clinical and molecular data of patients collected during the literature review were included in a database, and two expert clinicians separately evaluated the clinical features to provide a final diagnosis. In case of no agreement about the clinical description, a third expert opinion was required to make the final decision (n=18). Patients for which the clinical report provided a molecular diagnosis without a recognisable, clear phenotype were classified as 'other/undefined' patients in the present study.

Sample processing
Genomic DNA was extracted from peripheral venous blood, fresh or frozen biopsies and buccal swab samples using the QIAamp Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and quantified on a Bio Spectrometer Plus (Eppendorf, Hamburg, Germany). Genomic DNA samples extracted from skin biopsies of affected body regions were analysed in 128 patients. In 80 cases with a PROS phenotype characterised by either vascular malformation or naevi, the skin biopsy was performed in the affected body region. In 48 cases without an obvious vascular lesion or naevus (ie, (H/D)-MEG/FCD, FIL, macrodactyly, FAO, FH/MH, HHML), the skin biopsy was performed over the overgrown body region. Both blood and buccal swab DNA was available for 41 individuals; for 7 DNA was only available from blood, and in 1 from swabs only. For 21 patients, DNA samples extracted from skin biopsies were unavailable. Among these, 14 patients were tested on DNA derived from blood and swab, and 7 on blood DNA only.ù

Targeted next-generation sequencing
The next-generation sequencing (NGS) analysis was performed on genomic DNA from each sample type with two AmpliSeq Illumina Custom DNA Panels (2021 Illumina). The first custom panel included 21 genes involved in the PI3K/AKT/mTOR pathway (ie, *PIK3R1, PIK3R2, PIK3CA, PTEN, PDK1, PDK2, KRAS, AKT1, AKT2, AKT3, RICTOR, MAPKAP1, MLST8, MTOR, IRS1, GAB1, GAB2, THEM4, MAPK8I1, PTPN11, RAPTOR*).[18] Patients with a wild-type (*wt*) *PIK3CA* allele at first test underwent a re-analysis by NGS using a custom panel including also *RASA1*, *TEK*, *TSC2*, *GNAQ*, *TSC1*, *DEPDC5*, *CCND2*, *NPRL3* and *GNA11*. Sequencing runs were carried out according to the manufacturer's protocol on a MiSeq Instrument (Illumina, San Diego, California, USA). Data analysis was performed using Local Run Manager software V.3 (Illumina). Reads were aligned to the hg19 human reference genome, and alignments were visually verified with the software Alamut V.2.15 (Interactive Biosoftware). NGS was performed to obtain an average, unique on-target read depths >1000×. Such coverage is the minimum required in order to detect variants at very low levels (1% variant allele fraction (VAF) at a depth of 1000×). Our analysis produced for each sample a mean coverage ranging from 2500× to 3000×. The quality of the sequence run was monitored by Sequencing Analysis Viewer (Illumina, Hayward, USA), and BaseSpace, Variant Interpreter (Illumina) was used to interpret variants. Based on our experience, the Illumina Platform included the Pisces algorithm delivering confidence in discriminating variants from noise down to the VAF of 1%; only variants with a VAF >1% were considered and studied according to different levels of evidence supporting pathogenicity in cases of somatic mosaicism, as previously described.[18 41] In brief, assessed criteria included variant type, location in protein domain, in silico impact to protein, VAF and low allele frequency in the population. Variants with suggestive pathogenicity were further investigated, taking into consideration functional data in the literature and four publicly available databases of human genomic variation (ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/, dbSNP: https://www.ncbi.nlm.nih.gov/snp/, dbSNP, gnomAD: https://gnomad.broadinstitute.org and COSMIC: https://cancer.sanger.ac.uk/cosmic).

Statistical analysis
The χ^2 (or Fisher's exact when the categories examined had >20% of cells with expected frequencies <5) test was used to test for differences between phenotype groups. Differences were considered as significant at a p value <0.05. In order reduce type I errors, multiple testing corrections were performed using verifying p values with both Benjamini-Hochberg procedure post hoc Bonferroni test.

Results

Patient and level of mosaicism in PIK3CA by sample types

The study included a final sample of 150 unrelated patients (76 females and 74 males) aged 14.3±12.2 years (median 10.6 years, range 0.2–67.0 years). Patients' phenotypes are summarised in table 1. Thirty-nine (26%) patients were included in the CNS phenotype group and 111 (74%) patients in the non-CNS phenotype group. Overall, 93/150 patients (62%) were found to harbour a pathogenic variant in *PIK3CA*, while *PIK3CA* variants were not detected in 57/150 (38%) patients. Figure 1 and online supplemental table 1 detail the 81 pathogenic variants in *PIK3CA* described worldwide and the corresponding phenotypes of 1007 patients with PROS, including our cohort (case ID 915-1007) and a further 914 PROS cases from the literature review (case ID 1-914). Figure 2A displays the 10 most common variants in *PIK3CA* reported among the 1007 patients with PROS, responsible for >70% of cases. Figure 2B shows the distribution of phenotypes according to *PIK3CA* domains, and online supplemental table 2 details the distribution of the variants in the PROS phenotypes.

DNA extracted from blood or buccal swab was accepted as the primary sample for all cases in which a tissue biopsy could not be performed (n=22/146) and as secondary samples in cases first tested on tissue-extracted DNA (n=35 on blood and n=20 on buccal swab). Ninety-five patients (63%) had a single sample tissue tested: 47 (49%) had a pathogenic variant in *PIK3CA*, 9 (10%) in one of the angiogenic signalling pathways genes. Forty patients (27%) had two different tissues samples tested, with 33 (82%) with pathogenic variant in *PIK3CA*. Fifteen (10%) patients had three different sample tissues tested, of which 13 (87%) had a detected pathogenic variant in *PIK3CA*. Among the patients with a *wt PIK3CA* allele, this was confirmed on skin or blood DNA in six cases and one case, respectively. Fourteen of the 21 patients (67%) who did not have skin biopsy material available harboured a *PIK3CA* variant in blood (n=10, 5 on swab DNA as well) or in swab DNA (n=4, *wt* on blood). Figure 2C displays the percentages of somatic mosaicism (VAF) found in cases with multiple tissues tested (detailed in online supplemental table 3): 7/10 (70%) of cases with a variant detected in blood or swab DNA samples were MCAP/M-CM. Patients with *PIK3CA* variants had on average 2.5±1.4 different tissues affected by overgrowth vs 1.8±1.1 of the patients with *wt* variants (p=0.008). *PIK3CA* variants were found in 45.6% of patients with one tissue affected, 46.4% of those with two, 71.4% of those with three, 77.8% of those with four and 83.3% of those with more than four tissues affected (p<0.001). With respect to skin biopsy site, of the 80 patients biopsied over a skin vascular malformation or naevus we observed 53 cases with a positive molecular test (66.3%), whereas of the 48 biopsied on skin overlying an overgrown tissue (eg, muscular/lipomatous overgrowth) but without vascular malformation or naevus we reported 35 cases with a positive molecular test (72.9%, p=0.555).

Phenotype and correlations with the strength of activation and functional domains involved in *PIK3CA* variants

The distribution of the variants across the *PIK3CA* domains in the present study was different when compared with that observed in cases from literature (p<0.001), as we found fewer cases with variants in the helical domain and more spanning out over other *PIK3CA* domains (figure 2C). The distribution of CNS phenotypes (26/93, 30%) was similar to those reported in literature (278/914; 30.3%, p=0.722) (figure 2D). Based on variant strength already reported,[2] we observed that the p.His1047Arg variant,[2] established as the most common variant and known to have strong oncogenic activity, was distributed throughout all PROS phenotypes. Similarly, the second most common strong variant, p.Glu542Lys,[2] was detected in all PROS phenotypes but was more common in CLAPO/IVM and CLOVES. Likewise, p.Glu545Lys was widely spread out among phenotypes, with the exception of FH/MH.

MCAP/M-CM was associated with almost the entire mutational spectrum of the *PIK3CA* gene; conversely, some of the recurrent pathogenetic variants seem to exclusively result in MCAP/M-CM, such as the p.Arg88Gln, p.Pro449Thr, p.Pro471Leu, p.Gly1049Ser, in addition to other less frequent variants. Interestingly, we observed that some of the *PIK3CA* variants previously reported were exclusively associated with (H/D)-MEG/FCD phenotypes, including p.Ile112Asn, p.Asn354Ser, p.Pro449Ser, p.Arg770Gln and p.Thr1025Asn. Similarly, some *PIK3CA* variants were identified only among IVM/CLAPO: p.Phe83Ser, p.Glu109del and p.Ile143Val. The p.Val346insLys, p.Pro539Arg and p.Asn1044Ser variants were almost exclusively detected in KTS/DCMO cases, while the p.His450Arg, p.Cys901Phe and p.Gly914Ala variants were detected in CLOVES cases. A clear difference comparing CNS and non-CNS phenotypes was evident in the distribution of variants according to their strength: strong variants and hotspots[2] caused most of the non-CNS phenotypes (79%) and a small fraction of the CNS phenotypes (12%, p<0.001, figure 2E). Intermediate variants were more frequently found in the CNS subgroup than in non-CNS, weak variants were only present in non-CNS patients. However, this observation has limited value given that for many of the variants found strength has not been formally established.

All remaining variants with unknown functional impact on *PIK3CA* activity were observed with the highest percentage in CNS patients rather than in non-CNS patients (figure 2E).

In patients with detected *PIK3CA*-VAF <5% (ie, <10% of tissue involved), we did not observe a correspondence between tissue extension and phenotype severity (figure 3A-K). In contrast, the strong variants p.His1047Arg, p.Glu542Lys and p.Glu545Lys corresponded to severe phenotypes despite a very low VAF.

Based on functional studies addressing oncogenic activity classification from strong to weak,[4] we observed a skewed distribution of such variants in CNS and non-CNS phenotypes (p<0.001): strong variants were more commonly detected in the non-CNS-cohort compared with the CNS cohort (figure 2F).

Angiogenic signalling pathways genes variants and related phenotypes

Fifty-seven of the 150 patients had no detectable *PIK3CA* mutant alleles. Among 57 participants without a detectable *PIK3CA* mutant allele, 31 were screened using an angiogenic signalling pathways gene panel and 11/31 had detectable mutant alleles for one of the genes in the panel. This led to identification of a pathogenic variant in further 11 patients: 4/11 (36.3%) harboured a variant in *GNA11*, 3/11 (27.3%) in *RASA1*, 3/11 (27.3%) in *GNAQ* and 1/11 (9.1%) in *TEK* (table 2).

Among these, nine were tested on skin DNA and one on genomic DNA from a blood sample. Within individuals carrying the somatic p.Arg183Cys variant in the *GNA11* gene, two were clinically classified as KTS, and two as MCAP/M-CM. Whereas those patients harbouring variants in the *RASA1* gene (p.Lys661IlefsTer18, p.Trp181Ter and p.Tyr256Ter, two germinal and one somatic) were all clinically classified as KTS. Phenotypes of the patients with pathogenic variants in an angiogenic signalling pathways genes overlapped with patients carrying *PIK3CA* variants, including the clinical diagnostic criteria by Keppler-Noureil *et al* (figure 3L–O).[5]

Discussion

The wide phenotypic and molecular heterogeneity of PROS lends itself to an exploration of genotype/phenotype correlations. These important correlations, already highlighted in some studies,[9 10 13] appear rather weak, probably due to the somatic nature of PROS. Mirzaa *et al* first showed that several factors, such as tissue distribution, levels of mosaicism and variant strength, together impact on the wide spectrum of phenotypes.[10] FAO, HHML and macrodactyly were mostly linked to variants within the catalytic domain of *PIK3CA*, while CLOVES mutations were mostly in the coiled domain.[16] Variants in the *PIK3CA* cancer-related hotspots E542, E545 and H1047 were more often associated with CLOVES, while G914R mutations were mostly associated with MCAP.[9] Moreover, weak variants were mainly reported in patients with CNS involvement, while strong ones in cases without.[11]

In this study, we combined multiple cohorts from literature with the first Italian multicentric cohort looking for correlations on a large sample—>1000—patients with PROS. Based on clinical and molecular data review, we have drawn up a comprehensive list of all the 81 pathogenic variants described so far, relating them to their respective phenotypes.

Our data confirm that *PIK3CA* variants in PROS are spread out across the entire gene: 10 variants account for >70% of the total variant load, with the three most common mutational hotspots observed in cancer (p.His1047Arg, p.Glu542Lys and p.Glu545Lys) representing nearly 50% of cases. These data support a mutation-specific tailored molecular approach for the most frequent variants as a first-tier test, aiming at reducing analytic costs and turnaround time. In the small fraction (30%) of negative cases, the analysis should then be expanded with the NGS approach.

We observed some *PIK3CA* variants to be exclusive to some clinical entities within the PROS, such as (H/D)-MEG/FCD, IVM/CLAPO, KTS, MCAP and CLOVES. Conversely, some other variants are widely spread out across all the different phenotypes, hampering the definition of clear genotype-phenotype correlations. When analysing the variants with strength data available, we have replicated and confirmed previous observations[31]: strongly activating variants mostly clustered in the non-CNS phenotypes (79% vs 12% of the CNS group), while *PIK3CA* variants with moderate/intermediate strength were more common in CNS ones (13% vs 5%). Similarly, variants with unknown functional impact were over-represented in CNS phenotypes (75% vs 16% in the non-CNS ones, respectively). This skewed distribution might reflect a selective influence of *PIK3CA* activation in cells during the important step for fate cell lineage decisions during embryogenesis, implying that strong variants are not tolerated during CNS development.[7 42 43]

In most somatic disorders, a higher degree of mosaicism usually contributes to more severe disease.[44] Nevertheless, we observed some severe phenotypes with multiple tissue involvement and widespread overgrowth showing low VAFs of *PIK3CA* variants (<3%; <6% of mutated cells): this may be due to the selection of tissue for analysis (sampling bias) and underscores the importance of an optimal sample collection. The diagnostic yield increases using DNA directly from tissue samples of the affected body region rather than extracted from blood.[10 11] Our results confirmed this observation and suggests testing of multiple tissues and body sites improves the likelihood of finding variants.[28] As an exception to this rule, we confirm that in MCAP variants are frequently found in blood/swab-extracted DNA, as already reported.[5 10 11] The observation of severe phenotypes with low VAFs is intriguing, and allows to hypothesise that mutant cells might exert growth-promoting effects on unaffected cells near or far from them through cell-cell interactions and paracrine growth factors/exosomes.[7] Based on non-cell-autonomous actions, it cannot be excluded that the PI3K signalling could also be enhanced in non-mutated cells.[7]

By testing genes associated with vascular phenotypes in selected *PIK3CA* mutation negative cases, we obtained an increase in the diagnostic rate of >10%; in several cases with phenotypes overlapping PROS, we identified germinal/postzygotic pathogenic variants in *GNAQ*, *GNA11*, *RASA1* and *TEK*, as previously reported.[14 45–49] Specifically, in two patients with KTS/DCMO and in two with MCAP, we detected a somatic p.Arg183Cys pathogenic variant in *GNA11*, previously associated with DCMO, phakomatosis pigmentovascularis and congenital non-progressive hemangioma.[14] Also, three patients with *GNAQ:*p.Arg183Gln variants presented with KTS/DCMO. Furthermore, protein truncating variants in *RASA1*, reported in Parkes Weber syndrome,[46 50–53] were found in three patients (germline or somatic) with a phenotype partially overlapping KTS/DCMO, two of which without typical arteriovenous malformations. In this respect, it seems relevant to point out that the cases we studied have been accumulated over 10 years, a time period in which some clinical definitions have changed, such as that between KTS and DCMO, and this might have impacted on our clinical classification.[40]

Interestingly, *PIK3R1* variants have been recently shown to activate the *PI3K* pathway leading to phenotypes overlapping PROS.[54] Remarkably, we found no variant in such gene: either they are very rare, or this could be due to ascertainment bias

or differences in methodology. It would also be appropriate, in the light of recent evidence, to test cases with overgrowth and lymphatic/vascular anomalies for mosaic RASopathies using a NGS approach including genes of the RAS/MAPK pathways.[55 56]

Our observations underline that the clinical classification of PROS and overlapping disorders according to old nomenclature and nosology in diverse syndromes is burdened by several limitations. On the one hand, the allocation of patient into a diagnostic category is crucial to identify uniform medical implications and to help define phenotypes and facilitate support groups of patients with similar needs and goals. On the other hand, there are many indeterminate cases difficult to classify into a specific disorder, as well as cases with specific phenotypes that show genetic heterogeneity. In complex cases, it is more important to provide specific molecular characterisation and deep phenotyping than to pigeonhole any given clinical presentation into a specific syndrome. This has relevant clinical and patient implications: for example, for patient inclusion in trials criteria may necessitate the detection of *PIK3CA* variants notwithstanding the clinical presentation.[26] Over the past decade, molecular classification of PROS has paved the way to several novel targeted treatment options derived from oncology[57] including direct *PIK3CA* inhibitors,[58 59] such as the alpha-specific Alpelisib (BYL719) recently used in a compassionate access scheme in PROS.[26 60 61] Also drugs reducing oversignalling through the PI3K pathway have been successfully employed in PROS or related/overlapping conditions, such as miransertib (ARQ092), an oral, allosteric pan-AKT inhibitor[62–66] or rapamycin, the first identified mTOR inhibitor.[24 67–73]

The retrospective design of this study is a limitation. Ascertainment bias for the previously published cases reviewed in the paper is another limitation, in particular taking into consideration difficulties and heterogeneity in classifying phenotypes. Additionally, there was no longitudinal evaluation or systematic evaluation and quantification of the severity of the disease. This should be assessed in future studies and clarified to verify the evolution of the clinical picture over time and relate this to the different variants, fully defining their oncogenic potential, as well as defining the oncologic risk in PROS.

In conclusion, this work provides a comprehensive systematic review of the genotypes and phenotypes within the PROS. Clear-cut genotype/phenotype correlations for all PROS entities is not possible. Our analysis mostly confirmed previous observations and the few genotype-phenotype correlations already evidenced[9–32 74]: (a) some variants/domains interested seem to be exclusively associated with a specific phenotype; (b) strong variants are associated with non-CNS phenotypes, while weak/intermediate are associated with CNS ones; (c) there is often no correspondence between the VAF and the phenotype severity; (d) although in PROS *PIK3CA* variants are spread out across the entire gene, 10 hotspots are responsible for >70% of cases, making feasible a tailored first-tier approach to reduce analytic costs; (e) a molecular approach including testing for an angiogenic signalling pathways genes, than can be responsible for PROS-resembling phenotypes, leads to an increase in the diagnostic yield.

These observations will help guide clinicians in the challenging process of patients' diagnosis and stratification as well as in the follow-up. Given the promising outcomes with targeted therapies in PROS, stronger efforts to reduce the fraction of patients without a molecular diagnosis need to be made.

Data availability statement

All data relevant to the study are included in the article or uploaded as supplementary information. not applicable.

Ethics statements

Patient consent for publication

Consent obtained from parent(s)/guardian(s)

Ethics approval

Written informed consent was obtained from patients or guardians to perform the genetic tests for diagnostics and research purposes on peripheral blood, tissue biopsy, or other samples, according to the local ethic committee's policy (approval code study: 6743 N°0020062/05/03/2021, Policlinico of Bari, Italy and 00086/2022, Città della Salute e della Scienza of Torino, Italy). Written informed consent to publish photographs for clinical and research purposes was also collected for selected cases.

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Table 1
Phenotypes and mutated genes in our cohort of PROS patients

Phenotype	N	*PIK3CA*-mut	Vascular genes	Wild-type	Positive samples/total		
					Skin biopsies	Swab	Blood
MCAP/M-CM	34	26	2	6	17/20	12/20	7/21
(H/D)-MEG/FCD	5	3	–	2	2/2	0/1	2/3
CLOVES	5	5	–	–	5/5	0/2	0/2
KTS/DCMO	40	17	8	15	25/40	1/4	0/8
IVM	10	4	1	5	4/8	2/3	2/5
FIL	2	2	–	–	2/2	0/1	0/1
CLAPO	3	1	–	2	1/3	–	–
Macrodactyly	24	21	–	3	21/24	0/3	0/11
FAO	8	4	–	4	4/8	0/1	0/4
FH/MH	15	9	–	6	8/14	0/3	1/8
HHML	2	1	–	1	1/2	–	–
Epidermal naevus	2	–	–	2	0/2	–	–
Total	**150**	**93**	**11**	**47**	**93/130**	**15/38**	**12/63**

CLAPO, capillary malformation of the lower lip, lymphatic malformation of the face and neck, asymmetry and partial/generalised overgrowth; CLOVES, congenital lipomatous overgrowth, vascular malformations, epidermal nevi, scoliosis/skeletal and spinal syndrome; DCMO, diffuse capillary malformation with overgrowth; D-MEG, dysplastic megalencephaly; FAO, fibroadipose overgrowth; FCD, focal cortical dysplasia; FH, fibrous hyperplasia; FIL, facial infiltrating lipomatosis ; HHML, hemihyperplasia multiple lipomatosis; H-MEG, hemimegalencephaly; IVM, isolated vascular malformation; KTS, Klippel-Trenaunay syndrome; MCAP, megalencephaly-capillary malformation polymicrogyria syndrome; M-CM, macrocephaly-capillary malformation; MH, muscle hyperplasia; PROS, *PIK3CA*-related overgrowth spectrum.

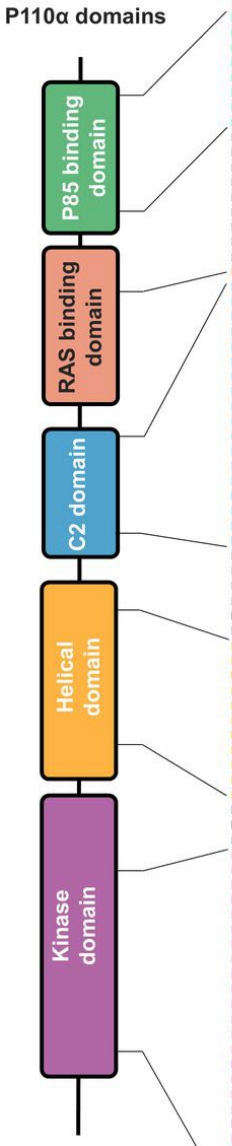
Table 2
Phenotypes and genotypes of the 10 presumptive PROS patients harbouring pathogenetic variants in 'vascular' genes

ID	Gene	Variant	Variant allele frequency % (tissue)	Phenotype	Details
88	*RASA1*	p.Lys661IlefsTer18	7.6% (skin)	KTS	Left lower limb hypertrophy, with ipsilateral superficial skin and muscular angiomas
96	*GNAQ*	p.Arg183Gln	9.0% (skin)	KTS	Complete hemi-hyperplasia with overlapping diffuse capillary malformation and venous malformation of the leg
108	*GNA11*	p.Arg183Cys	17.0% (skin)	KTS	Limb overgrowth with bone hyperplasia and capillary malformation
126	*GNA11*	p.Arg183Cys	4.0% (skin)	MCAP	Macrocephaly, syndactyly, overgrown limbs, diffuse capillary-venous malformation, congenital glaucoma with megalocornea
130	*GNAQ*	p.Arg183Gln	2.0%	KTS	Entire right body overgrowth with overlapping diffuse capillary malformation
131	*GNA11*	p.Arg183Cys	3.4% (skin)	KTS	Meningeal vascular malformation, macrocephaly, hemi-hyperplasia, diffuse capillary malformation of the trunk and leg
145	*GNAQ*	p.Arg183Gln	3.2% (skin)	KTS	Mild right lower limb hyperplasia with corresponding capillary malformation, dyspraxia
146	*GNA11*	p.Arg183Cys	1.8% (skin)	MCAP	Macrocephaly, diffuse capillary-venous malformation of the skin and *pia mater*
147	*TEK*	p.Leu914Phe	*4.8% (skin)* *7.6% (vascular malformation*)	IVM(V)	Isolated large capillary-venous malformation of the knee
148	*RASA1*	p.Trp181Ter	*46.5% (swab*)	KTS	Left upper limb hypertrophy with ipsilateral skin angioma also involving the left side of the trunk
149	*RASA1*	p.Tyr256Ter	*50.0% (blood*)	KTS	Valscular malformation of the trunk and arm, deep artero-venous fistula, bone overgrowth with arm hyperplasia

IVM(V), isolated vascular malformation (venous); KTS, Klippel-Trenaunay syndrome; MCAP, megalencephaly-capillary malformation polymicrogyria syndrome; PROS, *PIK3CA*-related overgrowth spectrum.

Figure 1

Distribution of the *PIK3CA* variants by functional domains and by frequencies in the respective *PIK3CA*-related overgrowth spectrum (PROS) phenotypes in the present study and in literature. Mutational hotspots (arbitrarily defined as variants with a frequency >2%) are displayed in bold. *Cases from literature with poor clinical characterisation or undefined phenotype were classified as 'other/undefined'. CLAPO, capillary malformation of the lower lip, lymphatic malformation of the face and neck, asymmetry and partial/generalised overgrowth; CLOVES, congenital lipomatous overgrowth, vascular malformations, epidermal nevi, scoliosis/skeletal and spinal syndrome; DCMO, diffuse capillary malformation with overgrowth; D-MEG, dysplastic megalencephaly; FAO, fibroadipose overgrowth; FCD, focal cortical dysplasia; FH, fibrous hyperplasia; FIL, facial infiltrating lipomatosis; HHML, hemihyperplasia multiple lipomatosis; H-MEG, hemimegalencephaly; IVM, isolated vascular malformation; KTS, Klippel-Trenaunay syndrome; MCAP, megalencephaly-capillary malformation polymicrogyria syndrome; M-CM, macrocephaly-capillary malformation; MH, muscle hyperplasia.



Amino acid change	Our serie	Total	% of the whole	MCAP/M-CM	(H/D)-MEG/FCD	CLOVES	FAO/FIL/HHML	KTS/DCMO	IVM /CLAPO	FH/MH	Macrodactyly	Epidermal Nevus syndrome	Other/undefined*
p.Glu81Lys	1	11	1.1%	90.9%		9.1%							
p.Phe83Ser		1	0.1%						100.0%				
p.Arg88Gln		7	0.7%	100.0%									
p.Arg93Gln		3	0.3%	66.7%									33.3%
p.Pro104Leu	1	11	1.1%	72.7%		18.2%				9.1%			
p.Gly106Arg108delinsIle		1	0.1%										100.0%
p.Gly106Val	1	5	0.5%	20.0%		20.0%		20.0%	20.0%	20.0%			
p.Arg108His	1	2	0.2%		50.0%				50.0%				
p.Glu109del		1	0.1%						100.0%				
p.Glu110del		9	0.9%	22.2%		55.6%		11.1%					11.1%
p.Lys111Asn114del		1	0.1%										100.0%
p.Ile112Asn		1	0.1%		100.0%								
p.Leu113del		1	0.1%						100.0%				
p.Arg115Pro	1	10	1.0%	60.0%					20.0%		10.0%		10.0%
p.Gly118Asp	2	11	1.1%	54.5%		18.2%		18.2%	9.1%				
p.Glu135Lys		1	0.1%	100.0%									
p.Ile143Val		1	0.1%						100.0%				
p.Glu218Lys		1	0.1%										100.0%
p.Val344Met	1	5	0.5%		60.0%			40.0%					
p.Asn345Ile		1	0.1%	100.0%									
p.Asn345Lys	1	5	0.5%			40.0%	40.0%				20.0%		
p.Asn345Thr		1	0.1%	100.0%									
p.Val346Asn347_Ins_Lys		1	0.1%				100.0%						
p.Val346insLys		1	0.1%					100.0%					
p.Asp350Asn	1	3	0.3%	33.3%	33.3%					33.3%			
p.Asp350Gly	1	5	0.5%	60.0%				40.0%					
p.Asn354Ser		1	0.1%		100.0%								
p.Val356Ile		1	0.1%										100.0%
p.Gly364Arg	1	4	0.4%	50.0%	25.0%			25.0%					
p.Glu365Lys	1	10	1.0%	60.0%	20.0%	20.0%							
p.Gly373Arg	1	1	0.1%	100.0%									
p.Cys378Arg		2	0.2%			50.0%							50.0%
p.Cys378Tyr	**4**	**26**	**2.6%**	**73.1%**	**3.8%**	**3.8%**		**7.7%**		**7.7%**			**3.8%**
p.Arg382Lys		1	0.1%										100.0%
p.Glu418Lys		5	0.5%	100.0%									
p.Cys420Arg	**2**	**26**	**2.6%**			**61.5%**	**3.8%**	**3.8%**	**15.4%**		**11.5%**		**3.8%**
p.Pro449Ser		1	0.1%		100.0%								
p.Pro449Thr		4	0.4%	100.0%									
p.His450Arg		1	0.1%			100.0%							
p.Glu453del	3	12	1.2%	75.0%	8.3%				8.3%	8.3%			
p.Glu453Lys	**3**	**29**	**2.9%**	**24.1%**		**31.0%**	**10.3%**	**10.3%**	**3.4%**	**3.4%**	**10.3%**		**6.9%**
p.Pro471Leu		3	0.3%	100.0%									
p.Pro539Arg		1	0.1%					100.0%					
p.Glu542Gly		1	0.1%	100.0%									
p.Glu542Lys	**6**	**129**	**12.8%**	**0.8%**	**3.9%**	**20.9%**	**8.5%**	**11.6%**	**38.8%**	**1.6%**	**11.6%**	**0.8%**	**1.6%**
p.Glu545Ala		4	0.4%	25.0%				25.0%	25.0%				25.0%
p.Glu545Asp		7	0.7%	71.4%		14.3%		14.3%					
p.Glu545Gly		13	1.3%	7.7%				23.1%				69.2%	
p.Glu545Lys	**4**	**109**	**10.8%**	**9.2%**	**7.3%**	**17.4%**	**1.8%**	**24.8%**	**30.3%**		**6.4%**		**2.8%**
p.Gln546Arg	1	7	0,7%			28,6%		42,9%		14,3%	14,3%		
p.Gln546His		3	0,3%			33,3%			33,3%				33,3%
p.Gln546Lys	2	11	1,1%	18,2%		9,1%	9,1%	27,3%	27,3%		9,1%		
p.Leu632*		1	0,1%										100,0%
p.Glu726Gly		1	0,1%	100,0%									
p.Glu726Lys	**9**	**42**	**4,2%**	**66,7%**		**7,1%**	**2,4%**	**16,7%**	**2,4%**	**4,8%**			
p.arg770Gln		1	0,1%		100,0%								
p.Cys901Phe		1	0,1%			100,0%							
p.Phe909Leu		2	0,2%	50,0%		50,0%							
p.Gly914Ala		1	0,1%			100,0%							
p.Gly914Arg	**3**	**43**	**4,3%**	**76,7%**	**7,0%**	**4,7%**	**2,3%**	**4,7%**		**4,7%**			
p.Asp939Gly	1	2	0,2%	50,0%				50,0%					
p.Gln958Lys		1	0,1%							100,0%			
p.Glu970Lys		4	0,4%	75,0%				25,0%					
p.Tyr1021Cys		7	0,7%	85,7%				14,3%					
p.Tyr1021His		2	0,2%	50,0%		50,0%							
p.Thr1025Ala	1	7	0,7%	42,9%		42,9%		14,3%					
p.Thr1025Asn	1	2	0,2%		100,0%								
p.Ala1035Thr		2	0,2%	100,0%									
p.Ala1035Val	1	10	1,0%	80,0%		10,0%				10,0%			
p.Met1043_N1044delinsTyr		1	0,1%							100,0%			
p.Met1043Ile	**1**	**22**	**2,2%**	**86,4%**	**4,5%**	**4,5%**		**4,5%**					
p.Met1043Val	2	6	0,6%	16,7%		50,0%	16,7%	16,7%					
p.Asn1044Lys	1	5	0,5%			40,0%	20,0%	20,0%		20,0%			
p.Asn1044Ser		1	0,1%					100,0%					
p.His1047Arg	**25**	**236**	**23,4%**	**1,3%**	**2,5%**	**15,3%**	**25,8%**	**5,9%**	**22,5%**	**7,2%**	**16,5%**	**0,4%**	**2,5%**
p.His1047Gln		2	0,2%	100,0%									
p.His1047Leu	**3**	**60**	**6,0%**	**1,7%**		**16,7%**	**26,7%**	**6,7%**	**20,0%**	**1,7%**	**25,0%**	**1,7%**	
p.His1047Tyr	**6**	**20**	**2,0%**	**65,0%**		**15,0%**		**5,0%**	**5,0%**	**5,0%**	**5,0%**		
p.Gly1049Ser		9	0,9%	100,0%									
p.His1065Tyr		1	0,1%	100,0%									
p.Ter1069Trpext*4		3	0,3%	66,7%		33,3%							

Figure 2

Frequency, oncogenic potential, and variant allele fraction (VAF) of *PIK3CA* variants and correlations with phenotypes. (A) The 10 most common *PIK3CA* pathogenic variants observed in the whole 1007 patient cohort (71.7% of total). (B) Enrichment of the *PIK3CA* domain mutated in the *PIK3CA*-related overgrowth spectrum (PROS) phenotypes (1007 patients). (C) Degree of mosaicism (VAF) of the *PIK3CA* variants by sample types (skin, blood and buccal swab) in the phenotypes. (D) *PIK3CA* functional domain mutated in the PROS clinical entities in our cohort (n=93) and in the 1007 cases from literature (p<0.001). (E) *PIK3CA* domain mutated in central nervous system (CNS) (30.1%) and non-CNS (69.9%) phenotypes (p<0.001). (F) Classification of the variant according to oncogenic potential (unknown, weak, intermediate, strong and strong hotspot) in CNS and non-CNS phenotypes (p<0.001). CLAPO, capillary malformation of the lower lip, lymphatic malformation of the face and neck, asymmetry and partial/generalised overgrowth; CLOVES, congenital lipomatous overgrowth, vascular malformations, epidermal nevi, scoliosis/skeletal and spinal syndrome; DCMO, diffuse capillary malformation with overgrowth; D-MEG, dysplastic megalencephaly; ENS, epidermal naevus syndrome; FAO, fibroadipose overgrowth; FCD, focal cortical dysplasia; FH, fibrous hyperplasia; FIL, facial infiltrating lipomatosis; HHML, hemihyperplasia multiple lipomatosis; H-MEG, hemimegalencephaly; IVM, isolated vascular malformation; KTS, Klippel-Trenaunay syndrome; MCAP, megalencephaly-capillary malformation polymicrogyria syndrome; M-CM, macrocephaly-capillary malformation; MH, muscle hyperplasia.

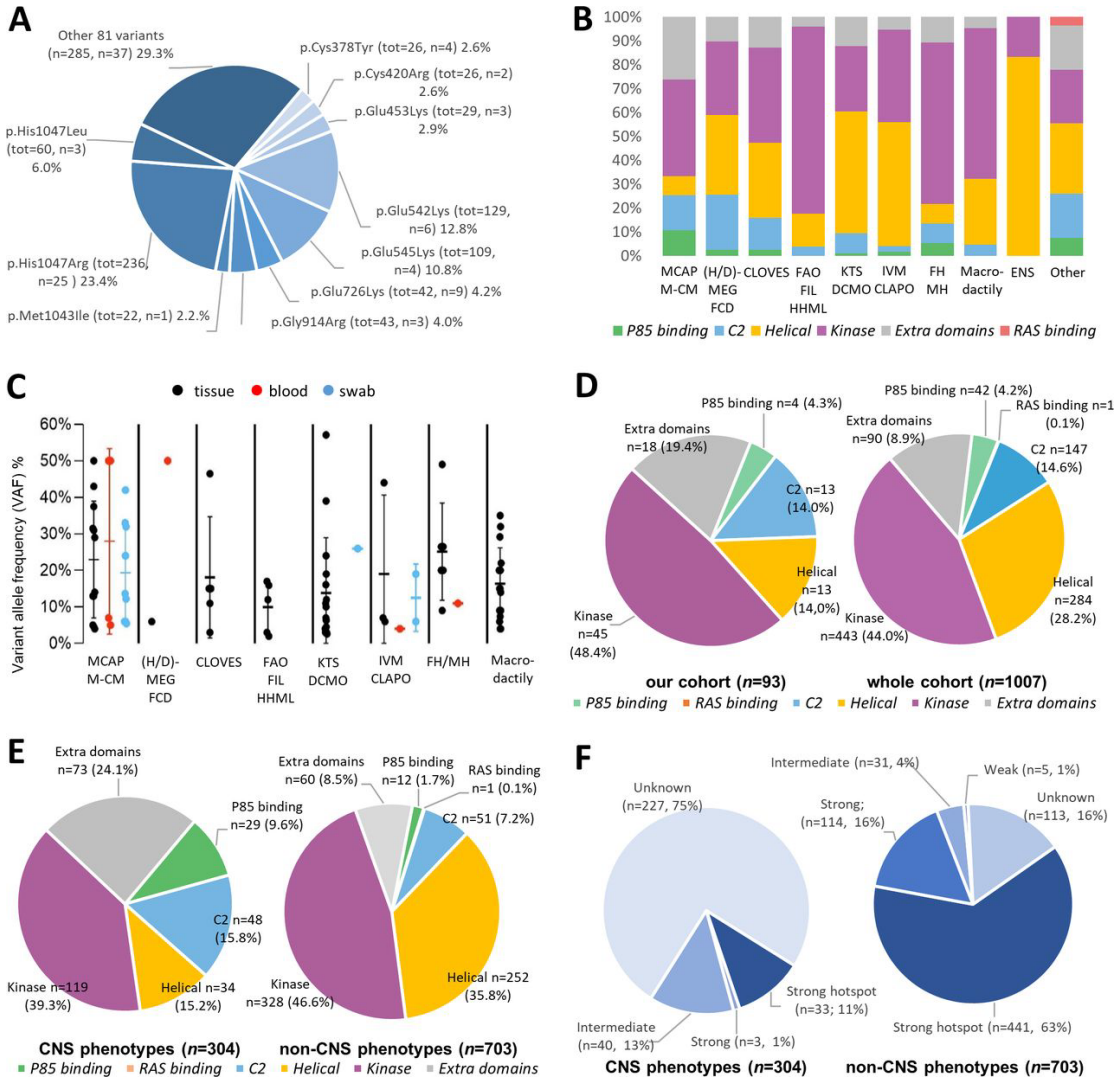


Figure 3
Low variant allele fraction (VAF) and related phenotypes. Patients with VAF <5% show a clinical disease expression largely overlapping that of patients with higher VAFs: (A, B) p.Arg115Pro at 4.6% on tissue DNA (blood DNA wild-type) in a girl aged 2 years with macrocephaly-capillary malformation syndrome (M-CM); (C) feet X-ray of a woman with Klippel-Trenaunay syndrome (KTS) and severe fibroadipose overgrowth (FAO) at the left side with a p.Met1043Val variant at 2.6% VAF on skin DNA; (D, E) a boy aged 11 years with congenital lipomatous overgrowth epidermal naevus syndrome (CLOVES) with a large adipo-lymphangioma of the trunk under the port-wine stain and with fibrous hyperplasia (FH) of the lower limbs with a p.Glu545Lys variant at 3.0% VAF on tissue DNA; (F) facial infiltrating lipomatosis in a girl aged 12 months with p.His1047Arg at 3.0% VAF on tissue DNA; (G, H) a boy aged 15 years with left KTS and syndactyly and a p.Glu453Lys variant at 3.0% VAF on tissue DNA; (I, J) a girl aged 7 years with right hemihyperplasia and a large vascular marformation and KTS with a p.Glu542Lys at 4.0% VAF on tissue DNA); (K) a boy aged 10 years with KTS and p.Cys378Tyr at 4.5% on tissue DNA. Patients with 'vascular' genes and phenotype overlapping *PIK3CA*-related overgrowth spectrum (PROS) (table 2): patient #130 (L), #149 (M) and #131 (N, O).