Tampere University

ANNAMARIA WEITZ-TUORETMAA

Low-Flow Vascular Malformations

Genetics and Quality of Life after Endovascular Sclerotherapy

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ACADEMIC DISSERTATION
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PunaMusta Oy – Yliopistopaino
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To my family

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Kokkola, June 2022
Annamaria Weitz-Tuoretmaa

ABSTRACT

Vascular anomalies represent a broad spectrum of disorders, often misdiagnosed, creating a very challenging patient entity. Management options for vascular anomalies have increased significantly during the last two decades. In addition, novel findings of genetic causes have opened an era for development of targeted precision therapies for these lesions.

The aim of our first study was to evaluate the efficacy of OK-432 sclerotherapy with long-term follow-up results in treatment of lymphatic malformations (LM). Next, we compared the effectiveness of two sclerosing agents polidocanol and ethanol in the treatment of venous malformations (VM) – both intra- and extramuscular – with special emphasis to quality-of-life results after endovascular sclerotherapy. Finally, we took part in an international multicenter genetic study to demonstrate the incidence of non-hotspot phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutations in congenital Lipomatous Overgrowth, Vascular malformations, Epidermal nevi, and Skeletal/spinal abnormalities and/or scoliosis (CLOVES) compared to common and combined LMs.

Thirty-six LM patients were treated with OK-432 in the Department of Otorhinolaryngology and Department of Radiology, Tampere University Hospital, during the years 1999 to 2009. Forty-one patients with a VM were treated with polidocanol in Tampere (n=23) and Turku (n=18) University Hospitals during 2008 - 2013 and the results were retrospectively analyzed by the author. In the first VM study, the results were compared with 44 patients previously treated with ethanol in Tampere University hospital between 1991 - 2001. Identical sclerotherapy technique by the same radiologists was used in both studies. In the second VM study, the results were compared between two groups: intra- and extramuscular malformations. In both malformations studies (LM and VM), all patients answered a symptom questionnaire, in which the patients evaluated the severity of pre-, and post-treatment symptoms. After sclerotherapy, patients answered a specific quality of life (QOL) questionnaire. The final study was an international multicenter study collecting samples from 143 patients with LMs. The prevalence, distribution, and

allele frequency of PIK3CA mutations in different phenotypes was analyzed and evaluated for any genotype-phenotype correlation.

In the first study, magnetic resonance imaging (MRI) results showed that 80% of the LM patients objectively benefitted from the OK-432 sclerotherapy. No serious complications were noted. According to the self-evaluating questionnaire 94% of the patients subjectively felt they benefitted from the treatment. The average follow-up period of 6 years is the longest so far published showing a long lasting effect of OK-432 in the treatment of macrocystic LMs.

According to the QOL questionnaire, 46% of the polidocanol treated VM patients subjectively benefitted from the treatment. However, the post-treatment overall results were significantly better in three of four dimensions of life in the ethanol group. In the ethanol group, there were three notable complications with none in the polidocanol group. With polidocanol treatment intramuscular VMs responded to the treatment comparably to extramuscular malformations. Post-treatment MRI findings did not correlate with either subjective symptoms or QOL-results. Polidocanol sclerotherapy was found to be effective, safe, and well tolerated treatment for VMs. Subjective symptoms and quality of life results are the most important parameters in evaluating the effectiveness of the treatment. Routine MRI for follow-up of VM patients appears redundant and may be omitted.

We found a statistically significant difference in the distribution of hotspot mutations and non-hotspot mutations between common and combined LM compared to the syndromes, to CLOVES, or to unclassified PIK3CA-related overgrowth syndrome (PROS), but not Klippel-Trenaunay syndrome (KTS). Based on our data, repurposing of PI3K signaling pathway inhibitors for the treatment of LMs, whether isolated, combined, or syndromic, have a sound epidemiological and pathophysiological basis. Diagnostic genotyping should thus not be limited to PIK3CA hot-spot mutations.

TIIVISTELMÄ

Veri- ja imusuoniepämuodostumat edustavat laajaa kudosten kehityshäiriökirjoa. Kliinisen monimuotoisuuden vuoksi diagnostiikka on vaikeaa ja potilaat muodostavat hoidollisesti varsin haastavan kokonaisuuden. Näiden epämuodostumien hoitovaihtoehdot ovat lisääntyneet merkittävästä viimeisen kahden vuosikymmenen aikana. Lisäksi viime aikoina löydetyt geneettiset syyt muutosten taustalla ovat avanneet uusia mahdollisuuksia täsmähoitojen kehitykselle.

Ensimmäisen tutkimuksemme tavoite oli arvioida OK-432 skleroterapian pitkäaikaisseurantatuloksia imusuoniepämuodostumien hoidossa. Seuraavaksi vertasimme kahden sklerosoivan aineen, polidocanolin ja etanolin, tehokkuutta laskimoepämuodostumien hoidossa. Vertasimme sekä lihasten sisäisten, että lihasaitioiden ulkopuolisten laskimoepämuodostumien hoitoa, painottaen erityisesti potilaiden elämänlaatua suonensisäisen skleroterapian jälkeen. Lopuksi olimme osana kansainvälistä monikeskustutkimusta, jonka tarkoituksena oli osoittaa non-hotspot PIK3CA mutaatioiden insidenssi CLOVE syndroomassa verrattuna imusuoniepämuodostumiin ja kombinoituihin imu- ja verisuoniepämuodostumiin.

Kolmekymmentä kuusi imusuoniepämuodostumapotilasta hoidettiin OK-342 skleroterapialla Tampereen Yliopistollisen sairaalan korva-nenä- ja kurkkutautien klinikassa sekä toimenpideradiologisessa yksikössä vuosina 1999–2009. 41 laskimoepämuodostumapotilasta hoidettiin polidocanolilla Tampereen (n=23) ja Turun (n=8) Yliopistollisissa sairaaloissa em. klinikoissa 2008–2013 välisenä aikana ja tulokset analysoitiin retrospektiivisesti. Ensimmäisen laskimoepämuodostuma-tutkimuksen tuloksia verrattiin vuosina 1991–2001 etanolilla Tampereen Yliopistollisessa sairaalassa hoidettuihin 44 potilaan tuloksiin. Molemmissa tutkimuksissa samat radiologit käyttivät identtistä skleroterapiatekniikkaa. Toisessa laskimoepämuodostumatutkimuksessa verrattiin lihasten sisäisten ja lihasaitioiden ulkopuolisten laskimoepämuodostumien hoitotuloksia keskenään. Sekä imusuoni-, että laskimoepämuodostuma-tutkimuksissa kaikki potilaat vastasivat oirekyselyyn, jossa he arvioivat oireiden vakavuutta ennen ja jälkeen hoidon. Skleroterapian jälkeen potilaat vastasivat spesifiseen elämänlaatukyselyyn. Viimeisessä monikeskustutkimuksessa kerättiin 143 imusuonipotilaalta kudosnäytteet. Esiintyvyys, levinneisyys ja PIK3CA mutaatioiden alleelitiheys erilaisissa

fenotyypeissä analysoitiin ja arvioitiin sisältäen kaikki mahdolliset genotyyppi-fenotyyppi korrelaatiot.

Ensimmäisessä tutkimuksessa MRI-tulokset osoittivat, että 80% imusuoniepämuodostumapotilaista hyötyi objektiivisesti OK-432 skleroterapiasta. Vakavia komplikaatioita ei havaittu. Oirekyselyn mukaan 94% potilaista koki subjektiivisesti hyötyneensä hoidosta. Tutkimuksen keskimääräinen seuranta-aika oli 6 vuotta, ollen näin pisin tähän asti julkaistuista. Tulokset osoittivat OK-432 hoidon pitkäaikaisen tehon imusuoniepämuodostumien hoidossa.

Elämänlaatukyselyn perusteella 46% polidocanolilla hoidetuista laskimoepämuodostumapotilaista hyötyi subjektiivisesti hoidosta. Kaiken kaikkiaan hoidon jälkeiset tulokset etanoliryhmässä olivat merkittävästi paremmat kolmessa neljästä elämänlaatuosa-alueista. Kolmella etanoliryhmän potilaalla todettiin merkittävä komplikaatio, kun taas polidocanolryhmän potilailla komplikaatioita ei esiintynyt. Polidocanolilla hoidetut lihaksen sisäiset laskimoepämuodostuma-potilaat hyötyivät hoidosta yhtäläisesti verrattuna potilaisiin, joilla epämuodostumat sijaitsivat lihasaitioiden ulkopuolella. Hoidon jälkeiset MRI-löydökset eivät korreloineet subjektiivisiin oireisiin eivätkä elämänlaatukyselyn tuloksiin. Polidocanolskleroterapia todettiin olevan vaikuttava, turvallinen ja hyvin siedetty laskimoepämuodostumien hoidossa. Subjektiiviset oireet ja elämänlaatukyselyn tulokset ovat tärkeimmät mittarit hoidon vaikuttavuutta arvioitaessa. Rutiininomainen MRI-tutkimus laskimoepämuodostumien seurannassa vaikuttaa tarpeettomalta ja täten siitä voidaan luopua.

Geneettisessä tutkimuksessa todettiin merkittävä tilastollinen ero hotspot ja non-hotspot mutaatioiden jakauman välillä yleisissä ja yhdistetyissä imusuoniepämuodostumissa verrattuna syndroomiin, CLOVE syndroomiin, tai määrittelemättömiin PRO syndroomiin, mutta ei KT syndroomiin. Tutkimuksen tuloksiin perustuen PI3K signaalia inhiboivalla lääkkeellä on epidemiologinen ja patofysiologinen peruste sekä isoloitujen, kombinoitujen, että syndroomisten imusuoniepämuodostumien hoidossa. Diagnostista genotyypitystä ei pitäisi siksi rajoittaa vain PIK3CA hotspot mutaatioihin.

CONTENTS

ABBREVIATIONS

AVF	Arteriovenous fistula
AVM	Arteriovenous malformation
CH	Congenital hemangioma
CIVIQ	Chronic Venous Insufficiency Questionnaire
CLOVES	Congenital Lipomatous Overgrowth, Vascular Malformations, Epidermal Nevi, Scoliosis/Skeletal and Spinal syndrome
CM	Capillary malformation
GLUT-1	Glucose transporter type 1
IH	Infantile hemangioma
ISSVA	International Society for the Study of Vascular Anomalies
KTS	Klippel-Trenaunay syndrome
LM	Lymphatic malformation
MRI	Magnetic resonance imaging
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PROS	PIK3CA-related overgrowth syndrome
QOL	Quality of life
US	Ultrasound
VAF	Variant allele frequency
VM	Venous malformation

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I Weitz-Tuoretmaa A, Rautio R, Valkila J, Keski-Säntti H, Keski-Nisula L, Laranne J. Efficacy of OK-432 sclerotherapy in treatment of lymphatic malformations: long-term follow-up results. Eur Arch Otorhinolaryngology. 2014;271:385-390.

II Weitz-Tuoretmaa A, Keski-Nisula L, Rautio R, Laranne J. Quality of life after endovascular sclerotherapy of low-flow venous malformations: the efficacy of polidocanol compared with ethanol. Acta Radiologica. 2018;59(8):946-952.

III Weitz-Tuoretmaa A, Keski-Nisula L, Rautio R, Laranne J. Quality of life and clinical results after endovascular sclerotherapy: A comparision between intra- and extramuscular low-flow venous malformations. Phlebology. 2020;36(3):226-232.

IV Brouillard P, Schlögel M, Sepehr N, Helaers R, Queisser A, Fastré E, Hamoir M, Clapuyt P, Hammer F, Dompartin A, Weitz-Tuoretmaa A, Laranne J, Pasquesoone L, Vilain C, Hendriks Y, Boon L, Vikkula M. Non-hotspot PIK3CA mutations are more frequent in CLOVES than in common or combined lymphatic malformations. Orphanet Journal of Rare Diseases. 2021;16(1):1-267.

The publications are referred to in the text by their roman numerals.

AUTHOR'S CONTRIBUTION

Publication I The author has taken part in study design. The author collected and analyzed symptom scores and all quality of life – data. The author has drafted and revised the manuscript and is the main author of this publication.

Publication II The author has taken part in study design. The author has collected the clinical data with co-authors and collected all symptom scores and quality of life data. The author has drafted and revised the manuscript and is the main author of publication.

Publication III The author has taken part in study design. The author has collected the clinical data with co-authors and collected all symptom scores and quality of life data. The author has drafted and revised the manuscript and is the main author of publication.

Publication IV The author has collected the medical information, tissue and blood samples from the Finnish patient population. The author visited Human Molecular Genetics, de Duve Institute, University of Louvain, Brussels to get acquainted with the laboratory methods of the study. The author has been involved in revising the publication and has given final approval of the version to be published. The author is the main contributor of the Finnish patient material in this publication.

1 INTRODUCTION

Vascular anomalies are categorized based on the classification established by the International Society of the Study of Vascular Anomalies (ISSVA) in two broad types: tumors and malformations (Wassef et al., 2015). Vascular tumors demonstrate endothelial proliferation and affect approximately 5% of the population. Vascular malformations are errors in the morphogenetic process that regulate vascular development between the 4th and 10th weeks of embryonic life. Vascular malformations are more uncommon than vascular tumors with an incidence of approximately 0,5%. This study concentrated on two types of low-flow vascular malformations: lymphatic and venous malformations.

Low-flow vascular malformations are congenital deformities and have a varied clinical presentation depending on whether the lesions are focal or diffuse. Low-flow malformations are deformities of lymphatic or venous vessels or both with an incidence of approximately 1 in 10,000 (Eifert et al., 2000). They are typically located in the neck and face followed by the extremities, trunk, internal viscera, bones, and skeletal muscle and generally present before two years of age (Kollipara et al., 2013). The two most commonly used imaging modalities for low-flow malformations are color Doppler US and MRI (Burrows et al., 1998; Lowe et al., 2012; Merrow et al., 2016). MRI is important in characterizing the extent of the malformation, categorizing the type, determing tissue involvement, and for the planning of therapy (Burrows et al., 1998; Yakes W et al., 1990).

Sclerotherapy is currently the primary treatment for most localized symptomatic macrocystic LMs and for VMs in most vascular anomaly centers (Cabrera J et al., 2003; Hein K et al., 2002; Legiehn & Heran, 2008; Rautio R et al., 2004). Macrocystic LM usually respond very well to sclerotherapy, which has a lower morbidity than with surgical treatment (Adams et al., 2012; S. N. Smith, 2019). Microcystic lesions respond more poorly to sclerotherapy. Using US guidance, it is possible to treat cysts or channels 5 mm or larger (Greene et al., 2005). Sclerotherapy results in endothelium disruption, intravascular coagulation, and inflammation of VM. This leads to fibrosis and contraction of the lesions. Often multiple injections are required to achieve the best result. It is most effective when performed as a series

of procedures, scheduled two to three months apart, until there is no further recanalization or swelling. Direct injection of sclerosing agents results in gradual shrinkage of most VMs (Mulliken J et al., 2013). Patient information plays a highly important role in treating patients with vascular malformations. Risks, benefits, and expectations should be discussed thoroughly. Patients should expect subtle improvements instead of radical changes in their symptoms.

QOL assessment is nowadays widely used to evaluate the efficacy of various treatments, especially when treating chronic illness or disorders. QOL data are usually collected by a set of generic, logical, and easily administered QOL questions. QOL measurements may be specific to a pathology or region of the body, or they may be more general and designed to evaluate health-related QOL. Vascular malformations are considered as chronic disorders. It is important not only to understand whether the illness is cured but also how the status and possible treatments affect everyday life. QOL questionnaire is useful for this purpose. We suggest that a pre- and post-treatment symptom and QOL questionnaire should be used with all vascular malformation patients, not exclusively in studies but in general clinical follow-up as well.

During the past twenty years knowledge of the genetic causes of vascular anomalies has increased considerably. Many of the identified genes cause inherited diseases, but the genetic anomalies underlying some sporadic malformations with postzygotic somatic mutations have also been unraveled. Sporadic lesions are mostly localized, whereas the inherited lesions are usually multifocal and mainly small. Genetic defects have been identified for hereditary hemorrhagic teleangiectasia, inherited cutanemucosal venous malformation, glomuvenous malformation, capillary malformation-arteriovenous malformation, cerebral cavernous malformation, and some isolated, and syndromic forms of primary lymphedema. Vascular endothelial growth factors, angiopoietins, and their endothelial tyrosine kinase are central regulators of angiogenesis and lymphangiogenesis (Lohela et al., 2009). In 2009 it was demonstrated that somatic genetic mutations are associated with sporadically occurring vascular anomalies: isolated venous malformations were discovered to have somatic TIE2/TEK mutations (Limaye, Boon, et al., 2009). Since then, the etiopathogenesis of various isolated and combined vascular malformations has been unraveled (Dekeuleneer et al., 2020; Schlögel et al., 2017). This has opened the era for development of targeted precision therapies for these lesions, especially using small molecule inhibitors. Rapamycin, an inhibitor of mTOR is the most studied compound for the treatment of vascular malformations. It has already been

tested in clinical trials and has demonstrated efficacy in selected cases (Boscolo, Limaye, et al., 2015; J. Hammer et al., 2018; X. Li et al., 2019; Seront et al., 2019).

Because of the relative rarity, complexity, and potential high morbidity vascular anomaly patients should be managed by multidisciplinary specialists.

2 REVIEW OF THE LITERATURE

2.1 Overview of vascular anomalies

2.1.1 Terminology and classification

Vascular anomalies are comprised of vascular tumors, demonstrating endothelial proliferation and vascular malformations, non-proliferative lesions consisting of dysplastic vascular channels due to errors in vascular morphogenesis (Fishman SJ & Mulliken JB, 1998). Vascular malformations are rheologically, and based on the structure of the anomalous channels, subcategorized into low-flow (capillary malformation, CM; lymphatic malformation, LM; venous malformation, VM) and high-flow lesions (arteriovenous malformation, AVM; arteriovenous fistula AVF) (Kollipara et al., 2013)(Figure 1). Nomenclature has been the main obstacle in understanding and management of vascular anomalies leading to misdiagnosis and inappropriate treatment (Greene, 2011; Hassanein A et al., 2011; Kilcline & Frieden, 2008). In 1982 Mulliken and Glowacki published the first classification system clarifying the field of vascular anomalies. A biologic classification categorizes vascular anomalies on their cellular features and clinical behavior (Finn MC et al., 1983; Mulliken JB & Glowacki J, 1982a, 1982b). A modified and expanded version of this classification was accepted by the International Society for the Study of Vascular Anomalies (ISSVA) in 1996. The classification of vascular anomalies continues to expand and has become more precise as the knowledge of these lesions evolves. The current classification has been approved by the ISSVA in 2014 and it was last revised in 2018. Vascular tumors are divided into benign, locally aggressive, or borderline and malignant (Wassef et al., 2015). Vascular malformations are divided into 4 groups: simple malformations, combined malformations, malformations of major named vessels, and malformations associated with other anomalies (Table 1). The ISSVA classification provides a comprehensive framework, encourages common terminology and aids in both management of affected patients and research.

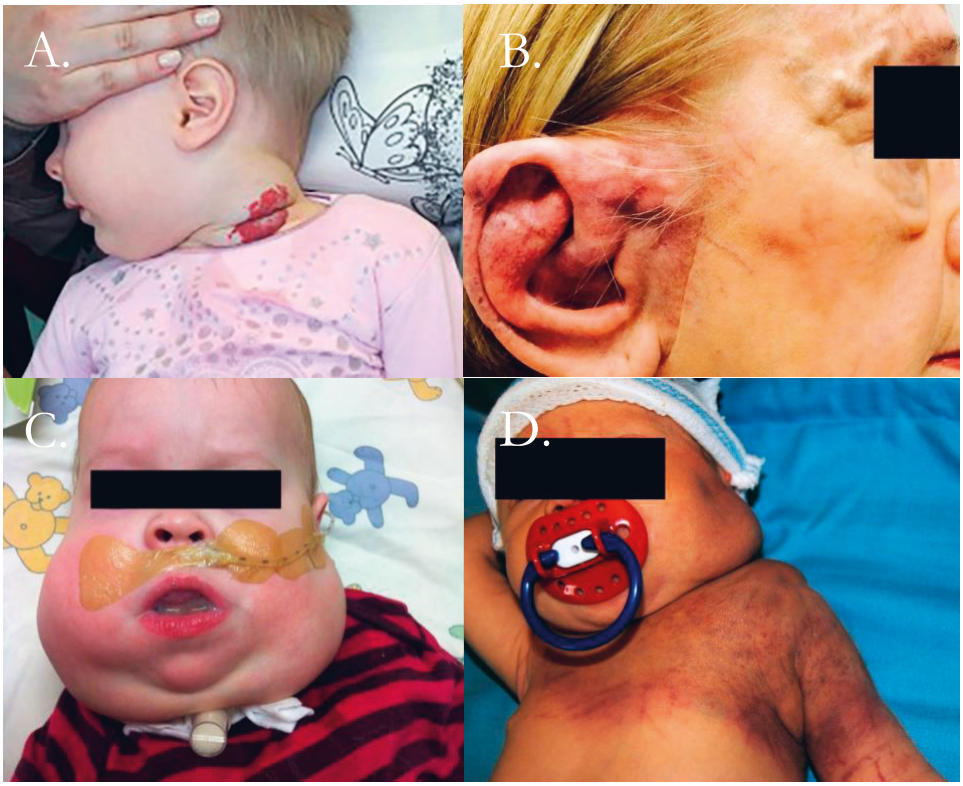
A small number of vascular anomalies remain unclassified. These lesions generally have overlapping features of a neoplasm and congenital malformations.

Table 1. The ISSVA Classification of Vascular Anomalies 2014 (Wassef et al., 2015).

Abbreviations: AVF: Arteriovenous fistula; AVM: Arteriovenous malformation; CH: Congenital hemangioma; CLOVES: Congenital Lipomatous Overgrowth, Vascular Malformations, Epidermal Nevi, Scoliosis/Skeletal and Spinal syndrome; CM: Capillary malformation; LM: Lymphatic malformation; VM: Venous malformation

Vascular Anomalies				
Vascular Tumors	Vascular Malformations			
	Simple	Combined	of major named vessels	Associated with other anomalies
Benign	CM			
Locally aggressive or borderline	VM LM	CM+VM CM+LM CM+LM+VM LM+VM	"Channel type" or "truncal" vascular malformations	Klippel-Trenaunay Parkes-Weber Servelle-Martorell Sturge-Weber Others
Malignant	AVM AVF	CM+AVM CM+LM+AVM CM+LM+AVM		CLOVES Bannayan-Riley-Ruvalcaba Others

Figure 1. Different types of vascular anomalies. A. Infantile Hemangioma. B. Arteriovenous malformation. C. Lymphatic malformation. D. Kaposiform hemangioendothelioma.



2.1.2 Vascular tumors

Vascular tumors are divided into benign, locally aggressive or borderline, and malignant (Table 1&2).

Table 2. Classification of Vascular Tumors

Vascular tumors	
Benign	Infantile hemangioma/hemangioma of infancy Congenital hemangioma Rapidly involuting (RICH) Non-involuting (NICH) Partially involuting (PICH) Tufted angioma Spindle-cell hemangioma Epithelioid hemangioma Pyogenic granuloma Others
Locally aggressive or borderline	Kaposiform hemangioendothelioma Retiform hemangioendothelioma Papillary intralymphatic angioendothelioma, Dabska tumor Composite hemagioendothelioma Kaposi sarcoma Others
Malignant	Angiosarcoma Epithelioid hemangioendothelioma Others

2.1.2.1 Infantile hemangioma

Infantile hemangioma (IH; also named hemangioma of infancy) is the most common tumor in infancy, with an incidence of approximately between 4% and 5%(Kilcline & Frieden, 2008). The prevalence of IH increases with low birth weight, from 1-4% in term infants to 23% in those of <1000g birth weight and decreasing gestational age (Chang et al., 2008; Goelz R & Poets CF, 2015; Léauté-Labrèze et al., 2017). The

majority of IHs are sporadic, but an autosomal dominant pattern has been described (Blei F et al., 1998). IHs are either undetectable at birth or minimally apparent with a precursor mark or discoloration. IH are classified into superficial, deep or mixed according to the depth of the lesion. IHs have a highly characteristic growth pattern, consisting of a rapid proliferative phase during the first 9 months of life. 80% of its size is achieved by the time the infant is 3 ± 2 months of age. It is followed by an involutive phase when the growth plateau and then the lesion begin slowly to regress. The appearance of the IH improves until 3.5 years of age. In 50% of children the lesion will no longer be visible after 3.5 years of age. After regression children may have some permanent deformity: anetoderma, residual telangiectasis, scarring, fibroflatty residuum, and redundant skin (Couto R et al., 2012). The exact etiopathogenesis of IH is unknown. It is known that IH is the result of dysregulation of both vasculogenesis and angiogenesis. IHs are histologically positive for the immunohistochemical marker, glucose transporter type 1 (GLUT-1). In the proliferative phase endothelial cells exhibit increased expression of proliferating cell nuclear antigen (PCNA), type IV collagenase, and proangiogenic factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The tissue inhibitor metalloproteinases (TIMP) is expressed only during the involuting phase (Storch & Hoeger, 2010). Figure 2 shows the stages and molecular markers of IH.

Typical complications are ulceration, disfigurement, airway obstruction and functional impairment and multifocal infantile hemangioma (Figure 3). IHs may be subclassified according to the morphology into localized, segmental, indeterminate or multifocal subtypes. The majority of IHs are localized, arising from one central focus. Segmental infantile hemangioma presents in developmental segments and are more frequently associated with developmental abnormalities and complications (Kwon et al., 2013; Olsen et al., 2020). Large segmental IHs can be associated with various anomalies, such as PHACES (posterior fossa malformations, hemangioma, arterial anomalies, cardiac anomalies, eye abnormalities, sternal and supraumbilical raphe syndrome) and LUMBAR (lower body infantile hemangioma, urogenital anomalies, myelopathy, bony deformities, anorectal malformations and arterial anomalies and renal anomalies) (Léauté-Labrèze et al., 2017; Olsen et al., 2020).

Most IHs typically regress spontaneously, thus treatment is required only for complicated cases. A nonselective beta-blocker, propranolol has become the treatment of choice for life-threatening, complicated IHs (Léauté-Labrèze et al., 2008) (Figure 4). Propranolol blocks the action of adrenaline on both β_1- and β_2-adrenergic receptors. The theories explaining the possible mechanism of action of

propranolol in treatment of infantile hemangiomas include vasoconstriction of the hemangiomas, inhibition of angiogenesis, induction of apoptosis and a reduction in vascular endothelial and epidermal growth factors (Burton, 2010; Storch & Hoeger, 2010). Other treatment methods for IH include systemic, intralesional and topical corticosteroids, vincristine, laser therapy and in some cases operative treatment. (Kwon et al., 2013; Léauté-Labrèze et al., 2017; Storch & Hoeger, 2010).

Figure 2. (a) Growth and regression curve of infantile hemangioma (IH). (b) Molecular markers in different phases of IH. Abbreviations: bFGF, basic fibroblast growth factor; GLUT-1, glucose transporter type I; PCNA, proliferating cell nuclear antigen; TIMPs, tissue inhibitor metalloproteinases; VEGF, vascular endothelial growth factor. (Storch & Hoeger, 2010)

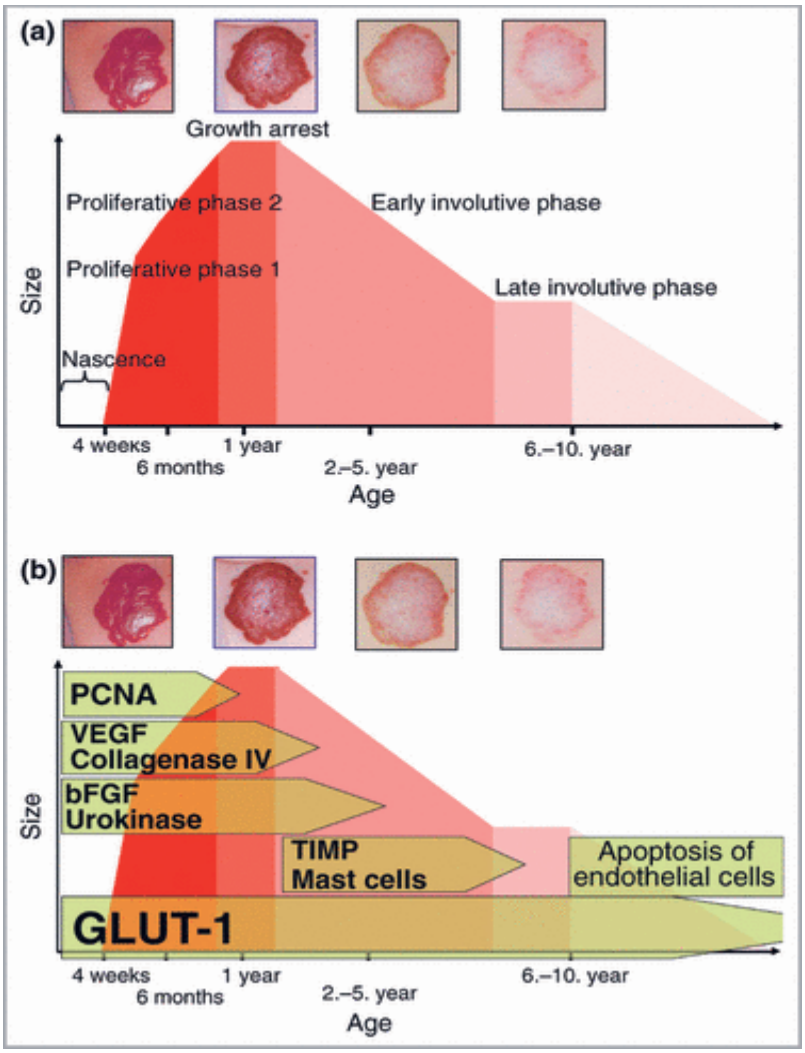


Figure 3. Complication of an infantile hemangioma. A. Ulceration of an infantile hemangioma. B. Response after propranolol treatment and scar formation because of ulceration.

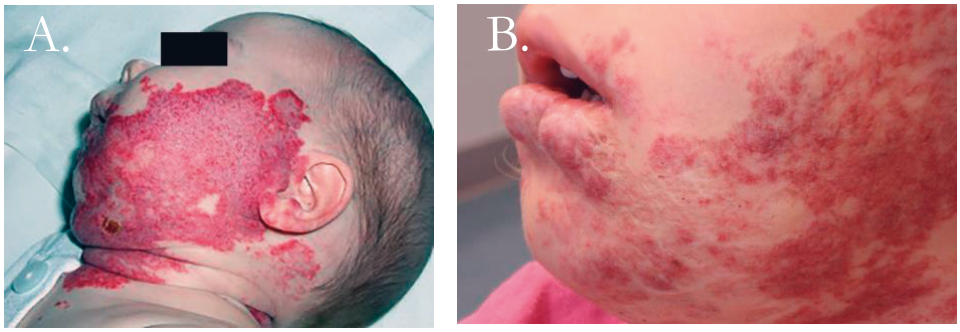
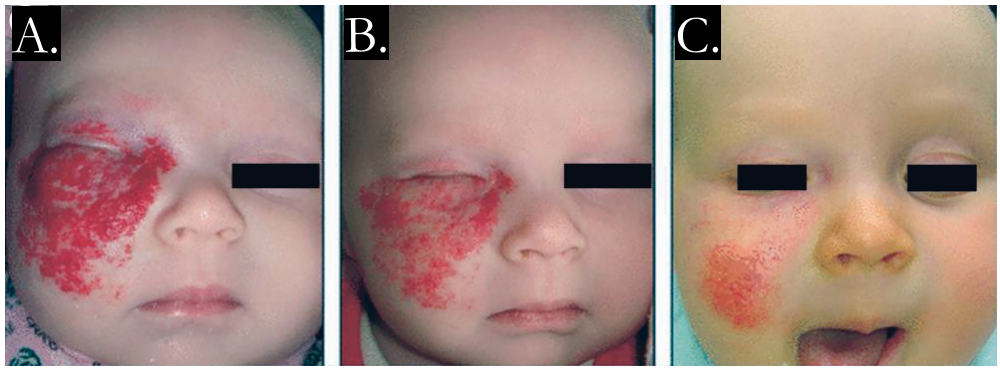


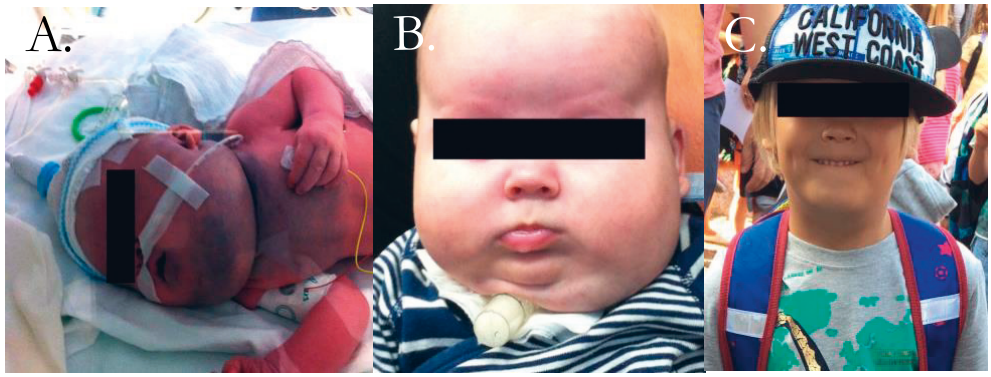
Figure 4. Infantile hemangioma. A. At age of 8 weeks before initiation of propranolol treatment. B. Response after one week of treatment. C. Response after 6 months of treatment.



2.1.2.2 Congenital hemangioma

Congenital hemangiomas (CHs) are less common. They are present and fully developed at birth and do not grow postnatally (Figure 5). CHs can be detected by imaging at the 12th week of gestation (Boon et al., 1996) (Figure 5). CHs tests specifically negative for GLUT-1 (Olsen et al., 2020). CHs tend to either regress rapidly (rapidly involuting congenital hemangioma, RICH), do not regress at all (non-involuting congenital hemangioma, NICH) or regress partially (partially involuting congenital hemangioma, PICH). Zein et al noted that histopathological features are similar in all these three subtypes, and they differ only in their involuting potential (el Zein et al., 2020). RICH involutes immediately after birth, showing complete regression at the age of 7 months in 50% of the patients and is generally fully involuted by 12-14 months of age (Greene A, 2013). This subtype can be associated with high flow, causing a fetal and postnatal high cardiac output state. RICH can also be associated with thrombocytopenia in the acute phase, due to the platelet damage or consumption in their large vascular bed. NICH are defined by a proportional growth with the child and does not regress at all. These two subtypes have similar morphology: they affect head and neck, limbs, or trunk and are differentiated by their clinical behaviour over the first months of life. PICHs represent a small subgroup demonstrating intermediate behaviours between the two extremes represented by RICHs and NICHs (Nasseri et al., 2014). Clinical inspection and patient history are crucial for the diagnosis. Gorincour et al analysed 26 CH patients with ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI). They noticed distinctive and diagnostic US imaging characteristics between CH, NICH and RICH cases. In congenital hemangiomas visible vessels and calcifications were seen statistically significantly more often than in the involuting cases. Based on this suggest that US might be the only imaging necessary to differentiate between these lesions (Gorincour et al., 2005). On doppler US hemangiomas appear as an echogenic mass and a fast flow pattern can usually be observed. A RICH rarely needs intervention because it rapidly involutes. Treatment options of CHs include surgical excision with or without embolization. Medical therapies are not effective in the treatment of CHs like they are in IHs.

Figure 5. Partially involuting congenital hemangioma. A. Congenital hemangioma of a newborn affecting the head and neck area and chest. B. At age of 3 months. C. At age of 7 years after several treatments with propranolol, siladenafil and sirolimus.



2.1.2.3 Tufted angioma and Kaposiform hemangioendothelioma

Tufted angiomas (TAs) are rare vascular tumors of infancy and early childhood. They are less common at birth (Mansfield et al., 2020). They often present as discolored or erythematous plaques of the head and neck, trunk, extremities, or retroperitoneum and are usually in a cutaneous/subcutaneous location. Kaposiform hemangioendothelioma (KHE) may affect the skin and subcutis but often involves the deep tissues, presenting as a locally aggressive tumor. Histopathologic examination is the gold standard for diagnosis of TA and KHE due to their characteristic features. KHE histologically resembles TA with larger and confluent tumor lobules, with a more spindled, infiltrating pattern (Chiu Y et al., 2012; Gibson & Barnacle, 2020; Herron et al., 2002; Johnson E et al., 2018). On immunostaining both are GLUT-1 negative. Both TA and KHE may be associated with Kasabach-Merritt Phenomenon (KMP), a consumptive coagulopathy and transient thrombocytopenia. Without treatment the coagulopathy can result in significant morbidity and mortality. Originally regarded as distinct lesions, these rare vascular tumors are now considered by many authors as part of a spectrum. Studies have shown differences in the immunostaining patterns of monoclonal antibody D2-40 that assists in in distinguishing these lesions from each other (Arai et al., 2006; Enjolras et al., 1997). Until recently, vincristine was the treatment of choice for TAs and KHE but sirolimus is now more commonly used (Blatt et al., 2010; Tasani et al., 2017).

2.1.2.4 Malignant vascular tumors

Malignant vascular tumors, epithelioid hemangioendothelioma (EHE) and Angiosarcoma (AS) are rare tumors. EHE may have a benign, indolent course or an aggressive one, showing an incidence of approximately 1 case per million (Rosenberg & Agulnik, 2018). The prevalence of AS is about 2 per million and these tumors are very aggressive. A malignant transformation of IH to AS has been described in children, but it is extremely rare (Jeng et al., 2014).

2.1.3 Low-flow vascular malformations

Low-flow vascular malformations are congenital deformities and have a varied clinical presentation depending on whether the lesions are focal or diffuse. Low-flow malformations are deformities of lymphatic or venous vessels or both with an incidence of approximately 1 in 10,000 (Eifert et al., 2000). They are typically located in the neck and face followed by the extremities, trunk, internal viscera, bones, and skeletal muscle and generally present before 2 years of age (Kollipara et al., 2013).

2.1.3.1 Capillary malformation

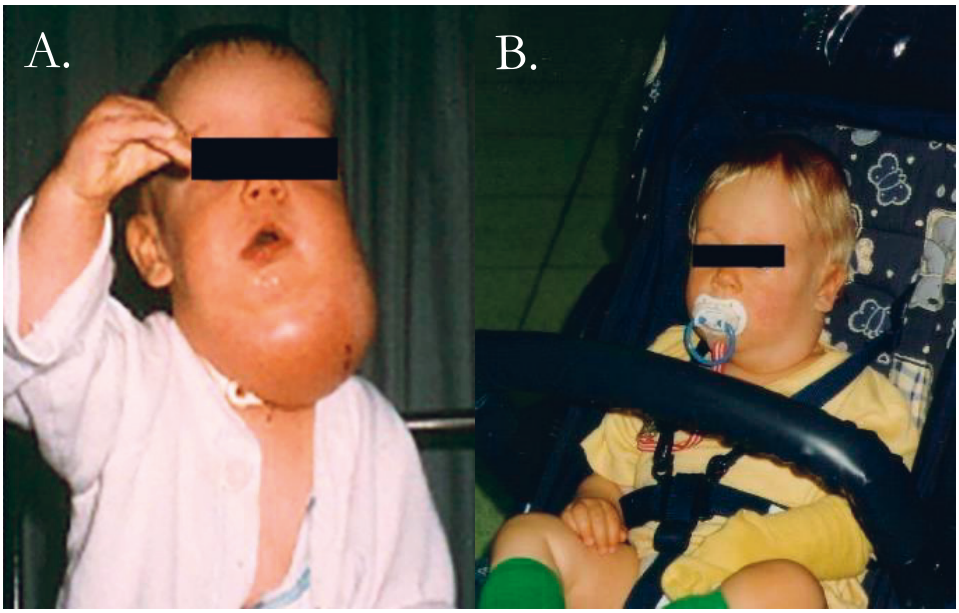
Capillary malformations (CMs) consist of dilated capillaries and/or postcapillary venules within the skin and mucosal membranes and need no further imaging modalities for diagnosis. It is the most common type of vascular malformation seen, with a prevalence of 0,3% in the new-borns (McCafferty, 2015). In most cases they occur sporadic, as unifocal lesion, but can also be inherited as an autosomal dominant trait with incomplete penetrance and variable expression. Autosomal dominant capillary malformation-arteriovenous malformation syndrome (CM-AVM) has been associated with mutations in RASA1 gene (Eerola et al., 2003). CMs are present at birth and generally persist throughout life. Over time the lesions may progress and become thicker, darker, and more purple. CMs are predominantly localized in the head and neck region. CMs may be associated with other vascular and nonvascular anomalies (Table 1). CMs may also be a hallmark of complex syndromes that exhibit hypertrophy, such as Sturge-Weber, Klippel-Trenaunay, Parkes-Weber or Proteus syndrome.

2.1.3.2 Lymphatic malformation

Clinical features

Lymphatic malformations (LMs) are due to errors in development of the lymphatic system resulting in numerous thin-walled cysts. LMs are compressible or noncompressible soft-tissue masses, overlying with normal or bluish skin (Figure 6). Approximately 50% of LMs are present at birth and 80%-90% of the lesions are noted by 2 years of age (Dubois J & Garel L, 1999; Zadvinskis et al., 1992). LMs are most commonly located in the head and neck region (70-80%), commonly in the posterior cervical triangle, or axilla. Other typical locations include the superior mediastinum, mesentery, retroperitoneum, pelvis, and lower limbs. LMs have no predilection for either sex or race (Greene A, 2013). LMs are divided into the following subtypes based on the size of the cysts; macrocystic, microcystic and mixed. There is no strict definition of the two types of cysts based on size. In the literature differentiation between microcystic and macrocystic lesions vary between 5mm to 2cm (Adams et al., 2012; McCafferty, 2015; S. N. Smith, 2019). Presumptively the most useful distinction between macro- and microcystic lesions is whether the cysts can be successfully aspirated and sclerosed. With microcystic lesions this is often ineffective (Mulliken J et al., 2013). LMs progress over time. Usually they grow slowly, but sudden enlargement of the lesions is an indication of bleeding or inflammation. LM can be combined with malformations of capillaries, veins, and/or arteries, such as in the Klippel-Trenaunay and Parkes Weber syndromes.

Figure 6. Macrocystic lymphatic malformation. A. After the first OK-432 sclerotherapy a marked soft tissue swelling in the neck and laryngopharyngeal region was noted. B. After four OK-432 injections a complete regression of the lymphatic malformation.



Etiopathogenesis

LMs are focal lesions, which occur sporadically, with no evidence of familial forms (Boon et al., 2011). Several theories for the formation of LMs exists. First in beginning of 20th Century Sabin proposed a theory of a venous origin with centrifugal spread. She believed that primordial lymphatic sacs sprout from developing central vein and propagate channels centrifugally (Sabin, 1909). At the same time Huntington and McClure and much later Kampmeier suggested a theory of a mesechymal origin with centripetal spread (Huntington & McClure, 1910; Kampmeier, 1971). They speculate that lymphatic channels form from mesenchymal spaces in the periphery of the embryo and the spaces spread centripetally by annexing other similar spaces. Van der Jagt proposed another theory of a combined venous-mesenchymal origin, he suggested that lymphatic structures initiate from the confluence of small venules and spaces in the adjacent mesenchyme (van der Jagt E,

1932). In 1980, van der Putte observed in microscopic sections of human embryos that the first phase of lymphatic development begins approximately at 6.5 weeks of gestational age, a short time after arterial and venous development in the jugular region. He came to the same conclusion with Sabin that the lymphatic primordia most likely have venous origin and believed in centrifugal growth and sprouting (van der Putte & van Limborgh, 1980). LMs may result from an aberrant bud arising from the primordial sac (R. J. H. Smith, 2004; Zadvinskis et al., 1992).

Recently one of the genes identified to be mutated in vascular malformations encodes the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA, also known as p110α). Mutations were first identified in rare syndromic patients with Congenital Lipomatous Overgrowth, Vascular malformations, Epidermal nevi, and Skeletal/spinal abnormalities and/or scoliosis (CLOVES) (Keppler-Noreuil et al., 2014). Subsequently, PIK3CA mutations were found in common LM (Boscolo, Coma, et al., 2015; Limaye et al., 2015; Luks et al., 2015; Osborn et al., 2015).

Phenotype

Macrocystic lesions are typically composed of multiple, either interconnected or separate, cysts. These contain a watery, amber fluid. They may be luctant and demonstrate trans-illumination (McCafferty, 2015; Mulliken J et al., 2013). They typically appear as soft non-pulsatile masses with normal overlying skin. Most commonly they are in the neck or axilla. They rarely improve spontaneously. Macrocystic lesions have a better prognosis than microcystic lesions, the large cysts can be accessed more easily and aspirated/sclerosed (Chen et al., 2009).

Microcystic lesions primarily affect the face and extremities. Microcystic LMs are firm and sponge-like. They may infiltrate tissues; most commonly mucous membranes and skin, but also bone and organs. Cutaneous microcystic vesicles may overlay the affected area and typically tend to bleed and leak lymphatic fluid, which may lead to complications such as episodes of bleeding and infection leading to acute enlargement of the affected area. Microcystic lesions are usually more challenging to treat, as the lesions are less amenable to aspiration and sclerotherapy than macrocystic lesions (de Serres L, 1995).

Approximately 50% of LMs are mixed cystic lesions containing both macro- and microcystic components (Renton & Smith, 2011). The prognosis and treatment are based on the ratio of the different types of the cysts (macrocysts vs microcysts).

Primary lymphedemas are considered a subtype of LM due to the primary dysgenesis of the lymphatic network and typically results from hypoplastic lymphatic development in an extremity, classically affecting the lower limb. Lymphedemas are divided into primary and secondary. Primary lymphedema is rare (prevalence approximately 1.15/100,000) and caused by congenital dysplasia of the lymphatic system. Primary lymphedema is divided in three types based on age at onset, congenital, pubertal, and late-onset lymphedema (Lazareth, 2002; K. Li et al., 2017). Secondary lymphedema results from a blockage of the lymph flow in the lymphatic system, which may be caused for example by an infection, surgery, trauma, or scar tissue formation. Five causal genes have been identified demonstrating that primary lymphedema is a complex heterogenous condition (Boon et al., 2011).

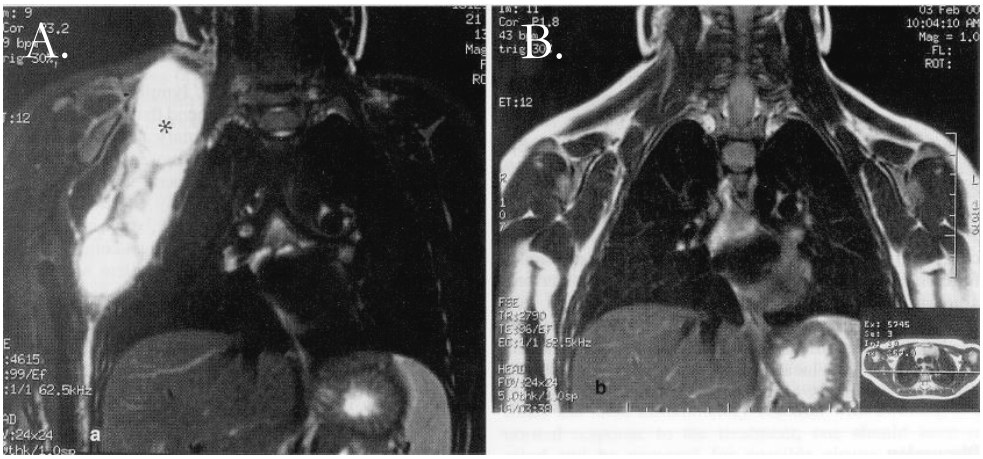
Gorham-Stout disease (GSD, also known as vanishing bone disease) is a rare, progressive osseus lymphatic anomaly that causes osteolysis of bone (Figure 7). GSD is most diagnosed in young children and in young adults without sex predilection or inheritance pattern. GSD is characterized by proliferation of thin-walled sinusoidal channels of lymphatic origin and by the replacement of bone with fibrous tissue and results in cortical loss (Lala et al., 2013). It can affect any and multiple bones in the body, commonly the maxilla, mandible, ribs, the cranium, clavicle, femur, and cervical spine (Dellinger et al., 2014)(Lala et al., 2013). The most common symptom is localized pain, due to the bone resorption it comes to pathologic fractures. Chylothorax is a rare complication, which may result in respiratory failure. It is due to the invasion of the thoracic duct or penetration of the lymphatic dysplasia into the pleural cavity and is reported with a 64% mortality rate (Chavanis et al., 2001). The etiology of GSD is still unclear. Two commonly used molecular markers Lyve-1, a receptor for the glycosaminoglycan hyaluronan, and podoplanin, a transmembrane glycoprotein recognized by the antibody D2-40 have been found in medullary and cortical regions of bones in GSD patients (Dellinger et al., 2014). There is no standardized treatment for GSD. Management of the disease involves weekly subcutaneous interferon injection and monthly intravenous bisphosphonate therapy. Sirolimus has lately been successfully used for GSD (Dellinger et al., 2014; F. Hammer et al., 2005; Ricci et al., 2019)

Generalized lymphatic anomaly (GLA) is a multisystem disorder characterised by the extensive proliferation of lymphatic vessels, affecting the skin and superficial soft tissue and abdominal and thoracic viscera. GLA often involves bone (85%). GLA typically involves the appendicular skeleton (the shoulders, pelvis, and upper and lower extremities). The mean number affected bones is 30 (Lala et al., 2013). In contrast to GSD, osseus lesions are generally nonprogressive, showing discrete lytic

areas confined to the medullary cavity and spares the bone cortical boundaries (Ozeki et al., 2016). Recently, the presence of somatic activating PIK3CA mutations in patients with GLA have been detected (Rodriguez-Laguna et al., 2019). Histopathologic findings are similar to those in GSD. In GLA the osteoclastic and osteoblastic activity with new bone formation and marrow fibrosis is minor to GSD (Lala et al., 2013). No standardized treatment for GLA has been established yet. Management strategies include surgery, radiation therapy, interferon, and bisphosphonates. Recently, low dose sirolimus in long-term course has been described to be successful in treatment of GLA (Dvorakova et al., 2018)

Kaposiform lymphangiomatosis (KLA) is a variant of generalized lymphatic anomaly causing thrompocytopenia and/or tissue hemorrhage. Most commonly patients present with respiratory symptoms (50%), pericardial (70%) and/or pleural (85%) effusions. KLA involves the mediastinum of almost all patients. Extrathoracic disease arises in bone, spleen and less often in peritoneum, abdominal viscera, or extremities. Histopathologic findings are abnormally dilated lymphatic channels and clusters or sheets of spindled lymphatic endothelial cells. KLA is treated with sclerotherapy and medical therapy, such as corticosteroids, vincristine, sirolimus and adjunctive zoledronate. Additional treatment includes drainage of symptomatic effusions, pleurodesis. The mortality rate of KLA is high despite aggressive multimodal therapy (Crane et al., 2020; Croteau et al., 2014).

Figure 7. T2-weighted MRI of a patient with thoracic wall lymphatic malformation. A. Before sclerotherapy *. B. After OK-432 sclerotherapy.



Diagnostic methods

Large macrocystic LMs can be diagnosed *in utero* already in the beginning of the second trimester. In most cases the diagnosis can be established based on clinical examinations and thorough patient history. The primary differential diagnosis is a venous malformation. Small superficial LMs can be diagnosed by simple ultrasound (US) but lesions extending into deep structures need more specific imaging. Imaging has also an important role in revealing intraosseous involvement, osteolysis and destruction as can be seen in GSD or GDA (Behr & Johnson, 2013).

Ultrasound is essential in the diagnosis of LMs., Being painless and non-invasive, it is an ideal means in examining children. On US, macrocystic lesions appear as hypoechoic spaces showing a multilocular cystic mass with no flow except in the septa. Microcystic lesions appear as echogenic areas on soft tissue thickening. Doppler US is a useful tool in distinguishing hemangioma from vascular malformations and low-flow lesions from high-flow lesions (Dubois J & Garel L, 1999; Paltiel et al., 2000).

Magnetic resonance imaging (MRI) is the imaging modality of choice as it can image in multiplanar fashion, and without ionizing radiation. Although US may be adequate to define subcutaneous cystic nonvascular prominent masses, MRI with

contrast is the preferred method to define the anatomic extent of the process and, importantly, its juxtaposition with other structures. Macrocystic LMs appear on MRI as well-defined cysts characterized by hypointense signal on T1-weighted imaging and marked hyperintense signal on T2-weighted imaging. The cysts of microcystic LM are usually too small to identify as discrete structures on MRI and mainly appear as diffuse areas of low T1 signal and increased signal on T2-weighted imaging, with the absence of or mild postcontrast enhancement. Fluid-fluid levels are commonly present (Blei & Bittman, 2016; Donnelly L et al., 2000; White et al., 2016). After gadolinium administration, septal and peripheral enhancement can be noticed, but the cystic spaces are not internally enhanced, which helps distinguish the lesion from VM. CT best demonstrates the bone distortion (Burrows et al., 1998).

Treatment

Treatment of patients with LMs depends on clinical presentation, size of the lesion, location of the lesion, and presenting complications. For complicated or extensive lesions, multimodal therapy is essential. The goal of the treatment is not necessarily cure, but improvement and reduction of functional and cosmetic complications, so as to increase the quality of life of these patients.

Conservative treatment

In a small percentage of patients (1.6% to 16%) with localized LMs, lesions appear to regress spontaneously (Zhou et al., 2011). This is likely due to a sclerosant effect on the LM related to trauma or infection. Therefore, only lesions that are persistently symptomatic, cause pain, functional limitation, or aesthetic disfiguration require intervention. Patients with malformations of the trunk and extremities benefit from tailored compression garments.

Sclerotherapy

Sclerotherapy is currently the primary treatment for most localized symptomatic macrocystic LMs. Macrocystic LMs usually respond very well to sclerotherapy, which has a lower morbidity rate than with surgical treatment (Adams et al., 2012; S. N. Smith, 2019). Microcystic lesions have tradionally responded poorly to

sclerotherapy. However, bleomycin has shown good results in the treatment of microcystic LMs (Chaudry et al., 2014; Yang et al., 2011). In some sporadic cases, such as in intraorbital LMs, the results with sodium tetradecyl sulfate have also been promising (Svendsen P et al., 2001). Using US guidance, it is possible to treat cysts or channels 5mm or larger (Greene et al., 2005).

Various sclerosing agents have been used in therapy of LMs, including OK-432, bleomycin, pingyangmycin, doxycycline, absolute ethanol, ethanolamine oleate, and Ethibloc®. Other sclerosants, such as, sodium tetradecyl sulfate has been used for intraorbital lesions and superficial cutaneous vesicles and acetic acid in treatment of LMs in different locations (Kok et al., 2012a; Svendsen P et al., 2001; Won et al., 2004). There still are no high-quality controlled studies available comparing these agents. The choice of sclerosant should be based on the location of the lesion, minimal toxicity, cost effectiveness and the institutional experience.

OK-432 is a preparation with lyophilized low-virulent group A Streptococcus pyogenes incubated with benzyl penicillin (Picibanil, Chugai Pharmaceutical Co, Tokyo, Japan). It was developed in Japan in the late 1960s and was primarily certified as adjuvant cancer therapy in 1975. It proved to be useful in pleurodesis for malignant pleural effusion (Ishida N & Hoshino T, 1985). OK-432 was first presented as a treatment method for LM by Ogita in 1985 and is now used widely as first-line treatment, especially for macrocystic LMs (Ogita S et al., 1994). It is thought to induce various cytokines and a local inflammatory response inducing sufficient endothelial damage (Närkiö-Mäkelä et al., 2011). OK-432 is a commonly used sclerosing agent and one of the most studied (Figure 8). Several studies indicate the effectiveness of OK-432 and its low complication rate as single therapy modality or when combined with surgery (Laranne J et al., 2002; Motz et al., 2014; Poldevaart M et al., 2009; Rautio et al., 2003; Smith M et al., 2009; R. J. H. Smith et al., 1996; Yabe & Takahashi, 2009; Yoo et al., 2009). Horbach et al published in 2016 a systemic review of sclerosing agents that included 204 patients from 9 retrospective studies. The studies indicate an overall response of between 50% to 95% with an average of two treatment sessions. In 28% to 57% of patients complete remission was observed. The prevalence of the complications reported was between 0% to 30% and included facial nerve palsy and local inflammation (Horbach, Lokhorst, et al., 2016).

Bleomycin is an antibiotic derivative and a well-known cytotoxic agent, produced the fermentation of Streptomyces. It was originally used in the therapy of different cancer types. Its cytotoxicity is mediated by DNA cleavage triggering single and double strand breaks. The precise mechanism is unclear, it causes endothelial damage

and leads to obliteration of the cysts (Chaudry et al., 2014; Horbach, Rigter, et al., 2016). In 2011, Sainsbury et al. reviewed the literature on intralesional bleomycin injection performed over the past 30 years. Data from over 850 patients with LM treated with bleomycin was analysed. The overall response rate was 44% to 100% and complete response rate 10% to 87% (Sainsbury et al., 2011). Bleomycin has proven its efficacy in treatment of microcystic lesions (Chaudry et al., 2014; Yang et al., 2011). Bleomycin treatment may cause minor complications, such as skin infection and ulceration, pain, swelling and flu-like illness. Pulmonary fibrosis is a major complication related to cumulative dose. It is usually reported with a lifetime dose of greater than 400 units, which is clearly much higher than the dose used for LM treatment (Acord et al., 2016).

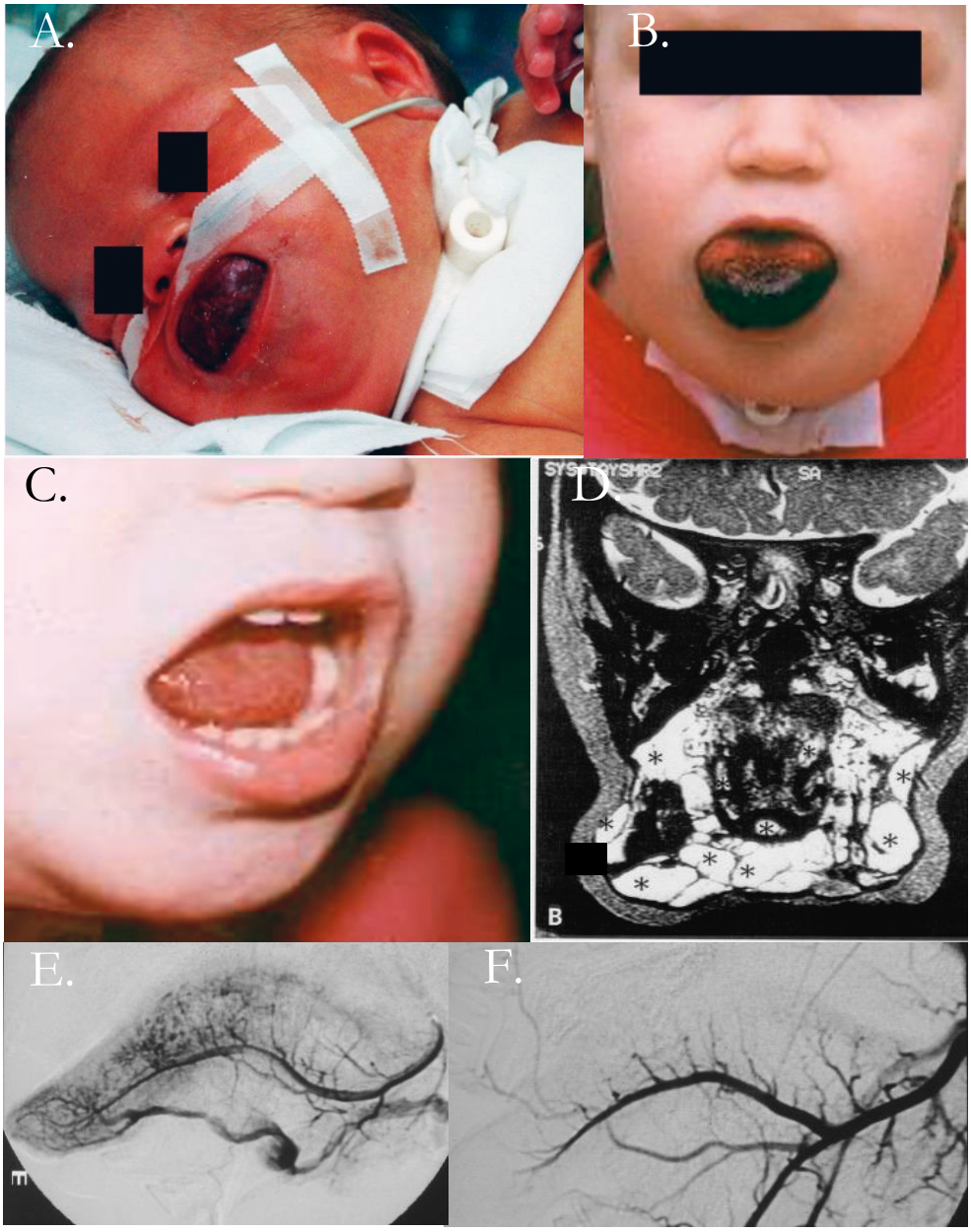
Pingyangmycin (bleomycin A5) is structurally similar to bleomycin. Discovered in Pingyang, China, it is a compound isolated from Streptomyces pingyangensisn cultures and is more cost-effective than Bleomycin. It is mostly used in China and has been reported to be effective and safe for patients with both macrocystic and microcystic LM. Yang et al. noted a size reduction greater than 90% in 81% of patients with macrocystic lesions and in 63% with microcystic LM (Yang et al., 2011). Bai et al reported 78% effectiveness in the sclerotherapy of microcystic LMs in oral and facial regions (Bai et al., 2009). Minor complications such as skin ulceration, tissue atrophy and mild fever were noted.

Doxycycline is an antibiotic of the tetracycline group and was originally reported in 1995 as an effective sclerosant agent in the treatment of LM by Molitch et al (Molitch H et al., 1995). In 2015, Cheng published a review article of doxycycline sclerotherapy in children with head and neck LMs in which five studies were included. At 84.2%, the overall success rate of these studies was high (Cheng, 2015). Also studying children, Burrows et al showed similar results but in different locations (cervicofacial, truncal or extremity) of lesions with an overall response of 95% (P. E. Burrows et al., 2008). Doxycycline has a better treatment response in macrocystic compared to microcystic LMs (P. E. Burrows et al., 2008; Nehra et al., 2008). The most common complications are swelling and skin blistering, with a complication-rate range of 2 to 10 % of patients. Complications are reported to be more common in microcystic and mixed lesions than in macrocystic LM and with higher doxycycline doses. Because doxycycline is a tetracycline antibiotic, there is a slight risk of tooth discoloration.

Ethanol is an aggressive sclerosant, causing instant damage of the endothelium of the vascular wall that results in obliteration of the lumen. It may be useful for treating relatively localized macrocystic LMs (Alomari A et al., 2006). There are no

publications on the success rates of ethanol in notable numbers of patients with LM (Burrows P & Mason K, 2004). The volume of absolute ethanol must be small to be safe (0.5-1ml/kg), making it ineffective in voluminous cysts. Furukawa reported in 2010 of two cases of adults with macrocystic LMs. Ethanol was injected into the lesion and aspirated after 5 min exposure. They noted that the injection and aspiration technique increased the efficacy of sclerotherapy (Furukawa et al., 2011). Ethanol sclerotherapy is associated with a significant risk for complications, such as permanent nerve injury and extensive skin necrosis. Because of the marked complication risk, it is not generally used as a sclerosant for treatment of LMs.

Figure 8. The first lymphatic malformation patient in Finland treated with OK-432 29.1.1999. A. As a newborn B. Before the first treatment with OK-432. C. After treatment with OK-432 and partial lingual artery embolization. D. ∗ LMs in T2-weighted MRI before the first treatment. E. Digital subtraction angiography (DSA) before embolization with polyvinyl alcohol (PVA) particles shows multiple branches of the lingual artery, hyperemic shunting lingual parenchyma and early filling of lingual vein. F. After embolization.



Sclerotherapy technique

Sclerotherapy is usually performed under general anesthesia, especially in pediatric patients and when treating malformations in the craniofacial area. Moderate sedation or local anesthesia may be used for adult patients with superficial lesions or lesions distant from critical structures (Azizkhan, 2013). Individual cysts are localized and cannulated under US guidance with a small angiocatheter of 19-22-gauge needle. Macrocysts can be opacified with a small amount of contrast medium, which is then aspirated. The desired sclerosing agent is then injected under US or fluoroscopic guidance. The volume of the sclerosant injected ranges between 50-75% of the volume aspirated from the cyst (Acord et al., 2016; Burrows P & Mason K, 2004; Elluru et al., 2014). It is also possible to use a 5-F pigtail catheter and inject and drain the cysts over several days (Shiels et al., 2009).

There is no consensus on the post-treatment use of prophylactic antibiotics. Broad spectrum antibiotics are mostly used for about 10 days post procedure when treated malformations in areas difficult to prepare with sterile techniques, such as the oral cavity or perineum (Acord et al., 2016; Burrows P & Mason K, 2004). Adequate analgesia is important to be provided postoperatively. Regression of the macrocystic lesions occurs slowly; changes in size may not be apparent until 4 weeks post-injection. The results should be assessed at about 6 weeks post-injection. If additional treatment is required, 6 to 8 weeks in is an appropriate interval (Elluru et al., 2014).

Surgical treatment

Historically, the first-line treatment for LM has been surgery. The challenging part of surgery is the requirement of a complete resection. Partial resection leads to recurrence and following salvage surgery carries a high risk of complications, morbidity, and poor cosmetic results-risks, which may be considered unnecessary to take in treatment of a benign lesion. Recurrence rates of partially or incompletely resected lesions are between 35 and 100% (R. J. H. Smith, 2004). With such a high co-morbidity and recurrence rates, surgery must be questioned as a first-line management of a benign condition like LM. Surgery is reserved for cases in which sclerotherapy fail or urgent intervention is needed.

2.1.3.3 Venous malformation

Clinical features

Venous malformations are the most common of the low-flow malformations with a prevalence of 1% in the general population (Eifert et al., 2000). Like other vascular malformations VMs are present at birth but become clinically evident later. They progress over time: 26% before adolescence, 75% prior to adulthood, and 93% during the patient's lifetime (Hassanein et al., 2012) (Figure 9). Progression is 2.6 times more likely during adolescence (61%) than during childhood (22%). There is no sex preponderance. VMs appear as faint blue patch or mass, the blue color being pathognomonic. The lesions are non-pulsatile, compressible and demonstrate filling on dependency (Figure 10). Phleboliths can often be palpated in large lesions. VMs can occur anywhere in the body but are frequently seen in the head and neck (40%), extremities (40%) and trunk (20%). VMs can involve multiple tissue types. Most commonly the lesions involve the skin, mucosa, and subcutaneous tissue. These superficial VMs are easy to diagnose clinically due to their characteristic appearance. However, 50% of VMs affect deeper structures, such as muscle, bone, joints, or viscera (Greene A, 2013). Intramuscular lesions present later, tend to be more painful and contain more phleboliths (Vogel et al., 2013). Lesions within the periphery are more likely to be associated with truncal abnormalities. VMs are often associated with localized coagulopathy, with low fibrinogen levels, leading to thrombosis and pain. Low fibrinogen associated with low factor XIII and elevated D-dimers can result in severe systemic coagulopathy and cause severe hemorrhage (Dompmartin A et al., 2008). Pain and cosmetic disadvantage are the most common symptoms associated with VMs. VMs may also induce neuropathy, chronic anemia (gastrointestinal VMs) and by muscle involvement, fibrosis and functional impairment. VMs affecting extremities can cause complications, such as length discrepancy, hypoplasia, pathologic fracture, hemarthrosis, and degenerative arthritis. The majority of VMs are sporadic and unifocal (93%), although 1% are multifocal. Inherited forms, familial venous malformation cutaneo-mucosals (VMCMs) (1%) and glomuvenous malformations (GVMs) (5%) are often multifocal (Dompmartin et al., 2010).

Figure 9. Venous malformation in the head and neck area. A. Patient at age 18 years. B. and C. Patient at age > 35v

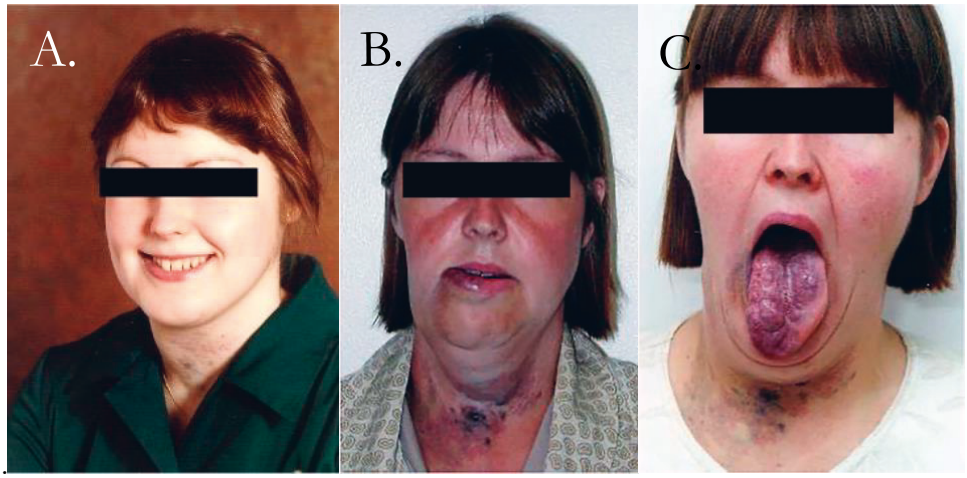
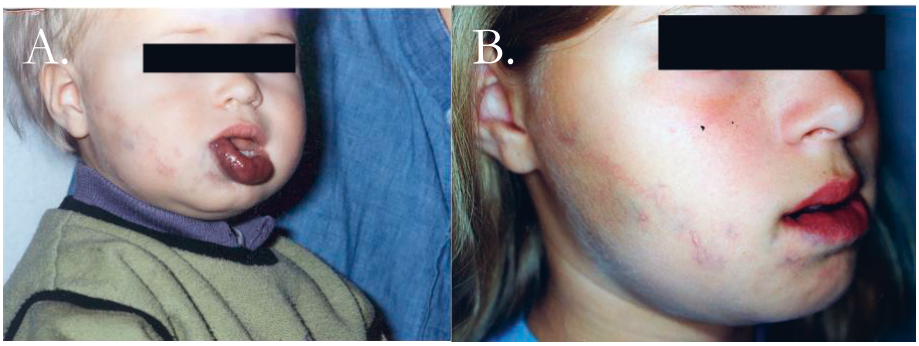


Figure 10. Venous malformation in the lower lip and right cheek. A. As a toddler before treatment. B. after several sclerotherapy treatments with ethanol and polidocanol and one surgery.



Etiopathogenesis

VMs are non-proliferative lesions consisting of dysplastic venous channels due to errors in vascular morphogenesis. Veins present a decreased amount of smooth muscle cells, and these are arranged in clumps rather than concentrically (Fishman SJ & Mulliken JB, 1998). In familial VMs following mutations have been detected:

glomulin, TIE2 and CCM1, CCM2 and CCM3. Approximately 50% of the sporadic lesions have a somatic mutation in the endothelial receptor TIE2 (Limaye, Wouters, et al., 2009). The VMs have been noted to have a higher risk of progression during puberty, so there is a presumption, that adolescent hormones stimulate VMs.

Phenotype

Common VM is the most typical type of VM (93%). The common VMs are subcategorized into intramuscular and superficial. Intramuscular VMs tend to be diagnosed later and they seem to cause more morbidity than superficial VMs. It is likely that the lack of skin or mucosal involvement makes dilated veins less apparent and the possible soft tissue mass in an infant is not suspected to be a vascular malformation (Scorletti et al., 2018). The primary reason for seeking treatment is pain and intramuscular VMs are also associated with more limitations to physical activity due to discomfort and swelling (Vogel et al., 2013). Intramuscular VM has a slight female predilection, as for superficial lesions there has not been noted a sex preponderance (Hein K et al., 2002). The other, more uncommon phenotypes are indexed below.

Blue rubber nevus syndrome (BRBNS) is an extremely rare disorder characterized by multiple small VM involving the skin, soft-tissue, and gastrointestinal tract. These gastrointestinal lesions are considered pathognomonic. 75% of BRBNS patients with gastrointestinal involvement have bleeding, which can lead to iron deficiency anemia. In rare cases other intestinal organs can be involved, such as bladder, liver, spleen, kidneys, and lungs (Dompmartin A et al., 2008). Recently it was discovered that BRBNS is genetically linked to TEK or TIE2 mutations (Soblet et al., 2017).

Cerebral cavernous malformations (CCMs) are rare sporadic or autosomal dominant inherited vascular lesions of the brain and spinal cord. CCMs may lead to hemorrhage, seizures, and neurologic deficits. The condition results from a loss-of-function mutations in 1 of 3 genes, CCM1 (originally called KRIT1), CCM2 (Malcavernin), or CCM3 (PDCD10). About 20% of cases are familial forms originating from germline mutations and usually a more severe disease presentation is noted than in sporadic CCM.

Familial venous malformation cutaneo-mucosal (VMCM) is rare inherited (autosomal dominant) lesion, caused by activating mutations in the TIE2 receptor (chromosome 9p) (Soblet et al., 2013; Wouters et al., 2010). VMCM are typically multifocal and small. One half of the lesions are located on the head or neck. Other typical locations are the extremities and the trunk. Skin and oral mucosa are the

typically affected areas, but VMCM are also seen in the muscle, brain, lung, and gastrointestinal tract. Lesions are mostly asymptomatic. No sex preponderance has been noted.

Glomuvenous malformation (GVM) is an autosomal dominant inherited malformation, which is caused by loss-of-function mutations in glomulin, comprised of abnormal smooth muscle and malformed venous channels lined by multiple layers of glomus cells (Brouillard et al., 2002). It commonly manifests as painful, bluish-purple, and firm multifocal skin nodules with a cobblestone surface. However, approximately 50% of the patients may have intramuscular lesions (Merrow et al., 2016).

Fibroadipose vascular anomaly (FAVA) is a unique lesion involving muscles in the extremities. FAVA typically affects the calf, followed by the thigh, forearm, gluteal area and ankle or foot. It is mostly diagnosed in children and young adults, and they present with pain and contracture (Cheung et al., 2020). FAVA causes a fibro-fatty solid mass which can displace normal muscle tissue and invade surrounding neurovascular structures (Wang et al., 2020). FAVA shares clinical features with intramuscular venous malformation and PTEN hamartoma and can commonly be mistaken as such. A clear distinction can be made by MRI. In MRI FAVA shows more fat or fibrosis, it is more heterogenous, has less defined channels, and displays nonspongiform-appearing vessels. On T2 images are not as bright as in VM. Histopathologically skeletal muscle is more infiltrated by fibroadipose tissue than in VM (Greene A, 2013).

Verrucous venous malformation (VVM, formerly verrucous hemangioma) is a sporadic, non-hereditary lesion. A somatic mutation of MAP3K3 is associated with VVM (Couto et al., 2015). It consists of aberrant clusters of malformed dermal venule-like channels underlying with variable degrees of hyperkeratosis. The lesions are typically hyperkeratotic, solitary, or multiple dermal plaques extending into the subcutis. 91% of lesions are localized in an extremity (usually legs) and the rest in trunk. Over time VVMs may become more hyperkeratotic, especially in response to injury, infection or subtotal resection, and bleeding can occur (Couto et al., 2015).

Diagnostic methods

90 % of VMs are diagnosed by history and physical examinations and the primary differential diagnosis is LM. Small and superficial VMs mostly do not need further diagnostic interventions.

US is mostly the first imaging modality used for diagnostics of VMs (Legiehn & Heran, 2008). On US VMs appear as heterogeneous and hypoechogenic clusters of compressible, dysplastic veins in every tissue layer. On color Doppler US, a slow flow pattern can be seen. Sometimes the flow can be undetectable and mistakenly interpreted as a sign of thrombosis. However, a VM can be compressed while a thrombosed vein is uncompressible. Also the differentiation with US between VMs and LMs can be difficult. Here the differential diagnosis can be done by puncture and aspiration. US has also its limits in the ability to evaluate extensive and deep lesions (Flis & Connor, 2005).

MRI has become the imaging modality of choice to demonstrate VMs and their extent, relationship to adjacent structures and in therapy planning (Burrows et al., 1998; Cooke-Barber et al., 2020; Dompmartin et al., 2010; Flis & Connor, 2005; Legiehn & Heran, 2008; Yakes W et al., 1990). MRI should be evaluated with fat suppression and contrast. VM lesions appear as either isointense or hypointense on T1-weighted images and hyperintense on T2-weighted sequences and can contain intra-lesional fluid-fluid levels. Intralesional phleboliths are a pathognomonic feature of VMs. They demonstrate a low-intensity signal on both T1 and T2-weighted sequences (Dubois J & Garel L, 1999; Flis & Connor, 2005). Phleboliths are detected only in 16% of the cases.

CT is of limited use in the diagnostic of focal VMs, because it usually provides poor lesion conspicuity relative to adjacent potentially critical structures and can underestimate lesion extent. CT is indicated in providing detailed anatomic information on osseus VMs (Legiehn & Heran, 2008).

Treatment

Because 93% of the VMs progress during the patient's lifetime, most patients who present with asymptomatic lesions ultimately will require intervention. Each patient should be considered individually by an experienced multidisciplinary team to determine the best management algorithm. A combination of multiple procedures, laser therapy, sclerotherapy and resection are often successful to achieve the best result. The size, location, and phenotype of the VM are the determinant factors in

choosing the treatment modality. The choice of treatment should be made after considering each modality's efficiency, chance of morbidity, and preservation of function (Hein K et al., 2002). Since curative treatment of VMs is seldom possible, the aim of the treatment must be to reduce and relieve symptoms, i.e., improve the patients' QOL.

Conservative treatment

VM patients with lesions in the extremities can benefit from well-tailored compression garments. These garments help to prevent blood stasis and reduces so swelling and pain. They are excellent for patients who are symptomatic only during activity. Patients suffering from painful superficial thrombophlebitis should be treated with anti-inflammatory drugs or low-molecular-weight heparin (Gibson & Barnacle, 2020; Glade et al., 2010).

Sclerotherapy

Sclerotherapy is the primary treatment for VMs in most vascular anomalies centers (Cabrera J et al., 2003; Hein K et al., 2002; Legiehn & Heran, 2008; Rautio R et al., 2004). Sclerotherapy results in endothelium disruption, intravascular coagulation, and inflammation of VM. This leads to fibrosis and contraction of the lesions. Often multiple injections are required to achieve the best result. It is most effective when performed as a series of procedures, scheduled two to three months apart, until there is no further recanalization or swelling. Direct injection of sclerosing agents results in gradual shrinkage of most VMs (Mulliken J et al., 2013). Most sclerotherapy complications are local. Low-flow vascular malformations are associated with localized intravascular coagulopathy (LIC), in which coagulation of stagnant blood in extensive lesions stimulates thrombin and initiates the conversion of fibrin to fibrinogen. It is characterized by elevated D-dimer, hypofibrinogenemia, and thrombocytopenia with a risk for both thrombosis and bleeding, however, rarely progressing to disseminated intravascular coagulopathy (DIC) (Aronniemi et al., 2016).

Detergent/surfactant sclerosants are a group of agents including sodium tetradecyl sulfate (STS), polidocanol, sodium morrhuate, and ethanolamine. Detergent sclerosants produce damage to the endothelial lining by multiple mechanisms associated with a decrease in endothelial cell surface lipids, disruption

of intercellular cement, and extraction of cell surface proteins (Duffy David M, 2010). These sclerosants have been shown to be efficient and safe in microfoam form (Bergan J et al., 2006; Cabrera J et al., 2003; Pascarella L et al., 2005). Microfoam is commonly produced by Tessari's method by mixing the sclerosant with air (or air and oily contrast medium). In this technique, a syringe containing 1 ml of 1% to 3% sclerosant is connected to a syringe containing 4 to 5ml room air by way of a three-way stopcock. Studies comparing the use of microfoam with liquid form sclerotherapy have confirmed microfoam to be more effective, having a lower rate of recanalization and a higher rate of obliteration of VMs. Another advantage is the possibility of reducing the amount of necessary sclerosing solutions as well as the concentration with a low complication rate (Cabrera J et al., 2001; Yamaki T et al., 2008). The assumption is, that the foam results in better and longer lasting contact with the vessel wall, leading to a more effective treatment, and more prolonged displacement of the intralesional blood (Burrows P & Mason K, 2004; Dompmartin et al., 2010). Other widely used sclerosing agents are ethanol, Ethibloc®, bleomycin and Pingyangmycin. Van der Vleuten et al. concluded in their review article, in which they compared the results of the five most used sclerosants; ethanol, bleomycin, polidocanol, STS, and Ethibloc®, that there are no differences concerning the efficacy of these sclerosants (van der Vleuten et al., 2014). The choice of the sclerosant should be based on minimal toxicity, cost effect, and institutional experience.

STS is a synthetic long-chain fatty acid widely employed industrially as an anionic surfactant (soap). It was first described in the 1940s. Historically, 3% liquid STS was injected percutaneous directly into the malformation. Today STS is mostly used in foam form, based on the better outcomes (Yamaki T et al., 2002, 2008). Microbubbles of air in the foam are coated with STS, which increases the displacement of blood from the lesion and increases the surface area, permitting better contact of the agent with the endothelium(Fowell et al., 2017). STS causes vessel thrombosis, erythrocyte sludging, intimal necrosis, adventitial fibrosis, and luminal collapse(Kok et al., 2012b) Van der Vleuten *et al.* published in a systemic review the data of two studies with VM patients treated with STS. The success rate ranged from 85 to 87%. The risk of systemic complications such as hemoglobinuria and oliguria can be reduced with hydration therapy during the procedure and in the recovery period (Barranco-Pons et al., 2012). STS foam sclerotherapy has been associated with transient ischaemic attack and stroke caused by emboli and macrobubbles (Parsi, 2012) Neuropathy has also been reported after STS sclerotherapy of VMs (Stuart et al., 2015)

Polidocanol, a mixture of ethanol (5%) and hydroxypolyethoxidodecain (95%), was first synthesized in 1936 and marketed as a topical and local anesthetic. It has been used as a sclerosing agent since the 1960s due to its ability to sclerose blood vessels without a significant risk of damage to surrounding tissue. The recommended concentration is in the range of 0.25-3%, giving the widest safety margins against extravasation necrosis of any potent sclerosing agent (Duffy David M, 2010). Cabrera and Yamaki were the first to report of the use of polidocanol in microfoam form(Cabrera J et al., 2001, 2003; Yamaki T et al., 2002) Cabrera *et al.* showed 46 of 50 patients (92%) having benefitted of the polidocanol microfoam sclerotherapy (Cabrera J et al., 2003). Reversible asystolic cardiac arrest and hemoglobinuria has been reported as a rare complication of polidocanol sclerotherapy (Marrocco-Trischitta et al., 2002). It does not incite as much endothelial damage as ethanol, STS, or ethanolamine oleate (Suzuki et al., 1992)

Sodium morrhuate is a fatty acid obtained from the liver of codfish. It damages the endothelial cell membrane and eventually leads to endosclerosis (Yildirim et al., 2005). Sclerotherapy with sodium morrhuate has been reported to cause ulceration and tissue necrosis of injected areas (Zhao et al., 2004). It has been found to be 1.5 to 4 times less effective than STS (Legiehn & Heran, 2008).

Ethanolamine oleate is an emulsion of fatty acids. The oleic acid portion induces an inflammatory reaction within the intima and penetrates the vascular wall, leading to an extravascular inflammatory reaction and activating coagulation. The ethanolamine portion suppresses fibrin clot organization. In combination, ethanolamine oleate allows fibrosis and sclerosis to replace the lesion that may progress over time and appear in delayed manner (Alexander et al., 2014; Legiehn & Heran, 2008; Uehara et al., 2009). Rodrigues et al noted a 100% effectiveness by 27 patients with oral vascular lesions treated with ethanolamine oleate (Rodrigues Johann et al., 2005). Seldom observed complications of ethanolamine are skin ulceration and necrosis. Ethanolamine can cause systemic complications, such as hemoglobinuria and hemolytic renal failure in higher doses(Kaji et al., 2009).

Ethanol (95%-98%) is the most effective sclerosing agent with the lowest recurrence rate, causing direct vessel-wall necrosis and disruption of erythrocytes, with subsequent inflammation and fibrosis of the intima (Rohlffs & Yakes, 2018; Yakes W et al., 1990). It was historically the most common sclerosing agent used but its high complication rate and dose limitations (0.5-1ml/kg) have made it less popular in recent years. Results of altogether 13 studies were analysed in a review article. The success rate of 695 patients treated with ethanol scleroherapy was 74% (range 27-100%) (van der Vleuten et al., 2014). Intravascular injected ethanol causes

pain and thus adequate general anesthesia is required during sclerotherapy (Gibson & Barnacle, 2020). Another disadvantage is the risk of extravasation which causes necrosis of the surrounding soft tissue. Severe complications such as nerve damage, lung embolism, pulmonary vasospasm, and heart arrhytmia have been reported with use of ethanol.

Ethibloc® is composed of zein solution, sodium amidotrizoate, oleum papaveris, and propylene glycol. Toxicity of ethanol is mainly due to the diffuseness of alcohol and the high therapeutic doses needed for the procedures. To reduce diffuseness, absolute ethanol has been mixed with these four solutions, leaving behind a non-resorbable mass (Dompmartin et al., 2010). The disadvantage of Ethibloc® is the usual need for general anesthesia, and the possible delayed extrusion of the embolic material to the skin surface.

Sclerotherapy technique

The technique of sclerotherapy of VM differs from that of LM. Prior to sclerotherapy the VMs morphologic characteristics must be considered. Several classification systems have been described in the literature. Dubois et al and Puig *et al.* categorize VMs, based on their venous drainage patterns, into one of three or four categories (J. M. Dubois et al., 1991; Puig et al., 2003). The types and sclerotherapy outcomes are listed in Table 3. Sclerotherapy is performed via direct percutaneous cannulation of vascular channels. Small, localized lesions can be injected with detergent sclerosants with use of regional anesthesia. Patients with extensive VMs, and those undergoing ethanol sclerotherapy, are anesthetized. Depending on the anatomy the lesion is accessed with one or more 20- to 27-gauge needles with a short low-volume connector tubing or a butterfly needle is connected to a saline-filled syringe by a three-way stopcock, often with use of US guidance. Appropriate contrast medium is injected to exclude arterial cannulation, to assess the nature of the lesion and its drainage, determine how much of the lesion is accessed, and to define the volume of distribution (Burrows P & Mason K, 2004; Legiehn, 2019; Legiehn & Heran, 2008; McCafferty, 2015). The chosen sclerosing agent is injected into the lesion. The procedure is terminated once an adequate volume of VM is treated, if the maximum allowable sclerosant dose is reached. If the VM is palpable, the operator should observe the degree of induration of the lesion over the course of sclerotherapy and limit administration as the lesion becomes firm. All sclerosing agents are radiolucent and may be opacified to facilitate visualization in fluoroscopic guidance before injection into the lesion.

Compression can be applied immediately post-procedure to allow vascular wall apposition and reduce intralesional volume and dilution effects. A compression bandage is applied for 24h. Analgesics, anti-inflammatory agents, and steroids are mostly prescribed. Patients with small, localized lesions can be discharged mostly on the same day, while the patients with extensive VMs must be monitored post-procedure overnight. For ideal results sclerotherapy frequently requires a course of treatment, rather than a single session and can be achieved by three to five sessions scheduled between six and eight weeks apart (McCafferty, 2015).

Table 3. Puig and Dubois classification of VMs

Type	Description	Sclerotherapy outcome
Type I	Isolated lesion without visible draining veins	Highest success rate
Type II	Isolated, well circumscribed lesion with drainage into normal veins and venous system	High success rate
Type III	Isolated, well circumscribed lesion with drainage via and into dysplastic veins	50% exclusion rate High complication rate
Type IV	Lesion is comprised entirely of ectatic and dysplastic veins	60% exclusion rate Highest complication rate

Laser therapy

Nd:YAG laser treatment was first used to treat hemangiomas in infancy. Endovenous laser ablation, using diode or Nd:YAG laser systems, has become a supplementary treatment modality in treating VMs. A bare laser fiber can be inserted through a catheter or large cannula to ablate the endothelium of VM under US guidance. The Success rate of three retrospective case series in all 270 patients was between 92.8%- 96%. In these studies, endovenous laser ablation was proven to be safe, with a very low incidence of complications, and minimal morbidity (Lu et al., 2011; Poetke et al., 2011; Sarig et al., 2006).

Pharmacological treatment

The mTOR inhibitor rapamycin integrates signals from the PI3K/AKT pathway to regulate multiple cellular processes, including cell growth and proliferation. Rapamycin suppresses TIE2-L914F-induced AKT phosphorylation and inhibits murine VM lesion expansion, although it fails to promote regression. Rapamycin has become a new therapeutic option for TIE2-mutated VM patients who fail the standard care (Boscolo, Limaye, et al., 2015; Seront et al., 2019). Ponatinib was combined with rapamycin and was reported to promote regression of murine VM (X. Li et al., 2019).

Surgical treatment

Complete surgical resection of extensive VMs is rarely possible, and it may require an extensive removal of surrounding unaffected tissue, leading to unwanted cosmetic and functional side-effects. Surgical excision is proposed in the following indications: small, well-localised, focal lesions that are thrombosed, and confined to a single muscle group or GVMs with possible complete surgical excision; VMs causing a neurologic or compression syndrome; or VMs with post-sclerotherapy fibrosis. Partial resection without preceding sclerotherapy is rarely performed because of the risk of a recurrence or surgical morbidity. Surgery is the treatment of choice for GVMs because it is more superficial and invades the adjacent structures less (Dompmartin et al., 2010; Hein K et al., 2002).

2.1.4 High-flow vascular malformations

High-flow vascular malformations represent a therapeutic challenge. These malformations are the only anomalies requiring diagnostic angiography in therapy planning and are most demanding among vascular malformations to be treated (Dubois J & Garel L, 1999; Yakes et al., 2016). High-flow vascular malformations are characterized by a cluster of arterial and venous channels without a significant, solid identifiable mass. They are divided into arteriovenous malformations (AVMs) and arteriovenous fistulas (AVFs) both shunting blood, AVM via nidus and AVF via direct arteriovenous connections. AVMs are typically congenital and located most often in the brain. Other typical locations are bone, muscle, and subcutaneous fat. AVMs contain a nidus between the arterial and venous portions of the malformation

that bypasses the normal capillary system, whereas AVFs lack the nidus. AVFs are usually acquired from venous hypertension and upregulated angiogenesis. In cerebrovascular AVFs feeding arteries are derived from the meninges, either pia or more commonly dura and shunt blood to small venules within a dural sinus. Histologically both AVMs and AVFs reveal dysplastic arteries that drain into arterialized veins. In US, high-flow lesions show enlarged vascular channels with a relative absence of a well-defined soft tissue mass (Legiehn & Heran, 2008). High-flow vascular malformations are differentiated from low-flow malformations by characteristic MRI features in T1-weighted and T2-weighted including serpiginous signal voids without a focal mass. Intraosseous lesions may benefit from contrast-enhanced CT (Baker et al., 1993).

2.1.4.1 Arteriovenous malformation

AVMs are rare, congenital vascular malformations associated with a variable degree of arteriovenous shunting through a nidus (abnormal channels bridging the feeding artery to draining veins). They result from an error in vascular development during embryogenesis. The prevalence of AVMs is estimated to have a range of 5 to 613 per 100,000 persons (Stapf c et al., 2001). It is relatively rare that AVMs cause clinical problems during childhood. Some of the lesions diagnosed in childhood may grow rapidly particularly around puberty. AVMs enlarge by dilation of existing channels and by the opening of collateral channels. Trauma, embolization, and resection may cause AVM to enlarge. Schobinger proposed a clinical staging system in 1990. It was published by Mulliken and collegues in 1997 (Table 4) (Kohout M et al., 1998). Clinical findings include a pulsatile mass with a thrill, bruit and occasionally local hyperthermia, skeletal overgrowth, trophic changes, congestive heart failure and functional impairment because of arterial steal and ischemia. Bleeding is common in AVMs at the skin or mucosal surface. Extracranial AVMs are typically located in head and neck, followed by the extremities, trunk, and viscera.

US is the first-line study to confirm the diagnosis. Magnetic resonance angiography (MRA) demonstrates feeding arterial vessels and early enhancement of draining veins and the location of the nidus. MRA is useful in the determination of the extense of the AVM and for developing a treatment plan. CT is helpful if the malformation involves bone (Azizkhan, 2013; Rosen et al., 2013; Sadick et al., 2018).

AVMs are undoubtedly of the vascular anomalies the most challenging to treat successfully. Embolization has become the treatment of choice for high-flow vascular malformations (J. Dubois & Garel, 2002) (Figure 11). Preoperative

embolization of the nidus combined with surgical resection offers in many cases excellent results (Rosen et al., 2013). The preoperative embolization will minimize the blood loss during surgery. The goal must always be to occlude or remove the nidus of the AVM. AVMs may be single, multiple, or part of a genetic disorder, such as Hereditary Hemorrhagic Telangiectasia (Osler-Weber-Rendu). It is an autosomal dominant heritable syndrome causing epistaxis, mucocutaneous telangiectasis and visceral arteriovenous malformations.

Figure 11. A. Residual orbital arteriovenous malformation after several previous treatments. B. T2 weighted MRI after five embolizations. Multiple tourtuous vessels of AVM. C. DSA after five embolizations demonstrates shunting via residual sinus, many peripheral branches of ophthalmic artery and early filling of facial vein. D. After the embolization nonopacification of the nidus.

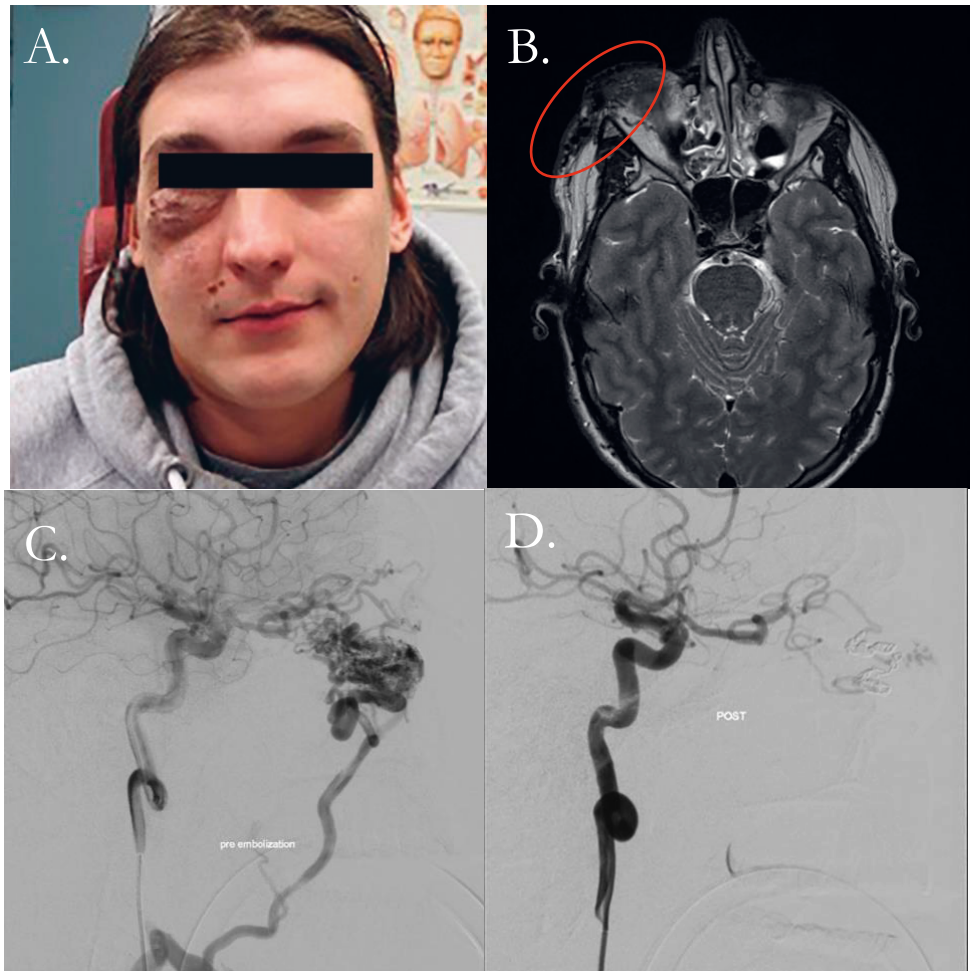


Table 4. Schobinger staging of arteriovenous malformations

STAGE	CLINICAL FINDINGS
I (quiescence)	Cutaneous blush/ warmth
II (expansion)	Bruit, audible pulsations, expanding lesion
III (destruction)	Pain, ulceration, bleeding, infection
IV (decompensation)	Cardiac failure

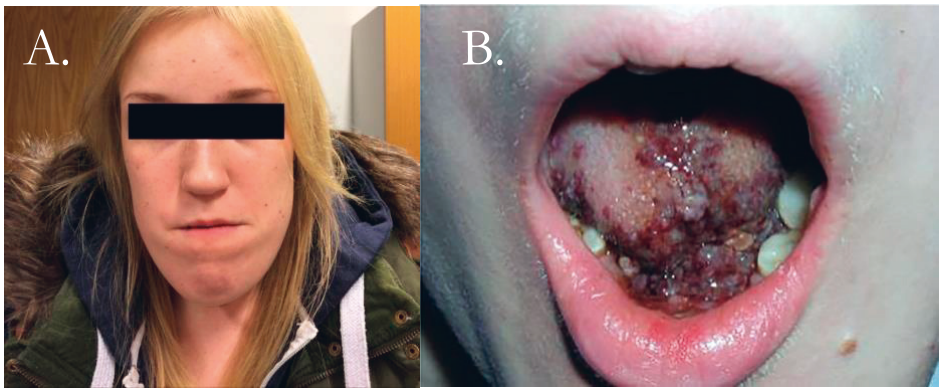
2.1.4.2 Arteriovenous fistula

An arteriovenous fistula (AVF) is an abnormal communication between an artery and a vein bypassing the normal capillary bed. AVF can be either a congenital or a traumatic lesion. AVF may be associated with other vascular and nonvascular anomalies, such as Parkes-Weber syndrome.

2.1.5 Combined vascular malformations

Combined vascular malformations are comprised of two or more vascular malformations in 1 lesion (Figure 12). Multiple combinations of low-flow and high-flow malformations are possible, causing distinct clinical findings; skin changes, orthopedic issues, and cosmetic impairment (Merrow et al., 2016; Sadick et al., 2018). Capillary-lymphatico-venous malformations (CLVMs) are the most common combined vascular anomaly (Azizkhan, 2013). For different possible combinations see Table 1.

Figure 12. Lymphatic-venous malformation. A. Originally treated surgically several times while sclerotherapy was not available. B. Current status, microcystic lesions treated with laser surgery when needed.



2.1.6 Malformations of major named vessels

Malformations of major named vessels are characterized by involvement of veins, arteries, or lymphatics of generally large caliber, often axial or conducting vessel. These following anomalies can influence major named vessels; origin, course, number, length, diameter (aplasia, hypoplasia, ectasia, aneurysm), valves, communication, and persistence (of embryonal vessels). For example, carotid artery anomalies associated with PHACES, and vein of Galen malformation are included in malformations of major named vessels (Merrow et al., 2016).

2.1.7 Vascular malformations associated with other anomalies

Vascular malformations may be associated with anomalies of bone, soft tissue, or viscera. This heterogenous group of disorders is mostly associated with overgrowth of soft tissue and/or bone or, rarely, undergrowth. The etiology of resulting symptoms, primarily pain and functional impairment, is also multifactorial and can be difficult to characterize and manage. Overgrowth can be focal or generalized (Blei, 2015; Uller et al., 2014). Recent discoveries have elucidated that specific genetic changes underlie overgrowth syndromes with vascular anomalies. These are associated with somatic mutations, particularly of PIK3CA. The improved knowledge has led to promising new targeted therapies for these patients (Bertino &

Chaudry, 2019; Eng et al., 2020). Most of these syndromic malformations are listed in Table 5.

Table 5. Vascular malformations associated with other anomalies

Klippel-Trenaunay syndrome: CM + VM +/- LM + limb overgrowth
Parkes-Weber syndrome: CM + AVF + limb overgrowth
Servelle-Martorell syndrome: limb VM + bone undergrowth
Sturge-Weber syndrome: facial + leptomeningeal CM + ocular anomalies +/- bone and/or soft tissue overgrowth
Limb CM + congenital nonprogressive limb hypertrophy
Maffucci syndrome: VM +/- spindle cell hemangioma + enchondroma
Macrocephaly-CM (M-CM) / megalencephaly-CM-polymicrogyria (MCAP)
Microcephaly-CM (MICCAP)
CLOVES syndrome: LM + VM + CM +/- AVM + lipomatous overgrowth
Proteus syndrome: CM, VM and/or LM + asymmetric somatic overgrowth
Brannayan-Riley-Ruvalcaba syndrome: AVM + VM + macrocephaly, lipomatous overgrowth

2.2 Genetics of vascular malformations

2.2.1 General genetics

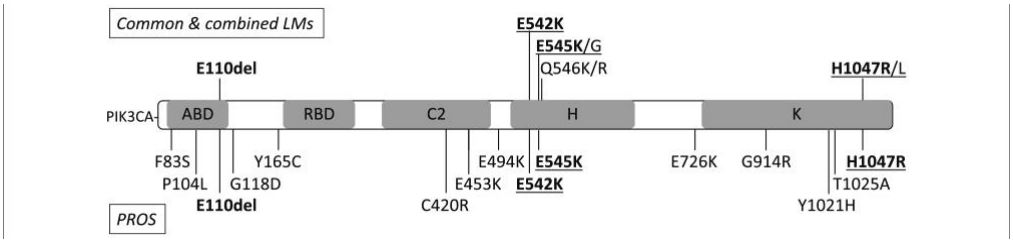
Most vascular malformations occur sporadically but for some there is an inherited predisposition. Sporadic lesions are mostly localized, whereas the inherited lesions are usually multifocal and mainly small. Genetic defects have been identified for hereditary hemorrhagic teleangiectasia, inherited cutanemucosal venous malformation, glomuvenous malformation, capillary malformation-arteriovenous malformation, cerebral cavernous malformation, and some isolated and syndromic forms of primary lymphedema. Vascular endothelial growth factors, angiopoietins, and their endothelial tyrosine kinase are central regulators of vasculogenesis, angiogenesis and lymphangiogenesis (Lohela et al., 2009). In 2009, it was demonstrated that somatic genetic mutations are associated with sporadically occurring vascular anomalies: isolated venous malformations were discovered to

have somatic TIE2/TEK mutations (Limaye, Boon, et al., 2009). Since then, the etiopathogenesis of various isolated and combined vascular malformations has been unraveled (Dekeuleneer et al., 2020; Schlögel et al., 2017). This has opened the era for development of targeted precision therapies for these lesions, especially using small molecule inhibitors. Rapamycin, an inhibitor of mTOR, has already been tested in clinical trials and has demonstrated efficacy in selected cases (Boscolo, Limaye, et al., 2015; X. Li et al., 2019; Seront et al., 2019).

2.2.2 PIK3CA

One of the genes identified to be mutated in vascular malformations encodes the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA, also known as p110α). Mutations were first identified in rare syndromic patients with Congenital Lipomatous Overgrowth, Vascular malformations, Epidermal nevi, and Skeletal/spinal abnormalities and/or scoliosis (CLOVES) (Keppler-Noreuil et al., 2014). Subsequently, PIK3CA mutations were implicated in common LM, and isolated VM without a TIE2/TEK mutation. Somatic/mosaic PIK3CA mutations have also been implicated in variable syndromic phenotypes that can associate vascular anomalies with hypertrophy, leading to name this spectrum "PIK3CA-Related Overgrowth Syndrome" or PROS. It includes CLOVES, Fibroadipose Hyperplasia, Fibroadipose infiltrating lipomatosis, Hemihyperplasia multiple lipomatosis, Klippel-Trenaunay syndrome, Megalencephaly-Capillary Malformation-Polymicrogyria syndrome, Macrodactyly, Hemimegalencephaly, and Muscle hemihyperplasia (Keppler-Noreuil et al., 2016). Subsequently, PIK3CA mutations were implicated in common LM, and isolated VM without a TIE2/TEK mutation (Boscolo, Coma, et al., 2015; Limaye et al., 2015; Luks et al., 2015; Osborn et al., 2015). PIK3CA is considered as an oncogene (Samuels et al., 2004). The encoded p110α subunit contains the adapter-binding domain (ABD), the Ras-binding domain (RBD), the C2-PI3K-type domain (C2), the helical domain (H), and the kinase domain (K) (Figure 2) (Burke et al., 2012). Mutations that spread over the five functional domains have been implicated in many cancers, with positions c. 1624G (p.E542), c.1633G (p.E545), and c.3140A (p.H1047) as hotspots. Similar mutations have been found in small series of lymphatic and vascular anomalies. Hotspot is the area in DNA where the risk for mutations is significantly elevated.

Figure 13. PIK3CA protein (Human, 1068aa) and mutations. Top, mutations found in common and combined lymphatic malformations (LM, LVM, CLVM9. Bottom, mutations found in patients with PROS (KTS, CLOVES, unclassified PROS). Shared mutations in bold. Hotspot mutations underlined. ABD, p85α-binding domain;RBD, Ras-binding domain; C2, C2-PIK3C-type domain; H, helical domain; K, kinase domain



2.2.3 TIE2

Activating mutations in the endothelial cell tyrosine kinase receptor TIE2 are a common cause for VM. The TIE/angiopoietin (ANGPT) family includes 2 receptors (TIE1 and TIE2) and 3 ligands (ANGPT1, ANGPT2, and ANGPT4) (Eklund & Saharinen, 2013). TIE2 is specially expressed in vascular endothelium of arteries, capillaries, and veins (Dumont et al., 1995). ANGPT1 and ANGPT2 bind to TIE2 and mediate, respectively, vascular maturation, and angiogenesis. In murine models, a knockout of TIE2 or ANGPT1 results in impaired blood vessel branching and deficient perivascular coverage. In the developing embryo a deletion of ANGPT1 produces a disorganized vascular network with an increased number of ectatic vessels (Dumont, Anderson, et al., 1994; Dumont, Gradwohl, et al., 1994). VMCM are inherited as an autosomal dominant trait and caused by mutation in TIE2. The most common inherited VMCM mutation is R849W (Vikkula et al., 1996). Most VM are sporadic (94%), solitary, and localized (Boon et al., 2011). Activating somatic mutations in TIE2 have been identified in approximately 50% of sporadic VMs (Limaye, Wouters, et al., 2009). These mutations differ from the inherited ones that cause VMCM. The most common one is L914F, which accounts for 85% of the lesions. It has not been identified as an inherited mutation, and this suggests that it is lethal in the germline (Limaye, Boon, et al., 2009; Limaye, Wouters, et al., 2009)

It has been demonstrated in murine model, and human subjects, that Rapamycin improves TIE2-mutated VM (Boscolo, Limaye, et al., 2015). Li et al combined

Rapamycin with Ponatinib and demonstrated a regression of VM in murine model (X. Li et al., 2019).

2.3 Quality of life

QOL assessment is nowadays widely used to evaluate the efficacy of various treatments, especially when treating chronic illness or disorders. QOL data are usually collected by a set of generic, logical, and easily administered QOL questions. QOL measurements may be specific to a pathology or region of the body, or they may be more general and designed to evaluate health-related QOL.

SF-36 is one of the most widely used and extensively validated generic self-report measurements (Aaronson et al., 1992, 1998; Gandek et al., 1998; Keller et al., 1998; Ware et al., 1998). The SF-36 was developed in the United States in the late 1980s as part of the Medical Outcomes Study. It was designed for use in clinical practice and research, health policy evaluations and general population surveys. SF-36 is composed of 36 questions and standardized response choices measuring each of eight health concepts: physical functioning, role limitations due to physical health, bodily pain, general health, vitality, social functioning, role limitations due to emotional problems, and mental health. SF-36 items and scales have been scored so that a higher value indicates a better health state (Aaronson et al., 1992).

The RAND 36-Item Health Survey 1.0 (RAND-36) includes the same items as those in the SF-36, but the recommended scoring algorithm is somewhat different from that of the SF-36 (Hays et al., 1993).

The Finnish version of the RAND-36 was presented in 1995 (Aalto A-M et al., 1995). Population reference values for the Finnish version of the RAND-36 have also been provided (Aalto et al., 1999).

Sickness Impact Profile-68 (SIP) was developed to provide a measure of functional limitations caused by general health status. Scores are calculated by adding the number of items answered affirmatively, with higher scores indicating greater disability. Scores range from 0 to 68 (Bergner M et al., 1981; de Bruin, Buys, et al., 1994; de Bruin, Diederiks, et al., 1994).

Nottingham Health profile (NHP) was developed in the 1970's in the United Kingdom. The NHP was developed to measure the perceived health for use in population surveys. It consists of 38 dichotomous items that are grouped into six scales, which each range from 100 to 0; 0 representing the best health status (Essink-Bot M-L et al., 1997; Hunt et al., 1986).

The Chronic Venous Insufficiency Questionnaire (CIVIQ), a specific QOL questionnaire, was developed by Professor Launois (Appendix). In the questionnaire, four dimensions are explored: pain, physical, social functioning and psychological and it includes 20 multiple-choice questions. The scores for each Constituent item are added, and the total score is obtained by summing the 20 items. The scores used in CIVIQ are converted into an index, analogous to the index used to score the SF-36. The value of the indices is directly proportional to the degree of deterioration of quality of life: 0 representing the highest quality of life and 100 the lowest (Launois et al., 1996, 2010).

2.4 Response shift theory

Response shifts are changes in self-evaluation that may be a direct or indirect result of the intervention being evaluated (Albrecht & Devlieger, 1999). The answers in the QOL questionnaires rely upon patient self-reporting and the results can be used for routine monitoring and assessment of treatment outcomes. This type of measurement is susceptible to the phenomena of response shift. There are three basic types of change: alpha, beta, and gamma (Howard et al., 2011; Schwartz et al., 2007).

- Alpha change is often considered to be "true change". Alpha change can be clearly observed and concretely measured using stable scales instrument
- Beta change is recalibration and describes a change in the scale against which the variable of interest may be measured
- Gamma change occurs when the dimension of interest is reconceptualized or redefined with a different meaning or interpretation by the individual.

3 AIMS OF THE STUDY

1. To evaluate the efficacy of treatment and long-term follow-up results of OK-432 sclerotherapy in lymphatic malformations. (I)

2. To compare the efficacy of two sclerosing agents: polidocanol and ethanol and to analyse the quality-of-life results after endovascular sclerotherapy of low-flow venous malformations. (II)

3. To evaluate the difference in treatment and quality-of-life results after polidocanol sclerotherapy of intra- and extramuscular low-flow venous malformations. (III)

4. To demonstrate the incidence of non-hotspot PIK3CA mutations in CLOVES compared to common and combined lymphatic malformations. (IV)

4 MATERIAL AND METHODS

4.1 Patients and methods

4.1.1 Study I

In Study I, the material comprised 36 LM patients treated with OK-432 in the Department of Otorhinolaryngology and Department of Radiology, Tampere University Hospital, Finland, during the years 1999 to 2009. Patients were diagnosed clinically and with an MRI examination. LMs were classified as macrocystic (cysts size of at least 2 cm^3), microcystic (cysts under 2 cm^3) or mixed, based on the radiological examinations. Patient demographics are shown in table 6. Patients of all ages were included in the study; ages ranged from 1 month to 47 years at the time of the first injection. OK-432 injections were performed with ultrasound and/or fluoroscopy guidance. OK-432 0.01 mg/ml was injected after aspirating intracystic fluid. The maximum volume of OK-432 injected was 10ml.

The treatment was finished after a stable clinical condition was reached or no response was observed. A late follow-up visit with an MRI scan was organized. The follow-up period was in average 6 years (range 5-13). During the visit patients were clinically examined. Results of immediate post-treatment MRI imaging were compared with the late follow-up MRI findings. Objective response to therapy was evaluated as the change in lesion size and graded as complete (90-100%), marked (50-90%), moderate (<50%) or no response.

Table 6. Patient demographics in studies I-III

	Study I (n=36)	Study II & III (n=41)
Sex		
Female	16	29
Male	20	12
Location		
Head and Neck	27	10
Trunk	9	0
Upper extremity	0	12
Lower extremity	0	19
Lymphatic malformation		
Macrocystic	33	
Microcystic	1	
Mixed	2	
Venous malformation		
Intramuscular		25
Extramuscular		16
Pre-treatment symptoms		
Pain	0	37
Cosmetic disturbances	31	17
Functional disturbances	16	38
No symptoms	2	0
Pre-sclerotherapy treatment		
No treatment	34	28
Surgery	2	12
Laser therapy	0	2

4.1.2 Study II-III

In study II and III, 41 patients with a VM were treated with polidocanol in Tampere (n=23) and Turku (n=18) University Hospitals during the years 2008 to 2013 and were retrospectively analyzed. In Study II the results were compared with the results of 44 VM patients treated with ethanol in Tampere University Hospital between 1991 and 2001. In study III the results were compared between two groups: intra- and extramuscular malformations. For patient demographics see table 6.

All clinically diagnosed patients underwent an MRI examination. Thirty-five patients were available for a post-treatment MRI after a mean follow-up time of three years. All pre- and post-treatment 1.5-T T2-weighted (T2W) fat-suppressed

axial, coronal, and sagittal MRI images were reviewed in consensus by two experienced radiologists. Pre-treatment size of the lesions, possible relation to muscle compartment, and modification in the post-treatment size and configuration were evaluated.

Polidocanol foam (3%) was generated by mixing the sclerosing liquid with air in a ratio of 1 to 4. Volume of the dosage was calculated with fluoroscopy or with US. The maximum dose did not exceed 2 mg/kg. The injections were performed under US and fluoroscopy guidance. Eleven patients were treated once, 14 patients twice, and 15 patients were treated three to seven times. One patient with a VM in the lower extremity was treated ten times. In all, there were 2.7 treatments/patient. The median interval between each session was five months (range 1-20 months).

4.1.3 Study IV

Study IV was an international multicenter study collecting samples from 143 patients with lymphatic malformations. From Finland 11 common LM patients were included to in this study. Altogether the samples included 105 common LM, 3 lymphatico-venous malformation (LVM), 1 capillaro-lymphatic malformation (CLM), 7 capillaro-lymphatico-venous malformation (CLVM), 4 unilateral capillaro-lympatico-venous malformation with hypertrophy (Klippel-Trenaunay-Syndrome, KTS), 14 CLOVES syndrome, 7 unclassified PROS, and 2 unclassified vascular anomaly syndromes (UVA). Clinical data regarding the phenotype, and localization (head & neck, trunk, and extremities), size (smaller or larger than 10 x 10 cm), and cystic structure (micro, macro, or mixed) of the lesion were collected. We used targeted next-generation sequencing (NGS) or digital droplet polymerase chain reaction (ddPCR) to screen for somatic PIK3CA mutations in DNA extracted from resected lesional tissue or lymphatic endothelial cells isolated from lesions. The prevalence, distribution, and allele frequency of PIK3CA mutations in these phenotypes was analyzed and evaluated for any genotype-phenotype correlation. So far, this study contains the largest cohort of isolated LMs.

4.2 Self-evaluation and quality of life (Studies I-III)

All patients answered a symptom questionnaire; patients were asked to evaluate the severity of pre-, and post-treatment symptoms and to estimate whether they had benefitted from the treatment or not. Symptoms were categorized as unchanged,

decreased, or symptom-free. After sclerotherapy, patients answered a specific QOL questionnaire. The QOL results from the study II were compared with the QOL results from the previous study with ethanol – this being the main reason for choosing this QOL questionnaire. In the questionnaire, four dimensions were explored with 20 multiple-choice questions: pain; psychological; physical; and social functioning. The scores for each constituent item were added and the total score was obtained by summing the 20 items. Absolute scores were converted into an index. The value of the indices is directly proportional to the degree of deterioration of QOL: 0 representing the highest and 100 the lowest QOL. This QOL questionnaire has been validated in an age-matched Finnish normal population (Aalto et al., 1999).

4.3 Ethics

All the study protocols were approved by the Ethical Committee of Pirkanmaa Hospital District, Tampere University Hospital. Study IV was also approved by the ethical committee of the Medical Faculty at the University of Louvain, Brussels, Belgium (ref B403201629786). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All participants were given oral and written information about the study protocol and gave their written consent.

4.4 Statistics

In study I the differences between groups in categorial variables were assessed with Fisher's exact test. Correlation between the continuous variables were analyzed with Spearman rank correlation test.

Studies II and III were retrospective, and all patients who met the inclusion criteria were included in the study. The difference between two-sample means was tested with Student's t-test. The Mann-Whitney U test was used to compare the two populations with unequal variance. For categorial data, Fisher's exact test, and Chi-Square test was used. Relationships between continuous variables were tested with correlation and simple linear regression analysis. A P value < 0.05 was considered to indicate a statistically significant difference.

In study IV data were analyzed to detect differences between groups using Fisher's exact test for two categorical variables, Pairwise Wilcoxon's rank-sum test for group comparisons of continuous variables, and the Kruskal-Wallis test for > 2 group comparisons of continuous variables. Nonparametric tests were used due to small sample size and non-normal distributions (tested with Shapiro test). Significance of *P* values: $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$. To control type I, Benjamini-Hochberg procedure was used. All analyses were performed using R, and graphs were generated with ggplot2. Descriptive statistics were computed using groupedstats R package. Variant allele frequency values for statistics were based on NGS results (average variant allele frequency values for patients with multiple sequencing) or ddPCR (for 10 samples tested only by ddPCR).

5 RESULTS

5.1 Study I

5.1.1 Clinical and radiological results

All patients showed a clinical response-local swelling and slight fever for 2-4 days-to the treatment. Three patients had marked swelling and a longer-lasting fever. MRI results showed that after follow up, 80% of the patients objectively benefitted from the OK-432 sclerotherapy (Table 7). Two patients (6%) relapsed after initially showing a complete or a moderate response. All patients with a complete response after the follow-up time had macrocystic lesions. The one patient with the microcystic lesion showed no response.

After the treatment two patients were diagnosed with a generalized LM disease and observed to have no response to the treatment after follow-up. No serious complications of OK-432 sclerotherapy were noted. There was not a correlation between the size of the LM, age of the patient at the beginning of the treatment and the final response.

Table 7. MRI outcome primarily and after follow-up (n=36)

Response	Outcome primarily	Outcome after follow-up
complete	17 (47%)	15 (42%)
marked	7 (19%)	8 (22%)
moderate	7 (19%)	6 (17%)
no response	5 (14%)	7 (19%)

5.1.2 Self-evaluation and quality of life

According to the self-evaluating questionnaire, 34 (94%) of the patients subjectively felt they benefitted from the treatment. Symptoms prior to treatment are shown in table 6. After the therapy and follow up, subjective symptoms were significantly decreased in 68% of the patients. Eleven (31%) of the patients still experienced cosmetic disturbances, 5 (14%) had functional symptoms post-treatment. Twenty-three (64%) were symptom-free after OK-432 therapy. 26/36 patients were available to answer the QOL questionnaire 5-13 years after the treatment. The results showed that a clear majority (24/26) did very well after sclerotherapy (Table 8). 16/26 patients had the lowest possible scores representing the highest quality of life measure. Two patients did clearly worse than the rest of the group. Both had persistent disease requiring further treatment.

Table 8. Dimensions in quality of life (n=26). 0 representing the highest and 100 the lowest QOL.

Patients	**(n)**	**Dimensions**			
Index	Total	Pain	Physical	Social	Psychological
0-19	24	23	24	23	24
20-39	1	0	1	2	1
40-59	1	3	0	0	1
60-79	0	0	1	1	0
80-100	0	0	0	0	0
Mean	5	8	4	5	5
SD	14	12	13	8	10
Minimum	0	0	0	0	0
Maximum	53	56	63	67	42

5.2 Study II

5.2.1 Clinical and radiological results

Thirty-five out of forty-one patients were available for the post-treatment MRI. The size of the VM appeared unchanged in 19 patients. In 10 patients, the size had decreased < 50% while a decrease > 50% was seen in 6 patients. In 12 patients the malformation was thrombosed partially and in four completely.

5.2.2 Self-evaluation and quality of life

According to self-evaluation, 19 (46%) patients subjectively benefitted from the treatment, and three of them were asymptomatic. Eighteen (44%) patients found no changes in their symptoms. QOL results showed that most patients did well after polidocanol sclerotherapy (Table 9). Mean index for total QOL was 22 (range=0-56), male mean 17, and female mean 24. The mean index compared to the Finnish normal population was equal; male mean 28, and female 25 (Aalto et al., 1999). Post-treatment pain was the single most significant factor having a poor impact on QOL. A statistically significant reduction in pre- vs. post-treatment pain was seen (P = 0.002).

Table 9. Dimensions in quality of life, polidocanol n=41, and ethanol n=42. 0 representing the highest and 100 the lowest QOL.

Patients	**(n)**		**Dimensions**							
Index	Total		Pain		Physical		Social		Psychological	
0-19	22	27	12	26	25	31	20	30	27	31
20-39	13	12	11	10	8	8	12	9	8	9
40-59	6	2	14	3	5	1	7	1	6	0
60-79	0	1	4	2	3	2	2	2	0	1
80-100	0	0	0	1	0	0	0	0	0	1
Mean	22	16	35	23	21	14	23	16	17	14
SD	16	16	19	20	20	17	20	18	16	14
Minimum	0	0	0	0	0	0	0	0	0	0
Maximum	56	74	63	81	63	69	75	75	53	78

5.2.3 Comparison between polidocanol and ethanol

Post-treatment QOL results in the ethanol group were better compared to the polidocanol group. The overall results were better in all measured dimensions of life (Table 9). The difference was noted to be significant in all dimensions, except the psychological. There were no complications in the polidocanol group. In the ethanol group there were three notable complications: a 1-cm diameter skin necrosis in the hypothenar; a transient paresis of the radial nerve; and a transient facial nerve paresis.

5.3 Study III

5.3.1 Clinical and radiological results

Forty-one VM patients were treated with polidocanol, 25 with intramuscular, and 16 with extramuscular lesions. In the pre-treatment MRI eleven intramuscular, and five extramuscular malformations were < 5cm. Fourteen intramuscular and eleven extramuscular were > 5 cm. The differences between pre- and post-treatment MRI findings were not found to be statistically significant.

5.3.2 Self-evaluation and quality of life

Altogether 23 of 41 patients (56%), 13 of 25 intra-muscular VM patients (52%), and 10 of 16 extramuscular patients (62.5%) felt to have benefitted from the treatment, according to the post treatment self-evaluation. Radiological changes in MRI examinations did not correlate with any of the subjective symptoms (*P*=0,43).

QOL results showed that most patients in both groups did well at the post-treatment evaluation (Table 10). Mean index for total QOL in the intramuscular group was 19 and in the extramuscular group, 28. The difference was not statistically significant. Mean index compared with the aged-matched Finnish normal population was equal (male=28, female=25).

Table 10. Dimensions in quality of life, intramuscular n=25, and extramuscular n=16. 0 representing the highest and 100 the lowest QOL.

Patients	**(n)**	**Dimensions**							
Index	Total	Pain		Physical		Social		Psychological	
0-19	15	7	7	5	16	9	14	6	18	9
20-39	9	4	7	4	5	3	8	4	5	3
40-59	1	5	9	5	3	2	3	4	2	4
60-79	0	0	2	2	1	2	0	2	0	1
80-100	0	0	0	0	0	0	0	0	0	1
Mean	19	28	33	38	18	32	19	32	13	23
SD	13	17	19	19	18	22	16	24	14	19
Minimum	0	3	0	0	0	0	0	0	0	0
Maximum	44	56	63	81	63	63	50	75	47	53

5.4 Study IV

PIK3CA mutations were found in 78/105 common LM (74.3%), 3/3 LVM (100%), 6/7 CLVM (85.7%), 4/4 KTS (100%), 12/14 CLOVES (85.7%), and 5/7 PROS (71.4%) (Table 12). No PIK3CA mutation was detected in the unique CLM, and the two unclassified vascular (lymphatic) anomaly syndrome (UVA) tested. Out of 108 mutations detected, 91 (85.1%) were in hotspot positions. There was a statistically significant difference in the distribution of hotspot mutations and non-hotspot

mutations. between common and combined LM compared to the syndromes (P=1.329 x 10^{-7}), to CLOVES (P=1.441 x 10^{-5}), or to unclassified PROS (P=1.207 x 10^{-4}), but not to KTS.

Within the mutated lesions, the variant allele frequency (VAF) varied strongly: from 0.54 to 25.33% for next-generation sequencing (NGS)-based analyses, and from 0.28% (14 single positive droplets (SPD)) to 22.7% for digital droplet PCR (ddPCR)-based analyses. Variability of VAF was studied in different samples of the same patient. From 16 patients 2, 3 or 4 samples were analysed. A mutation was found in 14. The detected allele frequencies had a mean average of 5.17% with an standard deviation (SD) of 2.36%. For each patient, the same mutation was identified in all tissues. The two patients without an identified somatic mutation, were each negative in 3 different samples. Overall, VAF varied from 0.54 to 25.33% between different phenotypes (Table 11). There was a statistically highly significant difference in VAF between common and combined LMs versus syndromes (PROS) (P=1.425 x 10-4), and versus CLOVES (P=6.510 x 10-5), as well as between common LMs versus syndromes (PROS) (P=6.765 x 10-5), and versus CLOVES (P=5.290 x 10-4).

There was no statistically significant difference between VAF or mutations (hotspot vs non-hotspot or presence vs absence) regarding LM localization size or cystic structure, except that a mutation is less often detected in small lesions (P=0.0129)

Table 11. Patients' cohort and PIK3CA mutations per pathology

Pathology	LM	LVM	CLM	CLVM	KTS	CLOVES	PROS	UVA	All
Cohort	105	3	1	7	4	14	7	2	143
Mutation	**78**	**3**	**-**	**6**	**4**	**12**	**5**	**-**	**108**
% With a mutation	74.3%	100%	0%	85.7%	100%	85.7%	71.4%	0%	75.5%
VAF median	3.71	4.59	-	7.34	8.12	12.90	11.09	-	4.05
Standard deviation	4.49	2.52	-	6.53	6.27	7.30	5.65	-	4.85
VAF rage	0.54-11.34	3.43-8.25	-	4.71-22.19	1.15-13.17	2.00-23.33	1.00-13.00	-	0.54-25.33
Hotspot mutations	74	3	0	6	3	5	1	0	92

6 DISCUSSION

6.1 Study I

The main finding in the study I is that OK-342 has a long lasting and safe effect in the management of macrocystic LMs. The longest so far published follow-up in this study showed that the response to OK-432 treatment was persistent in 83% of patients who demonstrated primarily a complete or marked response. Patients with no response initially remained unresponsive through the follow-up period; for patients showing no immediate response, it is reasonable to consider other treatment options if needed. Since microcystic lesions do not respond to OK-432 as well as the macrocystic LMs, bleomycin might be the preferred sclerosing agent for the microcystic lesions (Chaudry et al., 2014; Yang et al., 2011).

No serious side effects were noted in our series, which is consistent with previously published data (Motz et al., 2014; Poldevaart M et al., 2009; Smith M et al., 2009; S. N. Smith, 2019).

6.2 Study II

In study II we compared two different sclerosing agents, polidocanol and ethanol, for the treatment of low-flow VMs and compared their effect on QOL. The main result showed a significant reduction in subjective pre- vs. post-treatment pain scores, which is in accordance with a prospective study with a single sclerosant (ethanogel) (Wohlgemuth et al., 2017). Consequently, patients with pain especially benefit considerably from sclerotherapy and it should be offered to them.

Based on our reports and previous ones, ethanol is the most potent-but toxic-sclerosant available (Burrows P & Mason K, 2004; Fowell et al., 2017). Thus, polidocanol is probably the sclerosant of choice, at least when treating an area with a high risk of complications, i.e., subcutaneous lesions, proximity of a functionally significant nerve, or a major vein. An additional advantage of polidocanol is its local anesthetic effect, and thus no anesthesia is needed. Ethanol sclerotherapy is painful and requires adequate general anesthesia. No curative treatment exists at the moment

for VMs. The choice of the sclerosant should be based on minimal toxicity, cost effect and institutional experience (Duffy David M, 2010; van der Vleuten et al., 2014). For ideal results, multiple treatments are often necessary and can often be achieved by three to five sessions with a six- to eight-week interval between treatments (McCafferty, 2015). Age at the beginning of the polidocanol treatment did not influence QOL results. Consequently, patients benefit from the treatment at any time from infancy to adulthood.

Post-treatment MRI findings did not correlate with either subjective symptoms or QOL results. A routine MRI to control the results of treatment appears unnecessary. After sclerotherapy, changes in clinical appearance and MRI findings are often minimal and other methods to estimate the effectiveness of the treatment must be used. Based on our results, a subjective symptom and QOL questionnaire would be useful for this purpose. Systematic use of a pre- and post-treatment questionnaire provides a relatively objective tool with which to evaluate the results and effectiveness of the treatment and helps with the decision-making process while evaluating possible treatment options and schedules. We suggest that a pre- and post-sclerotherapy symptom and QOL questionnaire should be used with all VM patients, not exclusively in studies but in general clinical follow-up as well.

6.3 Study III

We compared the treatment results of extra- and intramuscular VMs after polidocanol sclerotherapy with the patient's subjective evaluation of the outcome and QOL after the treatment. Intramuscular VMs tend to be diagnosed later and they seem to cause more morbidity than extramuscular VMs. It is likely that the lack of skin or mucosal involvement makes dilated veins less apparent and the possible soft tissue mass in an infant is not suspected to be a vascular malformation (Scorletti et al., 2018). The primary reason for seeking treatment is pain and intramuscular VMs are also associated with more limitations in physical activity due to discomfort and swelling (Vogel et al., 2013). In our material, no difference were observed between the groups concerning QOL and specific dimensions, pain, functional problems or cosmetic appearance.

The main goals of the treatment are reduction of pain and discomfort, improvement of cosmetic aspect and reduction of the volume (Rabe E & Pannier F, 2013). Since, according to our results, there is no correlation between post-treatment MRI, subjective symptoms and QOL, a routine post-treatment MRI does not give

any meaningful information when considering the need for re-treatment. A similar result was also reported by Bianchini et al. in their series of 81 intramuscular VMs – no change or only moderate reduction in size was seen in 97,5 of their post-treatment MRIs (Bianchini et al., 2018).

6.4 Study IV

We identified a statistically significant association between mutation types and phenotypes. The non-hotspot mutations had a higher frequency in CLOVES and unclassified PROS compared to common and combined LMs. Similar finding has been noted for macrocephaly-capillary malformation in which PIK3CA mutations are more often non -hotspot mutations. Moreover, macrocephaly-capillary malformation patients tend to be more mosaic for the mutation, which is sometimes detectable in their blood. The level of mosaicism in human tissues may reflect the potential pathogenicity (the strength of downstream gain-of-function effects) of a given mutation. This suggests that the non-hotspot mutations that are more frequently seen in widespread PROS (such as CLOVES and macrocephaly-capillary malformation) may have weaker downstream effects and thus could appear earlier during fetal development, thereby affecting more extensive body parts. Interestingly, when KTS is defined as unilateral capillaro-lymphatico-venous malformation with hypertrophy, like in this study, it seems to have more hotspot mutations, like common and combined LMs, which are usually localized.

The implication of the same PIK3CA mutation in common LM and common VM reinforces the idea that the cell type in which mutations occur directly influences the pathology. LMs would be due to somatic mutations in lymphatic endothelial cells (LEC), whereas VMs would be due to a mutation in blood endothelial cells (BEC). Murine modelling demonstrated that the same somatic PIK3CA mutation activated in LECs can lead to macro- or microcystic lesions, depending on the time-point of induction of expression during development and growth (Martinez-Corral et al., 2020). Intra-uterine activation led to macrocystic LMs, whereas early post-natal induction led to microcystic LMs. This fits well with the concept of cell-type and time-dependent occurrences of somatic (hotspot)/mosaic (non-hotspot) mutations and explains the variability in phenotype (van Damme et al., 2020).

Our results indicate that PI3K-pathway inhibition could work in at least 75.5% of patients with LM. One fourth does not seem to have a PIK3CA mutation and this could be due to low representativeness of mutant endothelial cells (ECs) in the

studied tissue sample. Analysis of multiple lesional samples may increase the rate of molecular diagnosis (Zenner et al., 2019). In the retrospective clinical trials using Rapamycin on patients affected by various vascular malformations, response rates were high (Boscolo, Limaye, et al., 2015; J. Hammer et al., 2018; Hammill et al., 2011; Seront et al., 2019). Based on our and earlier data, PI3K signaling pathway inhibitors have a sound epidemiological and pathophysiological basis for the treatment of LMs.

6.5 Strengths and limitations

The Strengths of the studies I-III are that all patients were evaluated in a multidisciplinary meeting by the same experienced clinicians and interventional radiologists. Sclerotherapy, throughout all studies, was performed with a standardized technique by the same experienced interventional radiologists. In study II, which compared two different sclerosants, the QOL questionnaire was identical. The strengths of the study IV was the large number of patients and samples collected from various European centers and analyzed in one of the top and renowned institutes in this field.

Limitations in the studies I-III were the non-randomization and retrospective analysis of the QOL score. Due to the varying nature of vascular malformations, randomization of these patients would be difficult. Also the number of patients was relatively low. This is due to the low incidence of these malformations, making it difficult to gather a large number of patients from a single university hospital district. Limitation in the study IV was the low number of Finnish patients.

6.6 Future aspects

Since the incidence of vascular malformation patients is relatively low, for scientific and clinical purposes it would be beneficial if all university clinics in Finland followed a standard treatment and follow up protocol. We suggest that a pre- and post-sclerotherapy symptom and QOL questionnaire should be used in all clinics with VM patients. Further genetic research will open up new effective medical treatments of vascular malformations.

7 CONCLUSION

1. OK-432 is an effective and safe treatment for macrocystic lymphatic malformations. A long follow-up showed that effect of this sclerotherapy is long-lasting. Patient satisfaction after treatment is high.

2. Polidocanol sclerotherapy was found to be an effective, safe, and well-tolerated treatment option for venous malformations. Ethanol is the most potent but toxic sclerosant available. Due to its toxicity and need for anesthesia polidocanol is the sclerosant of choice. Routine MRI for follow-up appears redundant and may be omitted.

3. Intramuscular venous malformations responded to the treatment comparably to that of extramuscular malformations. Subjective symptoms and quality-of-life results are the most important parameters in evaluating the effectiveness of sclerotherapy

4. 75.5% of lymphatic malformations, whether common, combined, or syndromic, are caused by somatic activating PIK3CA mutations. There is a statistically significant difference in mutation types between common and combined lymphatic malformations versus syndromic lymphatic malformations (CLOVES and unclassified PROS), suggesting differential effects of the mutations. Diagnostic genotyping should thus not be limited to PIK3CA hotspot mutations.

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APPENDIX

Appendix. Questionnaire for patients with venous malformations

The following questions relate to a certain number of symptoms, sensations, or discomforts that can make everyday life more difficult or less difficult. For each symptom listed we ask you to answer the corresponding question in the following manner:

Please indicate whether you have experienced what is described in the sentence, and if so, to what intensity.

The following questions concern venous malformation

1.	If you have felt pain, what was the intensity of this pain?	No pain 1	Light pain 2	Moderate pain 3	Strong pain 4	Intense pain 5
2.	To what extent did you feel bothered/limited in your work or other daily activities because of your problem?	Not bothered/ limited 1	A little bothered/ limited 2	Moderately bothered/limited 3	Very bothered/ limited 4	Extremely bothered/limited 5
3.	Have you slept badly because of your problems, and how often?	Never 1	Seldom 2	Fairly often 3	Very often 4	Every night 5
	To what extent did your problems bother/limit you while doing the movements or activities listed below?	Not bothered/ limited at all	A little bothered/ limited	Moderately bothered/limited	Very bothered/ limited	Impossible to do
4.	Standing for a long time	1	2	3	4	5
5.	Climbing stairs	1	2	3	4	5
6.	Crouching, kneeling	1	2	3	4	5
7.	Walking briskly	1	2	3	4	5
8.	Travel by car, bus, plane	1	2	3	4	5
9.	Housework such as working in the kitchen, carrying a child, cleaning floors, doing handy work, ironing	1	2	3	4	5
10.	Going to discos, weddings, parties	1	2	3	4	5
11.	Sporting activities	1	2	3	4	5
	Problems can also have an effect on one's morale. To what extent do the following sentences correspond to the way you have felt?	Not at all	A little	Moderately	A lot	Absolutely
12	I feel nervous	1	2	3	4	5
13	I become tired quickly	1	2	3	4	5
14	I feel I am a burden to people	1	2	3	4	5
15	I must always be careful with my extremity	1	2	3	4	5
16	I am embarrassed to show my extremity	1	2	3	4	5
17	I get irritated easily	1	2	3	4	5
18	I feel handicapped	1	2	3	4	5
19	I do not feel like going out	1	2	3	4	5
20	I feel myself depressive	1	2	3	4	5

ORIGINAL PUBLICATIONS

PUBLICATION I

Efficacy of OK-432 sclerotherapy in treatment of lymphatic malformations: long-term follow-up results

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HEAD AND NECK

Efficacy of OK-432 sclerotherapy in treatment of lymphatic malformations: long-term follow-up results

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Abstract Lymphatic malformations (LMs) are rare congenital tumors of the lymphatic system often affecting the head and neck area. Because of cosmetic and functional symptoms most patients need to be treated. Traditionally surgical treatment has been considered to be the first-line treatment for LM. However, it is challenging because of the need for complete excision. The risk of poor cosmetic result and damage to surrounding structures is high. Since Ogita presented OK-432 as a treatment for LM in 1987, it has been widely used as the primary treatment. Many papers have been published on this topic but with relatively short follow-up times. We present a material of 36 LMs treated with OK-432 during the period of 1999–2009 and with an average follow-up time of 6 years. Immediate post-treatment results were compared with the late follow-up findings. Primary and late response to therapy was evaluated with an MRI scan by measuring the change in lesion size. At the follow-up visit, all patients were clinically examined and they answered a symptom questionnaire. Later 26/36 patients were also available for a quality of life questionnaire. Primarily 67 % demonstrated a complete or marked response. At the follow-up 64 % showed a complete or marked response, in 11 % the final response was better than the initially observed and only 2 patients had relapsed. The initial response predicted the long-term outcome accurately and the effect of OK-432 sclerotherapy seems to be long lasting. According to the MRI evaluation 80 % and subjectively 94 % of the patients benefitted from the treatment. Quality of life questionnaire showed high post-treatment satisfaction. We found OK-432 sclerotherapy to be a safe and effective treatment with a long lasting effect in the management of macrocystic LMs.

Keywords Lymphatic malformation · OK-432 · Macrocystic · Sclerotherapy

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Introduction

Lymphatic malformations (LMs) are comparatively rare congenital tumors of the lymphatic system. Approximately 75 % of LMs appear in the head and neck region. Nearly 50 % of patients present with an LM at birth and 90 % are diagnosed by the age of 2 years [1]. LMs are often functionally asymptomatic but with significant cosmetic concern. Usually they grow slowly but a rapid increase in size can be caused by an infection or internal bleeding even after minor trauma. In the head and neck area, LMs can cause significant compression on functionally important structures and interfere with breathing, swallowing and speech. Spontaneous regression occurs rarely.

Historically, the first-line treatment option for LM has been surgery. Challenging part of surgery is the requirement of a complete resection. Partial resection leads to recurrence and following salvage surgery carries a high risk of complications, morbidity and poor cosmetic results—risks which may be considered unnecessary to take in treatment of a benign lesion. Many different sclerosing agents—i.e., bleomycin, alcoholic solution of zein, doxycycline, hypertonic saline, 1 % polidocanol—have been used in therapy of LMs often with significant side effects and unpredictable results. The most studied agent is OK-432, followed by alcoholic solution of zein and bleomycin [2].

OK-432 is a preparation with lyophilized low-virulent group A Streptococcus pyogenes incubated with benzyl penicillin (Picibanil, Chugai Pharmaceutical Co, Tokyo, Japan). It was developed in Japan in the late 1960s and was primarily certified as adjuvant cancer therapy in 1975. It proved to be useful in pleurodesis for malignant pleural effusion [3]. OK-432 was first presented as a treatment method for LM by Ogita in 1985 and now it is used widely as first-line treatment especially for macrocystic LMs [4]. It has also been used for other benign cysts, such as ranulas, branchial cleft cysts and thyroglossal duct cysts, particularly in Asian countries [5, 6]. OK-432 induces a systemic inflammatory response. It has been shown that several cytokines, i.e., interleukin (IL) 6, 8, 12, interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha are produced locally in LM lesions and serum immune protein (IP)-10 levels are shown to increase after the sclerotherapy treatment. The exact sclerosing mechanism of OK-432 is still not totally clarified [7–10].

We have previously reported our short-term results concerning the use of OK-432 in the treatment of LMs. Now we report our series of 36 patients with an average 6-year follow-up period. Our aim was to find out the objective and subjective long-term results of OK-432 sclerotherapy.

Materials and methods

36 LM patients were treated with OK-432 between 1999 and 2009 at the Department of Otolaryngology and Department of Radiology, Tampere University Hospital. Patients were diagnosed clinically and with an MRI examination. Twenty-seven lesions were localized in the head and neck region, and the rest in the trunk. LMs were classified as macrocystic (cysts size of at least 2 cm^3), microcystic (cysts under 2 cm^3) or mixed based on the radiological examinations. There were 16 females and 20 males with an age range from 1 month to 47 years at the time of the first injection. Five of the patients were under 2 and seven were over 18 years old (Table 1).

OK-432 injections were performed with ultrasound and/or fluoroscopy guidance. OK-432 0.01 mg/ml was injected after aspirating intracystic fluid. The maximum volume of OK-432 injected was 10 ml.

The treatment was considered finished after a stable clinical condition was reached or no response was observed. A late follow-up visit with an MRI scan was organized. During the visit patients were clinically examined and they answered a symptom questionnaire. Results of immediate post-treatment MRI imaging were compared with the late follow-up MRI findings. Objective response to therapy was evaluated as the change in lesion size and graded as complete (90–100 %), marked (50–90 %), moderate (<50 %) or no response. In the symptom questionnaire patients were asked to evaluate the severity of pre- and post-treatment symptoms and to estimate whether they felt they had benefitted from the treatment or not. 26/36 patients were available to answer a specific quality of life questionnaire 5–13 years after the treatment. In the questionnaire four dimensions were explored: pain, physical, social functioning and psychological and it included 20 multiple-choice questions. The scores for each constituent item were added and the total score was obtained by summing the 20 items. Absolute scores were converted into an index. The value of the indices is directly proportional to the degree of deterioration of quality of life: 0 representing the highest quality of life and 100 the lowest [11].

Results

All patients showed a clinical response—local swelling and slight fever for 2–4 days—to the treatment. Three patients had marked swelling and a longer lasting fever. To prevent airway complications two patients were tracheotomised prior to treatment. No serious complications were observed.

The late follow-up time was in mean 6 years from the initial treatment.

Clinical and radiological results

Primary and late results of the sclerotherapy are shown in Table 1. MRI results showed that primarily 24 patients (67 %) demonstrated a complete or marked response, 7 (19 %) showed a moderate response and 5 (14 %) no response. After the follow-up 23 (64 %) showed a complete or marked response, 6 (17 %) a moderate response and 7 (19 %) no response. Thus, 80 % of the patients objectively benefitted from the therapy. After the follow-up

Table 1 Results of OK-432 therapy

Patient no./sex	Age at first injection	Location of LM	Type	Size (maximum diameter, cm)	Symptoms	Earlier treatment	No. of OK-432 treatments	Follow-up (years)	Outcome primarily	Outcome after follow-up	Symptoms post-treatment
1/F	19 years 1 month	Cheek	Macrocystic	3	Cosmetic and functional	No	2	6 years 7 months	Complete	Complete	No symptoms
2/M	1 year 4 months	Base of mouth	Macrocystic	4	Cosmetic	No	2	3 years 4 months	Complete	Complete	No symptoms
3/M	2 years 5 months	Neck	Macrocystic	4	Functional	No	1	9 years 2 months	Complete	Complete	No symptoms
4/M	12 years 8 months	Spleen	Macrocystic	7	No symptoms	No	2	4 years 2 months	Complete	Marked	No symptoms
5/M	12 years 11 months	Neck	Macrocystic	4	Cosmetic	No	3	8 years	Complete	Complete	No symptoms
6/M	10 years 2 months	Abdomen	Macrocystic	15	Functional	No	1	7 years 1 month	Complete	Complete	No symptoms
7/F	3 years 3 months	Abdomen	Macrocystic	30	Cosmetic and functional	No	3	4 years 6 months	Complete	Complete	No symptoms
8/F	2 years 8 months	Chest	Macrocystic	7	Cosmetic and functional	No	1	2 years	Complete	Complete	No symptoms
9/M	1 year 7 months	Neck	Macrocystic	10	Cosmetic	No	2	2 years 5 months	Complete	Complete	No symptoms
10/M	3 years 5 months	Neck	Macrocystic	5	Cosmetic	No	2	9 years 1 month	Complete	No	Cosmetic
11/F	6 years 2 months	Flank	Macrocystic	5	Cosmetic	No	3	8 years 5 months	Complete	Moderate	No symptoms
12/F	3 years 6 months	Neck	Macrocystic	5	Cosmetic	No	3	6 years 6 months	Complete	Complete	No symptoms
13/M	34 years 3 months	Neck	Macrocystic	4	Cosmetic	No	1	8 years 10 months	Complete	Complete	No symptoms
14/M	9 years 11 months	Neck	Macrocystic	11	Cosmetic and functional	No	2	7 years 10 months	Complete	Complete	No symptoms
15/F	5 years 8 months	Shoulder	Macrocystic	3	Cosmetic	Surgery	1	5 years 5 months	Complete	Complete	Cosmetic
16/M	4 years 5 months	Neck	Mixed	4	Cosmetic and functional	No	2	8 years 8 months	Complete	Moderate	No symptoms
17/M	6 years 9 months	Neck	Macrocystic	6	Cosmetic	No	1	3 years 8 months	Complete	Complete	No symptoms
18/M	12 years 10 months	Neck	Macrocystic	8	Cosmetic and functional	No	2	5 years 2 months	Marked	Marked	No symptoms
19/M	21 years 7 months	Orbita	Macrocystic	3	Cosmetic and functional	No	2	5 years 9 months	Marked	Marked	Cosmetic and functional

Table 1 continued

Patient no./sex	Age at first injection	Location of LM	Type	Size (maximum diameter, cm)	Symptoms	Earlier treatment	No. of OK-432 treatments	Follow-up (years)	Outcome primarily	Outcome after follow-up	Symptoms post-treatment
20/M	1 year 5 months	Head/ neck	Mixed	6	Cosmetic and functional	No	2	4 years 9 months	Marked	Marked	Cosmetic
21/F	3 years 1 month	Orbita	Macrocystic	4	Cosmetic	No	3	6 years	Marked	Marked	Cosmetic and functional
22/F	22 years 7 months	Neck	Macrocystic	4	Cosmetic and functional	No	2	4 years 3 months	Marked	Complete	No symptoms
23/M	10 years 9 months	Orbita	Macrocystic	3	Cosmetic and functional	No	1	2 months	Marked	Marked	No symptoms
24/M	5 years 6 months	Neck	Macrocystic	3	Cosmetic	No	8	2 years	Marked	Moderate	No symptoms
25/F	2 years 4 months	Neck	Macrocystic	10	Cosmetic and functional	No	11	9 years 4 months	Moderate	Marked	Cosmetic and functional
26/F	11 years 8 months	Face	Macrocystic	7	Cosmetic	No	3	10 years	Moderate	Moderate	No symptoms
27/F	24 years	Neck	Macrocystic	5	Cosmetic	No	3	8 years	Moderate	Complete	Cosmetic
28/M	13 years 6 months	Neck	Macrocystic	Generalized	Cosmetic	No	2	9 years 9 months	Moderate	No response	Functional
29/M	15 years 10 months	Cheek	Macrocystic	3	Cosmetic	No	3	3 years 5 months	Moderate	Moderate	Cosmetic
30/F	1 month	Base of mouth	Macrocystic	10	Cosmetic and functional	No	6	2 years 6 months	Moderate	Moderate	Cosmetic
31/M	8 months	Flank	Macrocystic	4	Cosmetic	No	1	3 years 2 months	Moderate	Marked	No symptoms
32/M	17 years 8 months	Head	Macrocystic	2	Cosmetic	No	1	4 years 6 months	No response	No response	Cosmetic
33/F	21 years 9 months	Neck	Macrocystic	7	Cosmetic	No	3	4 years 9 months	No response	No response	No symptoms
34/F	38 years 8 months	Back	Microcystic	10	Cosmetic and functional	Surgery	1	8 years 1 month	No response	No response	Cosmetic
35/F	6 years	Face	Macrocystic	3	No symptoms	No	1	6 years	No response	No response	No symptoms
36/F	47 years 9 months	Thorax, Spleen	Macrocystic	Generalized	Functional	No	3	4 years 7 months	No response	No response	Functional

period 26 (72 %) patients had the same result, 4 (11 %) had a better result and 4 patients (11 %) had a mild recurrence. Two patients (6 %) relapsed after initially showing a complete or a moderate response.

According to the patients subjective evaluation 34 (94 %) benefitted from the treatment. Prior to treatment most patients felt the LM affected them cosmetically and 16 patients had functional symptoms. Two patients were asymptomatic. After the therapy and follow-up, subjective symptoms were significantly decreased in 68 % of the patients. Even though still 5 patients (14 %) experienced that the LM has a negative impact on their quality of life. Seven patients were treated surgically after sclerotherapy. Three of these patients were complaining about poor cosmetic results.

One patient with a complete regression primarily had a mixed lesion, all other patients in this group had macrocystic lesions. All patients with a complete response after the follow-up time had macrocystic lesions. The one patient with the microcystic lesion showed no response. One patient showed a complete response primarily and after the follow-up no response; he got a residual after an infection and was operated afterwards. Two patients were diagnosed after the treatment with a generalized disease and observed to have no response to the treatment after the follow-up.

No correlation between the size of the LM, age of the patient at the beginning of the treatment and the final response was found.

Quality of life

Results from the quality of life questionnaire showed that a clear majority of the patients (24/26) did very well after sclerotherapy (Table 2). 16/26 patients had the lowest possible scores representing the highest quality of life measure. Two patients did clearly worse than the rest of the group. Both had persistent disease requiring further treatment.

Discussion

To our knowledge this series has the longest mean follow-up period published so far. Also there are no reports concerning the quality of life after sclerotherapy. The main finding of our study is that OK-432 sclerotherapy has a long lasting and safe effect in the management of macrocystic LMs. It is an efficient treatment regardless of the age of the patient or the size of the LM. After a mean follow-up time of 6 years, 64 % of the patients showed a complete regression and objectively measured 80 % of the patients benefitted from the treatment. It is also remarkable that subjectively 94 % of the patients were relieved of their functional and cosmetic symptoms. Post-treatment satisfaction was high as seen in the results of the quality of life questionnaire. Therefore, sclerotherapy seems to be the reasonable method to treat macrocystic LMs.

Two recent review articles concerning OK-432 treatment results have been published between 2009 and 2011. In the first review by Poldervaart et al. [12] results from 10 studies and 111 patients were analyzed; 88 % had excellent results and 8 % good results with a mean follow-up time of 21 months. In the second review from 2011 there were 318 patients from 23 studies, 56 % with excellent results and 17 % with good results [2]. All reports included in these reviews had a relatively short follow-up period.

The long follow-up in this study showed that the response to OK-432 treatment was persistent in 83 % of patients who demonstrated primarily a complete or marked response to therapy. In addition, 3/7 patients who had a moderate primary outcome demonstrated a complete or marked response after the follow-up. Patients with no response initially remained unresponsive through the follow-up period. Thus, with patients showing no immediate response it is reasonable to directly consider other treatment options if needed. On the other hand, patients who initially respond moderately can be followed since nearly

Table 2 Dimensions in quality of life (26 patients)

Index	No. of patients total index	Dimensions			
		Pain	Physical	Social	Psychological
0–19	24	23	24	23	24
20–39	1	0	1	2	1
40–59	1	3	0	0	1
60–79	0	0	1	1	0
80–100	0	0	0	0	0
Mean	5	8	4	5	5
SD	14	12	13	8	10
Minimum	0	0	0	0	0
Maximum	53	56	63	67	42

half of them showed an improved late response during the follow-up period.

No serious side effects were noted in our series, which is consistent with previous data. Serious side effects with OK-432 therapy seem to be rare since only three have been reported—an airway obstruction and an orbital compression and transient facial nerve paralysis after injection [13–15]. The inflammatory reaction that is typical for patients after the treatment was noted in all of our patients. The elevated temperature and swelling of the lesions is regarded to be an expected reaction to a successful injection.

Various sclerosing agents have been used for treatment of LMs, i.e., bleomycin, alcoholic solution of zein, doxycycline, hypertonic saline, 1 % polidocanol. All have been shown to have an effect but because of potentially remarkable side effects use of many of these agents has been abandoned. Bleomycin has shown comparable results to OK-432. However, it is posed with a theoretical risk of causing pulmonary fibrosis. Scar formation is the major negative side effect from treatment with alcohol. Doxycycline produces remarkable pain but it has shown promising results in the treatment of microcystic LMs [16]. Since microcystic lesions do not respond to OK-432 as well as the macrocystic LMs, doxycycline might be the preferred sclerosing agent for the microcystic lesions.

Surgery has been the first-line treatment option in the past. If a LM is operated it is necessary to achieve a complete removal. Recurrence rates of partially or incompletely resected lesions are between 50 and 100 % [13]. Complete excision without significant co-morbidity—such as seroma formation, infection, Frey and Horner's syndrome and cranial nerve damage—is often difficult to accomplish due to the relation of vital and important structures especially in the head and neck area. With such a high co-morbidity and recurrence rates surgery must be questioned as a first-line management of a benign condition like LM.

Conclusions

OK-432 is an effective and safe treatment for macrocystic LMs. A long follow-up showed that effect of this sclerotherapy is long lasting and in some cases an improvement to a mild initial response can be expected. Patient satisfaction after treatment is high. According to our study, to our knowledge with the longest follow-up so far, we recommend OK-432 sclerotherapy as primary treatment for macrocystic LMs.

Conflict of interest None.

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PUBLICATION II

Quality of life after endovascular sclerotherapy of low-flow venous malformations: the efficacy of polidocanol compared with ethanol

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Quality of life after endovascular sclerotherapy of low-flow venous malformations: the efficacy of polidocanol compared with ethanol

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Abstract

Background: Limited information is available on mid-term results and quality of life (QOL) after endovascular sclerotherapy of venous malformations.

Purpose: To compare two agents—polidocanol and ethanol—with a focus on the influence on QOL after sclerotherapy.

Material and Methods: Forty-one consecutive patients with a venous malformation in the head and neck area or in the extremities were treated with polidocanol between 2008 and 2013. Pre- and post-treatment magnetic resonance imaging (MRI) scans were compared. All patients completed a self-evaluation form on symptoms as well as a QOL questionnaire. The results were compared with previously obtained material during 1991–2001, comprising 44 consecutive, similarly located venous malformation patients subject to ethanol sclerotherapy.

Results: No significant clinical complications were observed. Subjectively, 19 (46%) of the patients benefitted from the treatment. QOL results showed that 85% of patients had an index < 39 – where 0 represents the highest and 100 the lowest QOL. Patients in the ethanol group had marginally better overall post-treatment QOL results. Post-treatment MRI in 35 patients showed the size of the malformation unchanged in 19 (54%) patients, in ten (29%) there was a decrease (<50%) while in six (17%) the decrease was more significant (>50%). Post-treatment MRI results did not correlate with either subjective symptoms or QOL results.

Conclusion: Polidocanol sclerotherapy were found to be an effective, safe, and well tolerated treatment option for low flow venous malformations. Routine MRI for follow-up appears redundant and may be omitted.

Keywords
Venous malformations, sclerotherapy, polidocanol, quality of life, pain

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Introduction

Low-flow venous malformations—the most common vascular malformations—are congenital non-proliferative lesions consisting of dysplastic venous channels due to errors in vascular morphogenesis (1). They are present at birth but often become clinically evident later, during adolescence or adulthood.

Most commonly affected anatomical locations are the head and neck area (40%), the extremities (40%), and the trunk (20%) (1,2). Symptomatic vascular malformations can cause pain, induce neuropathy, and the cosmetic disadvantage may be marked (Fig. 1).

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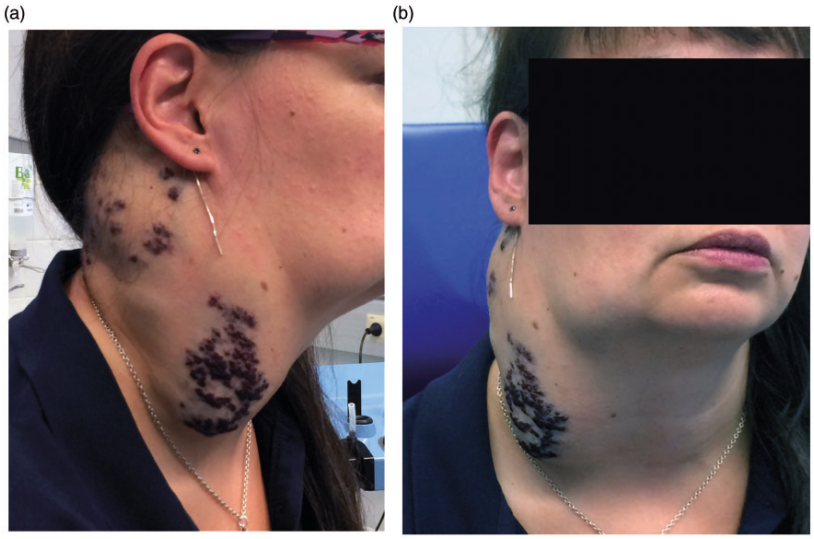

Fig. 1. Extensive low flow venous malformation of the neck.

In certain anatomical regions, i.e. the hypopharynx, venous malformations can impair speech, swallowing, and obstruct the upper airways.

Superficial venous malformations are easy to diagnose clinically due to their characteristic appearance. With deeper or parenchymal lesions, ultrasound (US) and magnetic resonance imaging (MRI) are the most important diagnostic tools. Both are suitable for characterizing and defining the extent, location, and relation to other anatomical structures.

Venous malformations have been treated with elastic compression garments, surgery, and embolotherapy with fibrosing materials. A complete surgical excision may require an extensive removal of surrounding unaffected tissue, leading to unwanted cosmetic and functional side effects. An incomplete removal usually leads to a recurrence. In most cases image-guided sclerotherapy alone or in combination with surgery is the current first-line treatment option (3). Since curative treatment of venous malformations is seldom possible, the aim of the treatment must be to reduce and relieve symptoms, i.e. improve the patients' quality of life (QOL). However, while a 100% effective sclerosant without any negative side effects has yet to be discovered, the challenge is to find a sclerosant that is a compromise between efficacy and toxicity.

Various sclerosing agents have been used for treatment of venous malformations, i.e. 99.5% ethanol, ethanolgel (ScleroGel), sodium tetradecyl sulphate (Sotradecol), alcoholic solution of zein (Ethibloc), and polidocanol. Intravascularly injected ethanol causes pain and thus adequate general anesthesia is required during sclerotherapy. Another disadvantage is the risk of extravasation causing necrosis of the surrounding soft tissue. Severe complications such as nerve damage, lung embolism, pulmonary vasospasm, and heart arrhythmia have been reported with the use of ethanol. Polidocanol, a mixture of ethanol (5%) and hydroxypolyethoxidodecain (95%), was first synthesized in 1936 and marketed as a topical and local anesthetic. It has been used as a sclerosing agent since the 1960s due to its ability to sclerose blood vessels without a significant risk of damage to surrounding tissue. The recommended concentration is in the range of 0.25–3%, giving the widest safety margins against extravasation necrosis of any potent sclerosing agent (4). Because of the anesthetic effect, polidocanol does not produce pain while being injected intravenously or perivascularly and thus no anesthesia is needed. Preparation of polidocanol foam facilitates its use for sclerotherapy. Cabrera and Yamaki were the first to report this technique (5–7).

We have used both 99.5% ethanol and polidocanol in the treatment of low-flow venous malformations. Although having had good results and minimal serious side effects with ethanol, the switch to polidocanol was done based on the previously mentioned practical reasons, i.e. painless injections with minimal risk of damage to surrounding tissue.

The main purpose of this study was to evaluate the QOL of patients with venous malformations after polidocanol sclerotherapy. Additionally, the results were compared with those from a previously published study of patients treated with ethanol (8,9).

Table 1. Ethanol and polidocanol groups.

	Ethanol (n = 44)	Polidocanol (n = 41)
Location		
Head and Neck	20	10
Upper extremity	11	12
Lower extremity	13	19
Sex		
Female	25	29
Male	19	12
Pre-treatment symptoms		
Pain	26	37
Cosmetic disturbances	9	17
Functional disturbances	4	38
Paresthesia	2	0
No symptoms	0	0
Pre-sclerotherapy treatment		
No treatment	25	25
Surgery	16	12
Laser therapy	4	2

Material and Methods

The study was approved by the ethical committee of Tampere University. All patients gave their written consent.

Forty-one patients with a venous malformation were treated with polidocanol in Tampere (n = 23) and Turku (n = 18) University Hospitals between 2008 and 2013. Results were compared with 44 patients treated with ethanol in Tampere University Hospital during the years 1991–2001 (Table 1) (8,9). Both hospitals are tertiary care centers, each serving a population base of approximately one million, caring for practically all low-flow venous malformation patients in need of treatment in their respective areas.

All clinically diagnosed patients underwent an MRI examination. Thirty-five patients (25 girls/women, mean age = 36 years, age range = 14–81 years) were available for a post-treatment MRI after a mean follow-up time of three years (range = 3 months–6 years).

All pre- and post-treatment 1.5-T T2-weighted (T2W) fat-suppressed axial, coronal, and sagittal MRI images were reviewed in consensus by two experienced radiologists (LK-N and RR). Pre-treatment size of the lesions, possible relation to muscle compartment, and modification in the post-treatment size and configuration were evaluated.

Polidocanol foam (3%) was generated by mixing the sclerosing liquid with air in a ratio of 1 to 4. Volume of the dosage was calculated with fluoroscopy or with US. The maximum dose did not exceed 2 mg/kg. Polidocanol injections were performed under US and fluoroscopy guidance. Eleven patients were treated once, 14 patients twice, and 15 patients were treated three to seven times. One patient with a malformation in the lower extremity was treated ten times between 2008 and 2013. In all, there were 2.7 treatments/patient. Eight patients had previously been operated. Two patients had been treated with an YAG-laser. The mean interval between each treatment session was six months (range = 1–20 months).

The patients were asked to evaluate in a self-evaluation questionnaire the severity of pre- and post-treatment symptoms and to estimate whether or not they felt they had benefitted from the treatment or not. Symptoms were categorized as unchanged, decreased, symptom-free, or relapsed. Further, after sclerotherapy, all patients answered a specific QOL questionnaire (10), which was identical to that used in our previous studies (8,9). In the questionnaire, four dimensions were explored with 20 multiple-choice questions: pain; psychological; physical; and social functioning (Suppl. Fig. 1). This questionnaire was developed by Professor Launois with an educational grant from Servier. The scores for each constituent item were added and the total score was obtained by summing the 20 items. Absolute scores were converted into an index. The value of the indices is directly proportional to the degree of deterioration of QOL: 0 representing the highest and 100 the lowest QOL. This QOL questionnaire has been validated in an age-matched Finnish normal population (11).

Statistical analysis

Difference between the two-sample means was tested with Student's t-test. Mann–Whitney U test was used to compare the two populations with unequal variance. For categorical data, Fisher's exact test was used. Relationships between continuous variables were tested with correlation and simple linear regression analysis. A P value < 0.05 was considered to indicate a statistically significant difference.

Results

Altogether, 41 patients were treated and subsequently followed.

MRI: size and configuration estimations

Pre-treatment MRI showed 16 malformations with a width <5 cm, while 25 were >5 cm. Twelve malformations were intra-muscular, 16 were extra-muscular, and 13 both intra- and extra-muscular. Altogether, in 35

post-treatment MRI examinations, the size of the malformation appeared unchanged in 19 patients. In ten patients, the size had decreased < 50% while a decrease of > 50% was seen in six patients. The configuration was unchanged in 19 patients, and in 12 patients the malformation was thrombosed partially and in four completely.

Patient self-evaluation

According to the 41 patients' subjective evaluation, 19 (46%) benefitted from the treatment, and three of them were asymptomatic. Eighteen (44%) patients found no changes in their symptoms. Four patients (10%) still experienced a persistent functional disadvantage with cosmetic impairment and pain and were evaluated subjectively doing worse after the treatment.

QOL results

QOL results showed that the majority of patients did well after polidocanol sclerotherapy. Mean index for total QOL was 22 (range = 0–56) (male mean = 17, range = 0–35, female mean = 24, range = 3–56). QOL mean index compared with the aged-matched Finnish normal population was equal (male = 28, female = 25) (11). Pain after treatment was the single most significant factor having a poor impact on QOL (male = 29, range = 0–56, female mean = 38, range = 6–69) (Table 2). In the Finnish normal population, mean QOL index for pain was 15 (male) and 20 (female).

In this material, a venous malformation had a more negative effect on QOL in women (mean = 24, range = 3–56) than in men (mean = 17, range = 0–35), but the difference was not statistically significant. Location of the malformation or relation to muscle compartment did not affect the outcome on post-treatment QOL. Age at the beginning of the treatment did not influence the post-treatment QOL.

Table 2. Dimensions in QOL (polidocanol, 41 patients).

Patients (n)		Dimensions			
Index	Total	Pain	Physical	Social	Psychological
0–19	22	12	25	20	27
20–39	13	11	8	12	8
40–59	6	14	5	7	6
60–79	0	4	3	2	0
80–100	0	0	0	0	0
Mean	22	35	21	23	17
SD	16	19	20	20	16
Min	0	0	0	0	0
Max	56	63	63	75	53

Correlations of pre- and post-treatment parameters

Initial size of the malformation did not affect the outcome when comparing pre- and post-treatment subjective symptoms ($P = 0.37$). A statistically significant reduction in pre- vs. post-treatment pain ($P = 0.002$) was seen. Otherwise changes in subjective symptoms were quite minimal. Radiological changes in MRI examinations did not correlate with any of the subjective symptoms (Fig. 2).

Size of the malformation and pain correlated with the QOL assessment. The poorest outcome was observed in patients with significant cosmetic (mean = 27, range = 0–59) or functional (mean = 22, range = 0–56) disadvantages before the treatment. The best QOL scores were among patients with no or only

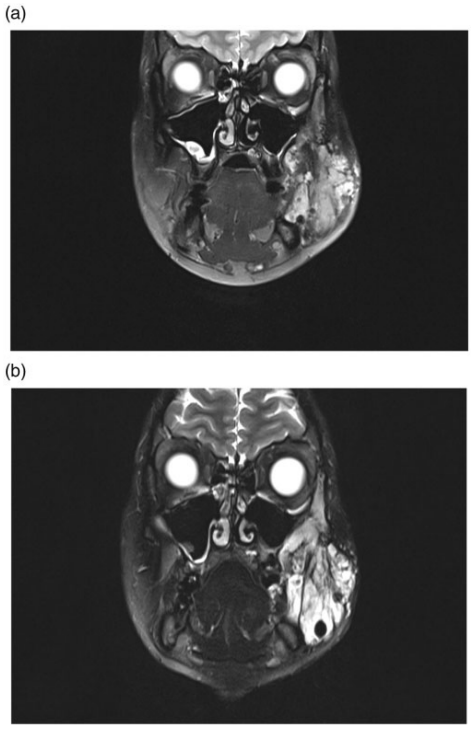

Fig. 2. (a) Pre- and (b) post-treatment T2W fat-suppressed coronal 3-T MR image of a buccal low flow venous malformation of a 22-year-old woman. Patient was treated three times with six-month intervals without changes in MRI appearance. Subjectively evaluated the patient experienced a marked decrease in swelling and an improvement in cosmetic result.

light pain at the beginning of the treatments (11). Similar results were also noted in the ethanol group (8,9).

Comparison between polidocanol and ethanol

After sclerotherapy, results of the treatment in the ethanol group were better than in the polidocanol group. The overall results were better in all measured dimensions of life (Table 3). In the ethanol group, average total index of QOL was 16 (range = 0–74) compared to 22 (range = 0–56) in the polidocanol group. In all dimensions—except the psychological—the difference was significant: total P value = 0.04; pain = 0.003; physical = 0.03; and social = 0.027.

When ethanol and polidocanol groups were compared concerning the change in subjective symptoms, the difference was not significant in any of them (Table 4).

Table 3. Dimensions in QOL (ethanol, 42 patients).

Patients (n)		Dimensions			
Index	Total	Pain	Physical	Social	Psychological
0–19	27	26	31	30	31
20–39	12	10	8	9	9
40–59	2	3	1	1	0
60–79	1	2	2	2	1
80–100	0	1	0	0	1
Mean	16	23	14	16	14
SD	16	20	17	18	14
Min	0	0	0	0	0
Max	74	81	69	75	78

Table 4. Change in subjective symptoms, ethanol vs. polidocanol.

	Treatment	
Changes in symptoms	Ethanol	Polidocanol
Unchanged		
n	11	19
%	24.4	46.3
Decreased		
n	29	16
%	64.4	39
Symptom-free		
n	2	2
%	4.4	4.9
Relapsed		
n	3	4
%	6.7	9.8

There were three notable complications in the ethanol group: a 1-cm diameter skin necrosis in the hypothenar; a transient paresis of the radial nerve; and a transient facial nerve paresis. The polidocanol group showed no complications.

Discussion

In this study, we compared two different sclerosing agents, polidocanol and ethanol, for the treatment of low flow venous malformations and compared their effect on QOL. To the best of our knowledge, no similar studies comparing two sclerosants and their effects on QOL have been previously published. The main result showed a significant reduction in subjective pre- vs. post-treatment pain scores, which is in accordance with a recently published prospective study with a single sclerosant (ethanogel) (12). Consequently, patients with pain especially benefit considerably from sclerotherapy and it should be offered to them.

MRI is important in characterizing the extent of the malformation, categorizing the type, determining tissue involvement, and for planning of therapy (13,14). We compared pre-and post-treatment MRI images and the results show that the size and structure of venous malformations do not essentially change after sclerotherapy. In addition, post-treatment MRI findings did not correlate with neither subjective symptoms nor QOL results. Therefore, a routine MRI to control the results of treatment appears unnecessary.

QOL assessment is nowadays widely used to evaluate the efficacy of various treatments, especially when treating chronic illnesses or disorders. As patients live longer with their maladies, it is important not only to understand whether or not their illness is cured or not but how the status and possible treatments affect their everyday life. QOL data are usually collected by a set of generic, logical, and easily administered QOL questions. The answers rely upon patient self-reporting and the results can be used for routine monitoring and assessment of treatment outcomes. This type of measurement is susceptible to the phenomena of response shift (15,16) – in this case especially of the beta-type. For example, this means that during the treatment period the patients' internal definition for pain changes due to the intervention and may result in over- or under-reporting of the true physiological change.

After sclerotherapy, changes in clinical appearance and MRI findings are often minimal and other methods to estimate the effectiveness of the treatment must be used. Based on our results, a subjective symptom and QOL questionnaire would be useful for this purpose. Systematic use of a pre- and post-treatment questionnaire provides a relatively objective tool with which to

evaluate the results and effectiveness of the treatment, and helps in the decision-making while evaluating possible treatment options and schedules. We suggest that a pre- and post-sclerotherapy symptom and QOL questionnaire should be used with all patients, not exclusively in studies but in general clinical follow-up as well.

Because of the retrospective analysis of our materials, one must be somewhat cautious in the conclusion that the difference between the two sclerosants was significant. However, based on our and previous reports, it is clear that ethanol is the most potent—but toxic—sclerosant available (17–19). A choice between ethanol and polidocanol can be made based on possible risks and side effects of the treatment. With ethanol, the eventual result might be slightly better. However, the change in subjective symptoms following the treatments can be expected to be equal. Thus, polidocanol is probably the sclerosant of choice, at least when treating an area with a high risk of complications, i.e. subcutaneous lesions, proximity of a functionally significant nerve, or a major vein. An additional advantage of polidocanol is its local anesthetic effect. Ethanol sclerotherapy is painful and requires adequate general anesthesia while polidocanol can be used without any. This obviously reduces the need for hospital resources and lowers the cost of a single treatment.

Age at the beginning of treatment did not influence QOL results. Consequently, patients benefit from the treatment at any time from infancy to adulthood. With a relatively safe sclerosant such as polidocanol, treatment of venous malformations can be started at an early age to prevent permanent functional and esthetic disadvantages.

Although the change in cosmetic symptoms in our series was not overly significant, it is obvious that patients wish for a positive change in the appearance of the malformation. Even a minor improvement, such as reducing the volume and appearance, can be of major significance to the patient. For ideal results, multiple treatments are often necessary and can often be achieved by three to five sessions with a six- to eight-week interval between treatments (3). In selected cases, surgery in addition to sclerotherapy might be considered (20).

The strengths of these data are that all patients were evaluated in a multidisciplinary meeting by the same experienced clinicians and interventional radiologists. Sclerotherapy, throughout both studies, was performed with a standardized identical technique by the previously mentioned interventional radiologists. Also, the QOL questionnaire was identical in both studies.

The shortcomings of this study were the non-randomization and retrospective analysis of the QOL score. Due to the varying nature of vascular malformations, randomization of these patients would be difficult. Also because of the potential toxicity of ethanol, randomization might be considered unethical in certain cases.

In conclusion, although a curative treatment for venous malformations is not currently available, the majority of patients benefit from sclerotherapy. Ethanol is an effective sclerosant but has the disadvantage of requiring general anesthesia and a potential to produce severe side effects. Polidocanol foam is nearly as effective as ethanol. It is well tolerated, does not require anesthesia when injected, and is therefore suitable for sclerotherapy of venous malformations at any anatomical location.

Declaration of Conflicting Interests

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PUBLICATION III

Quality of life and clinical results after endovascular sclerotherapy: A comparision between intra- and extramuscular low-flow venous malformations

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Quality of life and clinical results after endovascular sclerotherapy: A comparison between intra- and extramuscular low-flow venous malformations

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Abstract

Background: Based on clinical observations we hypothesized that patients with intramuscular venous malformations (VMs) did worse or needed more sclerotherapy sessions than patients with extramuscular VMs.

Purpose: To evaluate the difference in treatment and quality of life (QOL) results after Polidocanol sclerotherapy of intra- and extramuscular low-flow VMs.

Material and methods: Forty-one patients with a VM were treated with Polidocanol in two university hospitals. The results were retrospectively analyzed. Pre- and post-treatment magnetic resonance imaging (MRI) scans were compared. All patients completed a self-evaluating form on symptoms as well as a QOL questionnaire. The results were compared between two groups: intra- and extramuscular VM's.

Results: No statistically significant differences between intra- and extramuscular groups concerning QOL and specific dimensions pain, functional problems or cosmetic appearance were found. Radiological changes in MRI examinations did not correlate with any of the subjective symptoms. 56% of the patients benefitted from the treatment according to patient self-evaluation.

Conclusion: In this material intramuscular VM's responded to the treatment comparably to extramuscular malformations. Post-treatment MRI findings do not correlate with subjective symptoms or QOL results and thus, a routine post-treatment examination seems to be unwarranted. Subjective symptoms and QOL results are the most important parameters in evaluating the effectiveness of sclerotherapy.

Keywords

Intramuscular, extramuscular, venous malformation, quality of life, sclerotherapy

Introduction

Low flow venous malformations are congenital non-proliferative lesions consisting of dysplastic venous channels due to errors in vascular morphogenesis.[1] VM's can be located in the superficial and/or the deep venous system. They are present at birth but often become clinically evident later during adolescence or adulthood.

Most commonly affected anatomical locations are in the head and neck area (40%), the extremities (40%), and the trunk (20%).[1,2] Frequently the lesions are found in skin, mucosa, subcutaneous tissue and

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muscle. Presenting signs and symptoms include soft tissue swelling, skin or mucosal discoloration, discrete mass and pain. Superficial venous malformations are easy to diagnose clinically due to their characteristic appearance. However, up to 50% of VM's affect deeper structures, such as muscle, often in the head and neck area and the extremities.[3] Compared to extramuscular VM's, intramuscular VM's present later, tend to be more painful and contain more phleboliths.[4]Since extremity and truncal VM's are more likely to enlarge, particularly during puberty, intramuscular VM's tend to be highly symptomatic. They can cause significant functional limitations due to mass effect, neural involvement and muscle dysfunction.[5] Intramuscular VM's are considered to be associated with greater morbidity and to be more difficult to manage due to the severity of symptoms and poor overall response to therapy in general compared to other VM's.[6]

Patients who initially might present with an asymptomatic lesion usually will ultimately require intervention.[7] Venous malformations have been treated with elastic compression garments, surgery and sclerotherapy with fibrosing materials. Various sclerosing agents have been used but no difference in their effectiveness has been shown.[8] The choice of the sclerosant should be based on minimal toxicity, cost effect and institutional experience. In most cases image-guided sclerotherapy alone or in combination with surgery is the current first line treatment option.[9] Curative treatment of VM's is seldom possible and thus the aim of the treatment must be to reduce and relieve symptoms – i.e. improve the patients' quality of life (QOL).

We performed a retrospective analysis of 41 patients with extra- and intramuscular VM's treated in two University Hospitals in Finland. The aim of the study was to find out possible differences in treatment results and QOL after polidocanol sclerotherapy.

Material and methods

Patients

Forty-one patients with a VM treated with polidocanol at Tampere (n = 23) or Turku (n = 18) University Hospitals were retrospectively analyzed. The results were compared between two groups: intra- and extramuscular malformations (Table 1). Both hospitals are tertiary care centers, each serving a population base of approximately one million, caring for practically all low-flow VM patients in need of treatment in their respective areas. The study was approved by the local ethics committee.

Table 1. Intra- and extramuscular groups.

	Intramuscular (n = 25)	Extramuscular (n = 16)
Location		
Head and Neck	5	5
Upper extremity	7	5
Lower extremity	13	6
Sex		
Female	17	12
Male	8	4
Age		
Mean	32	39
Pre treatment symptoms		
Pain	22	15
Cosmetic disturbances	10	4
Functional disturbances	23	15
Treatment pre sclerotherapy		
No treatment	16	12
Surgery	9	3
Laser therapy	0	2

Diagnostic imaging

All 41 clinically diagnosed patients underwent an MRI examination. Thirty-five patients (25 women, mean age = 36 years, age range = 14–81 years) were available for a post-treatment MRI after a mean follow-up time of three years (range = 3 months–6 years).

All pre- and post-treatment 1.5-T T2-weighted (T2W) fat-suppressed axial, coronal, and sagittal MRI images were reviewed in consensus by two experienced radiologists (LK-N and RR). Pre-treatment size of the lesions, possible relation to muscle compartment, and modification in the post-treatment size and configuration were evaluated (Figures 1 and 2). The radiologists were blinded concerning the post-treatment self-evaluating and QOL results.

Treatment procedure

The procedure was identical in both Tampere and Turku University Hospitals. Polidocanol foam (3%) was generated by mixing the sclerosing liquid with air in a ratio of 1 to 4. Foam sclerotherapy was chosen due to its better and longer lasting contact with the vessel wall compared with a liquid form of the sclerosant. Volume of the dosage was calculated with fluoroscopy or with US. The maximum dose did not exceed 2 mg/kg to minimize the risk of toxic side effects. Polidocanol injections were performed under US and fluoroscopy guidance. Eleven patients were treated once, 14 patients twice, and 15 patients between 3 to 10 times during a span of 5 years (median 2 treatments/patient). The median interval between each treatment session was five months (range 1–20 months). Interval between

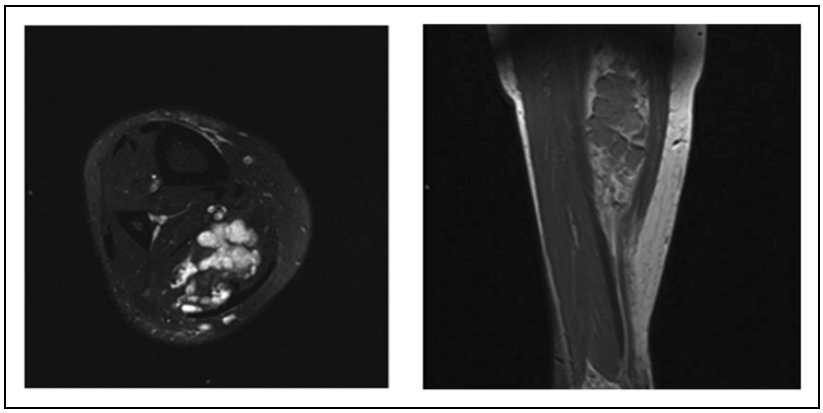

Figure 1. 35-year-old female, intramuscular low flow venous malformation in the soleus muscle.

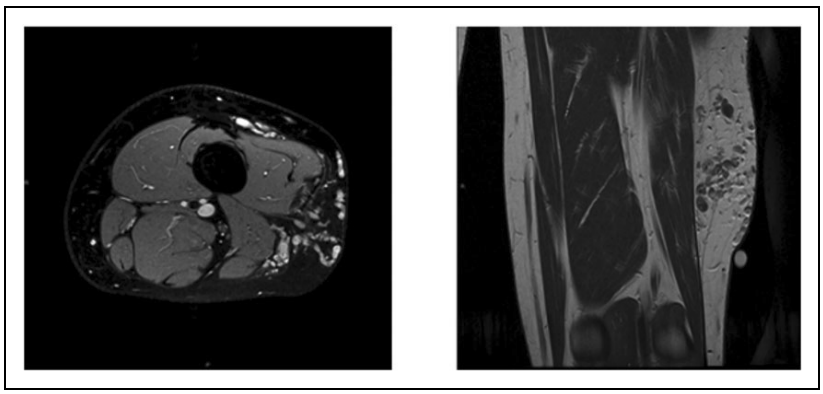

Figure 2. 32-year-old male, subcutaneous extramuscular venous malformation on top of musculus vastus lateralis and biceps femoris.

the sessions was not predetermined but was based on the patient's self-evaluation concerning worsening of physical or cosmetic symptoms. Thus, only patients who were symptomatic or with deteriorating symptoms were treated.

Self-evaluation and QOL

In a self-evaluation questionnaire the patients were asked to evaluate the severity of pre- and post-treatment symptoms and to estimate whether or not they felt they had benefitted from the treatment or not. Symptoms were categorized as unchanged, decreased, symptom-free. Further, after sclerotherapy, all patients answered a specific QOL questionnaire.[10] In the questionnaire, four dimensions were explored with 20 multiple-choice questions: pain; psychological; physical; and social functioning (Supplemental Figure 1) This questionnaire was developed by Professor Launois with an educational grant from Servier. The scores for each constituent item were added and the total score was obtained by summing the 20 items. Absolute scores were converted into an index. The value of the indices is directly proportional to the degree of deterioration of QOL: 0 representing the highest and 100 the lowest QOL. This QOL questionnaire has been validated in an age-matched Finnish normal population.[11]

Statistical analysis

Difference between the two-sample means was tested with Student's t-test. Mann–Whitney U test was used to compare the two populations with unequal variance. For categorical data, Chi-Square test and Fisher's exact test was used. Relationships between continuous

variables were tested with correlation and simple linear regression analysis. A P value < 0.05 was considered to indicate a statistically significant difference

Results

MRI: Size and configuration estimations

Pre-treatment MRI size was categorized as < 5 cm and > 5 cm (Table 2). The change in post treatment MRI size and configuration is presented in Table 3. The differences were not statistically significant between the two groups.

Patient self-evaluation

All 41 patients gave a post treatment evaluation considering the change in subjective symptoms (Table 4). In all 23 patients (56%) benefitted from the treatment with 2 asymptomatic patients in the intramuscular group. 4 patients in the intramuscular and 3 in the extramuscular group were painless at the follow-up. Eighteen patients (44%) found no changes in their symptoms. Radiological changes in MRI examinations did not correlate with any of the subjective symptoms. The relation to muscle compartment did not affect the outcome on subjective symptoms (P = 0,43). Age at the beginning of the treatment did not influence subjective symptoms or the number of treatment session

QOL results

QOL results showed that the majority of patients in both groups did well after polidocanol sclerotherapy. Pain after treatment was the single most significant factor having a poor impact on QOL (male = 29, female mean = 38). In the Finnish normal population, mean QOL index for pain was 15 (male) and 20 (female) (Table 5(a) and (b)). Mean index for total QOL was 22 and the mean index compared with the aged-matched Finnish normal population was equal (male = 28, female = 25).

In this material, an extramuscular venous malformation had a slight tendency towards a more negative effect on all QOL dimensions (mean = 28) compared with the intramuscular group (mean = 19). Age showed no significant difference between the groups (P = 0,56). Also, the number of treatment sessions did not affect QOL results.

Table 2. Pre-treatment MRI.

	MRI size	
	<5 cm	>5 cm
Intramuscular (n = 25)	11	14
Extramuscular (n = 16)	5	11
	16	25

Discussion

In a recent review concerning outcome measures of VM's after sclerotherapy Asdhal et al. stated that the past literature shows no clear method to evaluate the effect of sclerotherapy.[12] They also expressed that future studies should be related to anatomic location and depth of the malformation. In this study we compared the treatment results of extra- and intramuscular VM's after Polidocanol sclerotherapy with the patient's subjective evaluation of the outcome and QOL after the treatment.

In their review concerning effectiveness of VM treatment, C.J.M. van der Vleuten et al.[8] concluded that there are no differences concerning the efficacy of various sclerosants. Therefore, the choice of the sclerosant

Table 4. Change in subjective symptoms, intra- vs. extramuscular VM's.

Changes in symptoms	Intra	Extra
Unchanged		
n	12	6
%	48%	37.5 %
Decreased		
n	10	10
%	40%	62.5 %
Symptom-free		
n	3	0
%	12%	0%

Table 3. Post-treatment radiological findings.

MRI size	Unchanged n (%)	Decreased < 50% n (%)	Decreased > 50% n (%)
intra (n = 20)	12 (60%)	4 (20%)	4 (20%)
extra (n = 15)	7 (47%)	6 (40%)	2 (13%)
MRI configuration	Unchanged n (%)	Thrombosed partially n (%)	Thrombosed completely n (%)
intra (n = 20)	12 (60%)	6 (20%)	2 (20%)
extra (n = 15)	7 (47%)	6 (40%)	2 (13%)

Table 5. QOL results.

(a) Dimensions in quality of life (intramuscular)						(b) Dimensions in quality of life (extramuscular)					
						No. of patients		Dimensions			
Index Total index	Total	Pain	Physical	Social	Psychological	Index Total index	Total	Pain	Physical	Social	Psychological
0–19	15	7	16	14	18	0–19	7	5	9	6	9
20–39	9	7	5	8	5	20–39	4	4	3	4	3
40–59	1	9	3	3	2	40–59	5	5	2	4	4
60–79	0	2	1	0	0	60–79	0	2	2	2	1
80–100	0	0	0	0	0	80–100	0	0	0	0	1
Mean	19	33	18	19	13	Mean	28	38	32	32	23
SD	13	19	18	16	14	SD	17	19	22	24	19
Min	0	0	0	0	0	Min	3	0	0	0	0
Max	44	63	63	50	47	Max	56	81	63	75	53

should be based on minimal toxicity, cost effect and institutional experience.[13] Following this reasoning our clinics have chosen Polidocanol with good results and minimal side effects. Compared to a liquid sclerosant the foam form has the advantage of better and longer lasting contact with the vessel wall leading to a more effective treatment and a reduction of concentrations needed.[13]

Intramuscular VM's tend to be diagnosed later and they seem to cause more morbidity than extramuscular VM's. It is likely that the lack of skin or mucosal involvement makes dilated veins less apparent and the possible soft tissue mass in an infant is not suspected to be a vascular malformation.[6] Later in adolescence pubertal hormones, phlebolith formation and environmental factors such as trauma and infection may contribute to the expansion and transformation to a clinically diagnosable VM. The primary reason for seeking treatment is pain and intramuscular VM's are also associated with more limitations to physical activity due to discomfort and swelling.[4]

In our material no difference between the groups concerning QOL and specific dimensions pain, functional problems or cosmetic appearance was observed.

Horbach et al. reported outcomes of bleomycin sclerotherapy for 77 low flow vascular malformations. Similar to our study results were investigated in terms of QOL and patient-perceived changes in health. Their results showed that approximately half of patients experienced an improvement in pain and severity of symptoms. However, most patients only perceived little to moderate improvement to their overall symptoms. On the other hand, majority of patients were willing to have further treatment.[14] Our results are in close concordance to their material, whether treating intra- or extramuscular VMs. It is easy to agree with their reasoning that often the patient's expectations exceed the outcome that can realistically be expected leading to only moderate patient satisfaction scores. Thus, objective and truthful patient information is in a highly important role. Risks, benefits and expectations should be discussed thoroughly. Patients should be expecting subtle improvements instead of radical changes in their symptoms. No curative treatment exists at the moment and therefore VMs should be considered as a chronic condition often requiring lifelong follow-ups and multiple consecutive therapeutic sessions.

In this study post-treatment MRI findings did not to correlate with neither subjective symptoms nor QOL results. In addition, the size and configuration of the malformation remained unchanged in most cases and without significance concerning the localization of the malformation. A Similar result was also reported by Bianchini et al. in their series of 81 intramuscular VMs – no change or only moderate reduction in size was seen in 97,5% of their post treatment MRIs.[15] Accordingly, routine MRI follow-up seems to be redundant. As van der Vleuten et al. stated in their review, effectiveness of VM therapies is uncertain and the evaluation of results is experience rather than evidence based. Patients with minimal symptoms and no functional limitations can be followed and offered supportive care.[5] The main goals of the treatment are reduction of pain and discomfort, improvement of cosmetic aspect and reduction of the volume.[16] Since, according to our results, there is no correlation between post-treatment MRI, subjective symptoms and QOL, a routine post-treatment MRI does not give any meaningful information when considering the need for re-treatment. If the patient is symptomless repeating the sclerotherapy is not beneficial regardless of the MRI finding – and vice versa – a symptomatic patient should be offered re-sclerotherapy whether the size of

the malformation in MRI has diminished or not. In conclusion change in subjective symptoms and QOL of the patient are of great interest and importance when evaluating the effectiveness of VM sclerotherapy.

Because of the relative rarity, complexity and potential high morbidity VM patients should be managed by multidisciplinary specialists. All patients in this study were evaluated in a multidisciplinary meeting by experienced clinicians and interventional radiologists. The treatment interval was not fixed since indication for treatment was subjective cosmetic or physical symptoms or worsening of the symptoms after previous therapy. Thus, only symptomatic patients were treated making the patients evaluation of the efficacy of the treatment and on QOL reliable. Sclerotherapy was performed by two experienced radiologists (LK-N and RR) with a standardized technique. Also, the MRI images were reviewed in consensus by the same radiologists, blinded concerning the self-evaluation and QOL results. These might be considered as strengths of our study.

Limitations of this study were the relatively low number of patients and retrospective setup and analyzation of the QOL score. This type QOL- measurement is susceptible to the phenomena of response shift[17] – in this case of the beta- type. This means, that during the treatment period, the patients' internal definition of – e.g. pain- changes due to the intervention and may result in over- or under-reporting of true physiologic change. This could be avoided by study design using pre- and post-treatment testing. However, at the beginning of this study routine pre-treatment testing had not yet started at our institutions. Due to the number of patients in this study statistical conclusions should probably not be drawn. Nevertheless, our overall results seem to be in concordance with the previous literature and comparisons between treatment results of extra- and intramuscular VM's have not been previously published.

Conclusion

Polidocanol sclerotherapy is an effective treatment for both intra- and extramuscular VM's in any location. In this material intramuscular VM's responded to the treatment comparably to extramuscular malformations. Since post treatment objective clinical findings are mostly indistinct, subjective symptoms and QOL results are important parameters in evaluating the effectiveness of sclerotherapy.

Declaration of Conflicting Interests

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

Guarantor

None.

Contributorship

None.

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Supplemental material

Supplemental material for this article is available online.

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PUBLICATION IV

Non-hotspot PIK3CA mutations are more frequent in CLOVES than in common or combined lymphatic malformations

Brouillard P, Schlögel M, Sepehr N, Helaers R, Queisser A, Fastré, Boutry S, Schmitz S, Clapuyt P, Hammer F, Dompmartin A, Weitz-Tuoretmaa A, Laranne J, Pasquesoone L, Vilain C, Boon L, Vikkula M

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Non-hotspot PIK3CA mutations are more frequent in CLOVES than in common or combined lymphatic malformations

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Abstract

Background: Theragnostic management, treatment according to precise pathological molecular targets, requests to unravel patients' genotypes. We used targeted next-generation sequencing (NGS) or digital droplet polymerase chain reaction (ddPCR) to screen for somatic *PIK3CA* mutations on DNA extracted from resected lesional tissue or lymphatic endothelial cells (LECs) isolated from lesions. Our cohort (n = 143) was composed of unrelated patients suffering from a common lymphatic malformation (LM), a combined lymphatic malformation [lymphatico-venous malformation (LVM), capillaro-lymphatic malformation (CLM), capillaro-lymphatico-venous malformation (CLVM)], or a syndrome [CLVM with hypertrophy (Klippel-Trenaunay-Weber syndrome, KTS), congenital lipomatous overgrowth-vascular malformations-epidermal nevi -syndrome (CLOVES), unclassified PIK3CA-related overgrowth syndrome (PROS) or unclassified vascular (lymphatic) anomaly syndrome (UVA)].

Results: We identified a somatic *PIK3CA* mutation in resected lesions of 108 out of 143 patients (75.5%). The frequency of the variant allele ranged from 0.54 to 25.33% in tissues, and up to 47% in isolated endothelial cells. We detected a statistically significant difference in the distribution of mutations between patients with common and combined LM compared to the syndromes, but not with KTS. Moreover, the variant allele frequency was higher in the syndromes.

Conclusions: Most patients with an common or combined lymphatic malformation with or without overgrowth harbour a somatic *PIK3CA* mutation. However, in about a quarter of patients, no such mutation was detected, suggesting the existence of (an)other cause(s). We detected a hotspot mutation more frequently in common and combined LMs compared to syndromic cases (CLOVES and PROS). Diagnostic genotyping should thus not be limited to PIK3CA hotspot mutations. Moreover, the higher mutant allele frequency in syndromes suggests a wider distribution in patients' tissues, facilitating detection. Clinical trials have demonstrated efficacy of Sirolimus and Alpelisib in treating patients with an LM or PROS. Genotyping might lead to an increase in efficacy, as treatments could be more targeted, and responses could vary depending on presence and type of *PIK3CA*-mutation.

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Keywords: Lymphatic malformation, Isolated, Gene, Mutation, Somatic, PI3K, Epidemiology, Theragnostic, Allele, Frequency

Background

Vascular malformations are usually congenital and localized. They slowly develop with the growth of the child. Treatments are mostly limited to laser, sclerotherapy, embolization and surgical resection. A malformation can affect any part of the lympho-vascular system (Fig. 1). A pure malformation affects only one compartment, e.g. a common lymphatic malformation (LM), whereas combined vascular malformations associate at least two components in the same lesion, e.g. a capillaro-lymphatic malformation [1]. Patients with isolated lesions have no other associated signs, whereas syndromic-forms affect at least one other organ.

In the princeps study in 2009, it was demonstrated that somatic genetic mutations are associated with sporadically occurring vascular anomalies: isolated venous malformations (VM) were discovered to have somatic *TIE2*/TEK mutations [2]. Since then, the etiopathogenesis of various isolated and combined vascular malformations has been unravelled [3, 4]. One of the genes identified to be mutated in vascular malformations encodes the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA, also known as p110α). Mutations were first identified in rare syndromic patients with Congenital Lipomatous Overgrowth, Vascular malformations, Epidermal nevi, and Skeletal/spinal abnormalities and/or scoliosis (CLOVES) [5]. Subsequently, *PIK3CA* mutations were implicated in common LM (43 out of 47 lesions) [6–8], and isolated VM without a *TIE2*/TEK mutation (27 out of 50 lesions) [9]. Somatic/mosaic PIK3CA mutations have also been implicated in variable syndromic phenotypes that can associate vascular anomalies with hypertrophy, leading to name this spectrum "PIK3CA-Related Overgrowth Syndrome" or PROS [10]. It includes CLOVES, Fibroadipose Hyperplasia (FH), Fibroadipose infiltrating lipomatosis, Hemihyperplasia multiple lipomatosis (HHML), Klippel-Trenaunay syndrome (KTS), Megalencephaly-Capillary Malformation-Polymicrogyria syndrome (MCAP), Macrodactyly, Hemimegalencephaly, and Muscle hemihyperplasia [10].

PIK3CA is considered as an oncogene [11]. The encoded p110α subunit contains the adapter-binding domain (ABD), the Ras-binding domain (RBD), the C2-PI3K-type domain (C2), the helical domain (H) and the kinase domain (K) (Fig. 2) [12]. Mutations that spread over the five functional domains have been implicated in many cancers, with positions *c.1624G* (p.E542), *c.1633G* (p.E545) and *c.3140A* (p.H1047) as hotspots. Similar mutations have been found in small series of lymphatic and vascular malformations.

In this study, we analysed for the presence of a *PIK3CA* mutation in a large cohort of lymphatic malformations, including common LM, lymphatico-venous malformation (LVM), capillaro-lymphatic malformation (CLM), capillaro-lymphatico-venous malformation (CLVM), unilateral capillaro-lymphatico-venous malformation with hypertrophy (KTS), CLOVES syndrome, unclassified PROS and unclassified vascular anomaly syndrome (UVA). This study contains the largest cohort studied so far for isolated lymphatic malformations. We analysed the prevalence, distribution and allele frequency of *PIK3CA* mutations in these phenotypes and evaluated for any genotype–phenotype correlation. This study provides firm epidemiological data, essential for the design of diagnostic testing, prospective clinical trials and drug development.

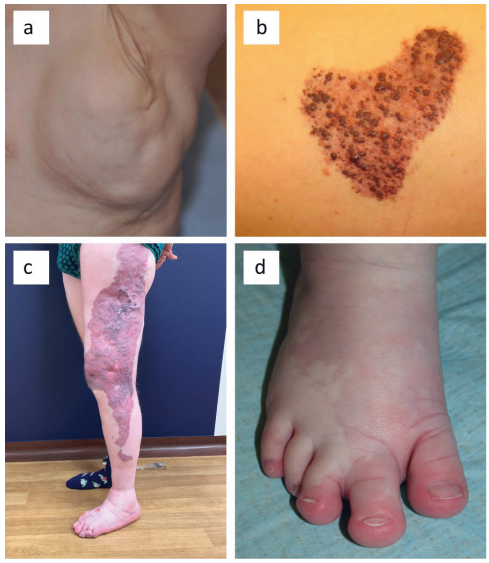

Fig. 1 Clinical characteristics of representative patients. **a** Mixed LM of left thorax (mutation p.Glu545Lys at 5% by NGS). **b** CLVM of left thorax with visible lymphatic vesicles (mutation p.His1047Arg at 5% by NGS and 5% by ddPCR). **c** Unclassified PROS (mutation p.Cys420Arg at 2% by NGS). **d** Macrodactyly and syndactyly 2–3 with small sandal gap and CM of right foot of a CLOVES syndrome patient

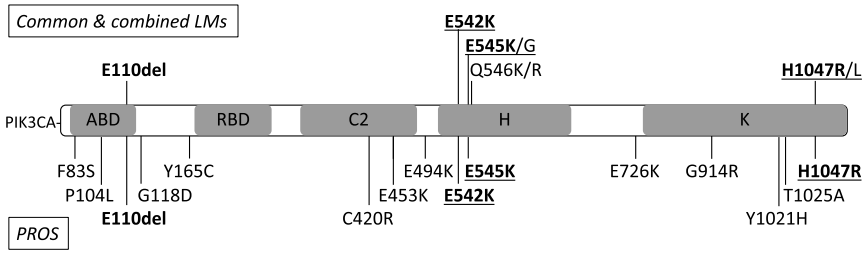

Fig. 2 PIK3CA protein (Human, 1068 aa) and mutations. Top, mutations found in common and combined lymphatic malformations (LM, LVM, CLVM). Bottom, mutations found in patients with PROS (KTS, CLOVES, unclassified PROS). See distribution in Table 1. Shared mutations in bold. Hotspot mutations underlined. ABD, p85α-binding domain; RBD, Ras-binding domain; C2, C2-PIK3C-type domain; H, helical domain; K, kinase domain

Results

To assess the contribution of PIK3CA mutations in common, combined and syndromic LM (Table 1), we screened DNA extracted from frozen tissues or isolated cells from 143 patients: 105 common LM, 3 LVM, 1 CLM, 7 CLVM, 4 KTS, 14 CLOVES, 7 PROS and 2 UVA. Within the mutated lesions, the variant allele frequency (VAF) varied strongly: from 0.54 to 25.33% for NGS-based analyses, and from 0.28% (14 SPD) to 22.7% for ddPCR-based analyses. The median VAF was 4.04% (STDEV = 4.57%), with 60% of patients having ≤ 5% VAF. In addition, we had 8 common LMs inconclusive for the presence of hotspot mutations (5 to 9 mutant reads, but only 0.24–0.52% VAF, not confirmed by ddPCR due to lack of DNA).

PIK3CA mutations were found in 78/105 common LM (74.3%), 3/3 LVM (100%), 6/7 CLVM (85.7%), 4/4 KTS (100%), 12/14 CLOVES (85.7%) and 5/7 PROS (71.4%) (Table 1). No *PIK3CA* mutation was detected in the unique CLM and the two UVA tested. Out of the 108 mutations detected, 92 (85.1%) were at hotspot positions: 31 p.E542K (*c.1624G* > *A*), 34 p.E545K (*c.1633G* > *A*), 1 p.E545G (*c.1634A* > *G*), 23 p.H1047R (*c.3140A* > *G*) and 3 p.H1047L (*c.3140A* > *T*). The rest was covered by 14 distinct non-hotspot mutations in 16 samples (Table 1). We also isolated primary cells from two resected tissues (one LM and one CLVM), to locate the population harbouring the mutation (Table 2). Strong enrichment of the somatic mutation was observed within the isolated endothelial cells (ECs) and especially lymphatic ECs.

We detected a statistically significant difference in the distribution of hotspot mutations and non-hotspot mutations between common and combined LM compared to the syndromes (p value = 1.329×10^{-7}), to CLOVES (p value = 1.441×10^{-5}) or to unclassified PROS (p value = 1.207×10^{-4}), but not to KTS (Fig. 3 and Table 3). Our meta-analysis of data collected from published reports detected a similar but weaker difference for CLOVES (p value = 0.0023) [6–8, 10, 13, 14]. When combining our data with the literature, the mutation distribution difference was significant between common and combined LMs versus PROS (p value = 7.742×10^{-5}) and versus CLOVES (p value = 5.575×10^{-7}). All p values adjusted for multiple testing remained significant (Table 3). As earlier reports focussed on hotspot screenings, it probably explains the weaker association in the literature data. Out of the altogether 367 patients reported by us and others, 54 (14.7%) are PIK3CA-negative: 38 LM, 1 CLM, 3 CLVM, 1 KTS, 4 CLOVES, 5 PROS and 2 UVA. Some of these could just be under the threshold of detection.

We also studied variability of VAF in different samples of the same patient. We had 16 patients for which 2, 3 or 4 samples were analysed (Table 4). A mutation was found in 14. The detected allele frequencies had a mean average of 5.17%, with an SD of 2.36%. For each patient, the same mutation was identified in all tissues. The two patients without an identified somatic mutation, were each negative in 3 different samples.

Overall, VAF varied from 0.54 to 25.33% between different phenotypes (Table 1). There was a statistically highly significant difference in VAF between common and combined LMs versus syndromes (PROS) (p value = 1.425×10^{-4}, Fig. 4a) and versus CLOVES (p value = 6.510×10^{-5}, Fig. 4b), as well as between common LMs versus syndromes (PROS) (p value = 6.765×10^{-5}, Fig. 4c) and versus CLOVES (p value = 5.290×10^{-4}, Fig. 4d). There was no statistically significant difference between the other entities (Additional file 1: Table S1).

The VAF for hotspot mutations varied between 0.54 and 22.19% (n = 92, median: 3.88%, SD = 3.72), whereas for the non-hotspot mutations it varied between 1 and 25.33% (n = 16, median = 8.71%, SD = 7.27). There was no statistically significant

Table 1 Patients cohort and PIK3CA mutations per pathology

Pathology	LM	LVM	CLM	CLVM	KTS	CLOVES	PROS	UVA	All
Cohort	105	3	1	7	4	14	7	2	143
Mutation	**78**	**3**	–	**6**	**4**	**12**	**5**	–	**108**
No mutation	19	–	1	1	–	2	2	2	27
Inconclusive	8	–	–	–	–	–	–	–	8
% with a mutation	74.3%	100%	0%	85.7%	100%	85.7%	71.4%	0%	75.5%
VAF median	3.71	4.59	–	7.34	8.12	12.90	11.09	–	4.05
Standard deviation	4.49	2.52	–	6.53	6.27	7.30	5.65	–	4.85
VAF range	0.54–11.34	3.43–8.25	–	4.71–22.19	1.15–13.17	2.00–25.33	1.00–13.00	–	0.54–25.33
Hotspot mutations	**74**	**3**	**0**	**6**	**3**	**5**	**1**	**0**	**92**
Non-hotspot mutations	4	0	0	0	1	7	4	0	16

PIK3CA Domain	Mutation	LM	LVM	CLM	CLVM	KTS	CLOVES	PROS	UVA	All
ABD	F83S							1		1
ABD	P104L						1			1
	E110del	1				1				2
	G118D							1		1
	Y165C						1			1
C2	C420R							1		1
C2	E453K						1			1
	E494K							1		1
Helical	**E542K**	**26**	**3**				**1**	**1**		**31**
Helical	**E545K**	**27**			**3**	**3**	**1**			**34**
Helical	**E545G**	**1**								**1**
Helical	Q546K	2								2
Helical	Q546R	1								1
	E76K						1			1
Kinase	G914R						1			1
Kinase	Y1021H						1			1
Kinase	T1025A						1			1
Kinase	**H1047R**	**17**			**3**		**3**			**23**
Kinase	**H1047L**	**3**								**3**

Bold: hotspot mutations. Protein domains based on https://www.uniprot.org/uniprot/P42336: ABD, p85α-binding domain (amino acids 16–105); C2, C2-PIK3C-type domain (330–487); Helical, Helical domain (517–694); Kinase, Kinase domain (797–1068). See also Fig. 2

Table 2 PIK3CA variants allele frequencies in tissues and isolated cells

Disease	Sample type	Mutation	VAF (%)
CLVM	Tissue	p.His1047Arg	5
	CD31-positive cells (EC)	p.His1047Arg	47
	CD31-negative cells	p.His1047Arg	0
LM	Tissue	p.Glu542Lys	11
	Unselected cells	p.Glu542Lys	4
	CD34-positive cells (BEC)	p.Glu542Lys	17
	CD31-positive and CD34-negative cells (LEC)	p.Glu542Lys	32
	CD31- and CD34-negative cells	p.Glu542Lys	0
	Fibroblasts	p.Glu542Lys	0

VAF, variant allele frequency; EC, endothelial cell; BEC, blood endothelial cell; LEC, lymphatic endothelial cell

difference between these two groups (p value = 0.0844) (Fig. 4e) or with individual hotspots (Fig. 4f, Additional file 1: Table S1). Finally, we analysed associations between VAF (Fig. 4g–i and Additional file 1: Table S1) or mutations (hotspot vs non-hotspot or presence vs absence) in regard to LM localization (Table 5), size (Table 6) or cystic structure (Table 7). There was no statistically significant difference identified in adjusted p values, except that a mutation is less often detected in small lesions (p value = 0.0129) (Table 6).

Discussion

We used deep sequencing and ddPCR to study a series of lymphatic malformations. Variant allele frequency ranged from 0.54 to 25.33%, suggesting that the most

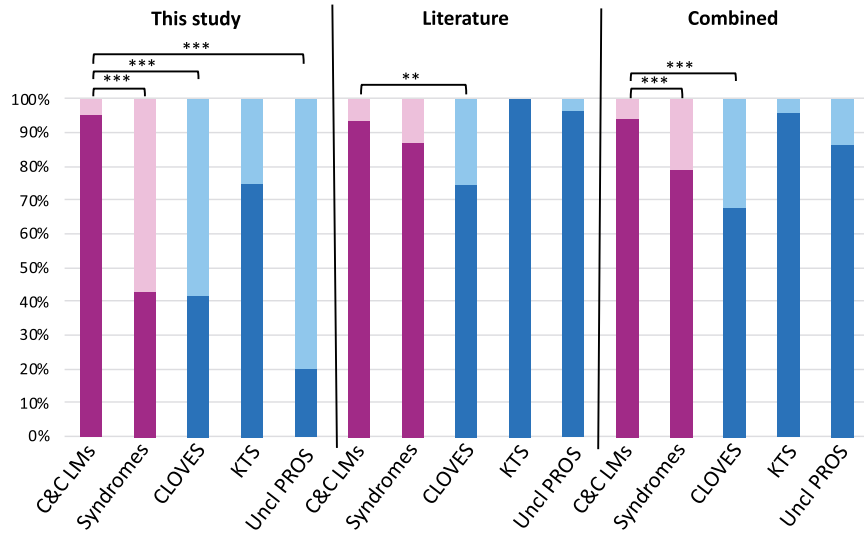

Fig. 3 Genotype distribution of PIK3CA mutations between patients with common and combined LMs or PROS. Distribution of hotspot mutations (dark colours, affecting amino acid positions 542, 545 or 1047) and non-hotspot mutations (light colours, all other amino acid positions) in patient cohorts. C&C LMs (Common and combined LMs) includes LM, LVM and CLVM. PROS, includes CLOVES, KTS and unclassified (Uncl) PROS (shown separately in blue). Literature: from references [6–8, 10, 13, 14]. Combined: this study and the literature. See Table 3 for values. ***p value < 0.001. **p value < 0.01

Table 3 Comparison of the mutation types between the different cohorts

	Number of patients	Pathology	Hotspot	Non-hotspot	Fisher's Exact Test	p value	Adjusted p value	Significance
This study	n = 87	C&C LMs	83	4				
	n = 21	PROS	9	12	C&C LMs vs PROS	1.329e-07	1.5948e-06	***
	12	*CLOVES*	*5*	*7*	*C&C LMs vs CLOVES*	*1.441e-05*	*5.764e-05*	***
	4	*KTS*	*3*	*1*	*C&C LMs vs KTS*	*0.2055*	*0.274*	
	5	*Uncl PROS*	*1*	*4*	*C&C LMs vs Uncl PROS*	*0.0001207*	*0.0002896*	***
Literature	n = 107	C&C LMs	100	7				
	n = 98	PROS	85	13	C&C LMs vs PROS	0.1562	0.2343	
	47	*CLOVES*	*35*	*12*	*C&C LMs vs CLOVES*	*0.002331*	*0.004662*	**
	20	*KTS*	*20*	*0*	*C&C LMs vs KTS*	*0.5955*	*0.7146*	
	31	*Uncl PROS*	*30*	*1*	*C&C LMs vs Uncl PROS*	*0.6832*	*0.7453*	
Combined	n = 194	C&C LMs	183	11				
	n = 119	PROS	94	25	C&C LMs vs PROS	7.742e-05	2.232e-04	***
	59	*CLOVES*	*40*	*19*	*C&C LMs vs CLOVES*	*5.575e-07*	*3.345e-06*	***
	24	*KTS*	*23*	*1*	*C&C LMs vs KTS*	*1*	*1*	
	36	*Uncl PROS*	*31*	*5*	*C&C LMs vs Uncl PROS*	*0.1434*	*0.2343*	

C&C (common and combined) LMs: LM, LVM, CLVM. PROS: includes CLOVES, KTS and unclassified (Uncl) PROS. Detailed values of PROS subgroups are given in italic. Literature: from references [6–8, 10, 13, 14]. Combined: this study and the literature. **p value < 0.01; ***p value < 0.001. The distributions between hotspot and non-hotspot are shown in Fig. 3

clonal surgical resections contained up to 50% of heterozygous mutant cells. In contrast, in the least clonal lesion detected, the frequency was as low as 1% of cells. VAF in common and combined LMs had a median of

Table 4 Patients with multiple samples analysed

Sequenced samples	Common code	Pathology	Mutation	VAF (%)	Average VAF (%)	SD VAF (%)
VA-10	VA-10	LM	E545G	10.01	8.28	2.45
VA-47.pKPT			E545G	6.55		
VA-1124.pKPT	VA-1124	CLOVES	E453K	13.51	8.64	6.89
VA-1133.pKPT			E453K	3.76		
VA-1126.pKPT	VA-1126	CLOVES	–	–	–	–
VA-1126.pRASO2			–	–		
VA-1126-T.pCMAVM			–	–		
VA-1158-T.pKPT	VA-1158-T	CLOVES	E545K	12.16	8.66	4.17
VA-1158-T-1.pTVPP			E545K	9.77		
VA-1158-T-2.pTVPP			E545K	4.05		
VA-230.pKPT	VA-230	LM	H1047L	2.52	2.50	0.04
VA-298.pKPT			H1047L	2.47		
VA-24.pKPT	VA-24	LM	–	–	–	–
VA-240.pKPT			–	–		
VA-986.pKPT			–	–		
VA-312.pKPT	VA-312	LM	E110delE	1.06	1.90	0.82
VA-339.pKPT			E110delE	1.95		
VA-403.pKPT			E110delE	2.70		
VA-364.pKPT	VA-364	LM	E542K	1.11	2.74	2.30
VA-421.pKPT			E542K	4.36		
VA-50.pKPT	VA-50	LM	E542K	4.95	6.56	2.28
VA-790.pKPT			E542K	8.17		
VA-528.pKPT	VA-528	LM	E545K	4.3	4.93	2.39
VA-714.pKPT			E545K	6.24		
VA-830.pKPT			E545K	7.32		
VA-913.pKPT			E545K	1.87		
VA-711.pKPT	VA-711	LM	H1047R	5.66	8.33	3.77
VA-829.pKPT			H1047R	10.99		
VA-756.pKPT	VA-756	LVM	E542K	8.25	7.07	1.67
VA-979.pKPT			E542K	5.89		
VA-1037.pKPT	VA-886	LM	Q546R	2.16	3.06	1.27
VA-886.pKPT			Q546R	3.96		
VA-868-T.pRASO2	VA-868	LM	E542K	2.11	3.78	2.36
VA-1245.pKPT			E542K	5.45		
VA-869-T.pRASO2	VA-869	LM	E545K	4.0	2.87	1.61
VA-1243.pKPT			E545K	1.73		
VA-1041.pKPT	VA-916	LM	H1047R	3.86	3.10	1.08
VA-916.pKPT			H1047R	2.33		

VAF, variant allele frequency; SD, standard deviation; mean of averages = 5.17%; mean of SDs = 2.36%

3.50% (SD = 3.24); it was more than double in PROS samples (median = 8.78%, SD = 6.47). These are similar to the ranges previously reported [13, 14]. The higher mutant allele frequency detected in PROS patients suggests broader mosaicism.

We identified a statistically significant association between mutation types and phenotypes. The non-hotspot mutations had a higher frequency in CLOVES and unclassified PROS compared to common and combined LMs (Fig. 3). Similar has been noted for macrocephaly-capillary malformation (M-CM) in which PIK3CA mutations are more often non-hotspot mutations [15]. Moreover, M-CM patients tend to be more mosaic for the mutation, which is sometimes detectable in their blood [15]. The level of mosaicism in human tissues may reflect the potential pathogenicity (the strength of downstream

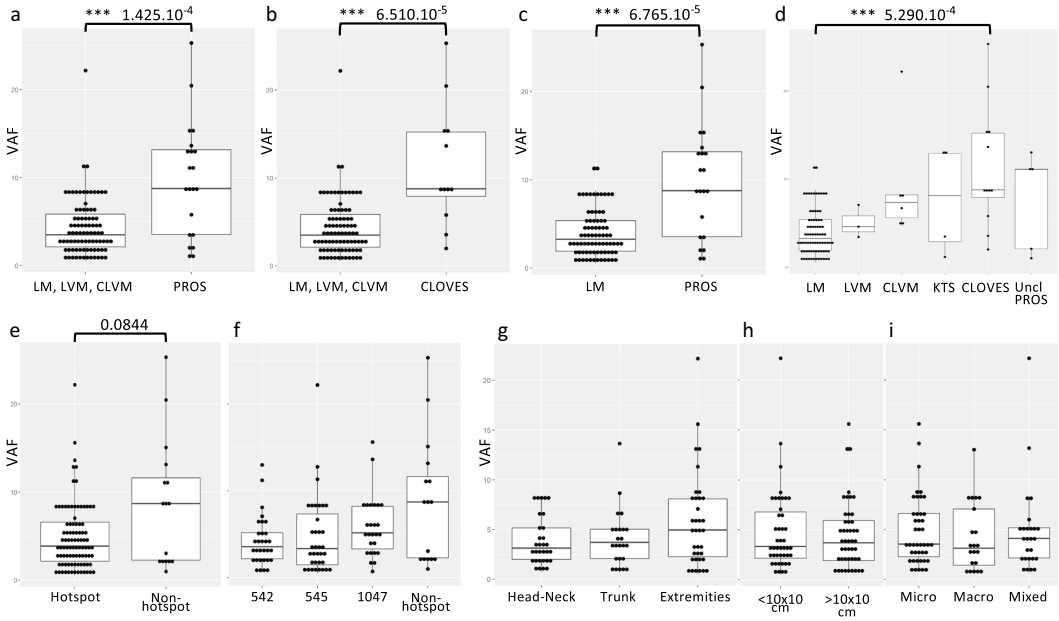

Fig. 4 VAF distributions. **a** Highly significant difference in VAF between common and combined LMs (LM, LVM,CLM) versus syndromes (PROS), and **b** versus CLOVES. No statistically significant difference between common and combined LMs versus unclassified PROS or KTS, nor between LVM or CLVM versus syndromes. **c** Significant difference between common LM versus syndromes (PROS), and **d** versus CLOVES. There was no statistically significant difference between any other entities (Additional file 1: Table S1). **e, f**, no statistically significant difference between VAF and hostpot/non-hotspot or each of the hotspots individually (Additional file 1: Table S1). **g–i** no statistically significant differences between VAF and LM localization (**g**) (Additional file 1: Table S1), size (**h**) (Additional file 1: Table S1) or cystic structure (**i**) (Additional file 1: Table S1). ***p value < 0.001

Table 5 Comparison of mutations versus localisation

Localisation	Mutation	Hotspot	Non-hotspot	No mutation
Input data				
Head & Neck	32	31	1	12
Trunk	25	23	2	11
Extremities	40	37	4	8
group_1	**group_2**	***p* value**	**Adjusted *p* value**	
Fisher's exact test Mutation/no mutation				
Head & Neck	Trunk	0.8068	0.8068	
Head & Neck	Extremities	0.3118	0.4677	
Trunk	Extremities	0.1877	0.4677	
Fisher's exact test Hotspot/non-hotspot				
Head & Neck	Trunk	0.5762	0.8643	
Head & Neck	Extremities	0.3732	0.8643	
Trunk	Extremities	1	1	

gain-of-function effects) of a given mutation. This suggests that the non-hotspot mutations that are more frequently seen in widespread PROS (such as CLOVES and M-CM) may have weaker downstream effects and thus could occur earlier during fetal development, thereby affecting more extensive body parts. Interestingly, when

Table 6 Comparison of mutations versus lesional size

Size	Mutation	Hotspot	Non-hotspot	No mutation
Input data				
< 10 × 10 cm	46	45	1	23
> 10 × 10 cm	50	44	6	8
Unknown	1	1	0	0
group_1	**group_2**	***p* value**	**Significance**	
Fisher's exact test Mutation/no mutation				
< 10 × 10 cm	> 10 × 10 cm	0.01285	*	
group_1	**group_2**	***p* value**		
Fisher's exact test Hotspot/non-hotspot				
< 10 × 10 cm	> 10 × 10 cm	0.1134		

*p value < 0.05

Table 7 Comparison of mutations versus cystic structure

Cystic structure	Mutation	Hotspot	Non-hotspot	No mutation
Input data				
Microcystic	48	43	5	14
Macrocystic	21	21	0	13
Mixed	28	26	2	4
Unknown	11	2	9	4
group_1	**group_2**	***p* value**	**Adjusted *p* value**	
Fisher's exact test Mutation/no mutation				
Microcystic	Macrocystic	0.1536	0.2304	
Microcystic	Mixed	0.2815	0.2815	
Macrocystic	Mixed	0.0241	0.0722	
group_1	**group_2**	***p* value**	**Adjusted *p* value**	
Fisher's exact test Hotspot/non-hotspot				
Microcystic	Macrocystic	0.3132	0.7500	
Microcystic	Mixed	1.0000	1.0000	
Macrocystic	Mixed	0.5000	0.7500	

Kruskal-Wallis chi-squared = 5.906, df = 3, p value = 0.1163

KTS is defined as unilateral capillaro-lymphatico-venous malformation with hypertrophy, like in this study, it seems to have more hotspot mutations, like common and combined LMs which are usually localized.

The same amino acid substitutions are found in cancers resulting in activation of p110α [11]. As vascular anomalies do not transform into malignancy, the activating p110α mutations in ECs are not able to induce oncogenesis. Nevertheless, a study on 122 patients with CLOVES syndrome demonstrated a higher risk of developing a Wilms tumour (WT) [16]. This underscores the likely presence of CLOVES-associated mutations in cell types other than EC. Interestingly, two studies on patients with KTS reported that the risk of cancers in children and adults was not higher than in the general population [17, 18]. These reports fit with our notion that especially CLOVES patients tend to have non-hotspot mutations with higher allelic frequencies. A similar risk may also be true for other PROS patients that have a wider (bilateral) phenotype.

The implication of the same PIK3CA mutation in common LM and common VM reinforces the idea that the cell type in which mutations occur influences directly the pathology [9]. LMs would be due to somatic mutations

in lymphatic endothelial cells (LEC), whereas VMs would be due to a mutation in blood endothelial cells (BEC). The cell-type specificity of mutations was underscored by the undetectable presence of the somatic PIK3CA mutation in our LM-derived fibroblasts and our CD-31 negative cells derived from a CLVM. Moreover, there was an increased detection rate of the tissular mutation (11%) in the LM-derived LECs (32%) (Table 2). This reinforces the idea that the somatic mutations occur in ECs or their precursors, determining the phenotype, and contrasts with CLOVES in which fibroblasts also harbour the mutation [19].

Intercellular signalling (mutant and wild-type) and cell–matrix interactions are also likely to play an essential role in the development of vascular malformations. This is highlighted by the single mutant cell type being at very low frequency (< 1%) in the lesion. Moreover, murine modelling demonstrated that the same somatic PIK3CA mutation activated in LECs can lead to macro- or microcystic lesions, depending on the time-point of induction of expression during development and growth [20]. Intrauterine activation led to macrocystic LMs, whereas early post-natal induction led to microcystic LMs. This fits well with the concept of cell-type and time-dependent occurrence of somatic (hotspot)/mosaic (non-hotspot) mutations explaining variability in phenotype [21].

Our results indicate that PI3K-pathway inhibition could work in at least 75.5% of patients with a lymphatic malformation. In the three published prospective clinical trials using Rapamycin, an mTOR inhibitor, on patients affected by various vascular malformations, response rates were high [22–25]. In one study, all 6 patients had a somatic mutation activating PI3K signalling and the response rate was 100%. In the other two studies, somatic genotyping had been performed only for a minority of the patients, yet > 85% had at least a partial response after 12 months of treatment. A PIK3CA inhibitor, BYL719 or Alpelisib, was tested on 19 patient with PROS, also showing good outcomes [26]. Similarly, the Pan-ALK inhibitor ARQ 092 (Miransertib) showed promising results on a cohort of 6 patients with PIK3CA-Related Overgrowth Syndrome [27]. These results demonstrate the positive impact of repurposing oncology drugs to patients suffering from benign vascular anomalies with a proven or likely somatic mutation that activates PI3K.

Genotyping is likely crucial in future clinical trials to increase efficacy. The high frequency of mutations identified in this study (75.5%) suggests that LMs are mostly caused by a somatic *PIK3CA* mutation. However, one fourth does not seem to have a *PIK3CA* mutation. This could be due to low representativeness of mutant ECs in the studied tissue sample. Analysis of multiple lesional samples may increase rate of molecular diagnosis [14]. Yet, we confirmed negativity in additional samples when available (n = 2 patients), and in the other 14 patients that were sequenced two, three, or four times, the mutation was found in all samples, albeit with variability in VAFs (Table 4).

Conclusion

In conclusion, our systematic data on a large cohort of patients with lymphatic malformations demonstrate that 75.5% of LMs, whether common, combined, or syndromic, are caused by somatic activating PIK3CA mutations. There is a statistically significant difference in mutation types between common and combined LM versus syndromic LMs (CLOVES and unclassified PROS), suggesting differential effects of the mutations. Non-hotspot mutations need to be looked for especially in the more wide-spread PROS phenotypes. Based on these and earlier data, repurposing of PI3K signalling pathway inhibitors for the treatment of LMs, whether isolated, combined or syndromic, have a sound epidemiological and pathophysiological basis.

Methods

Aim

Identification of prevalence of PIK3CA mutations and genotype–phenotype correlations in pure and combined lymphatic malformations.

Design

Tissues were collected from the leftover of programmed surgeries and snap-frozen in liquid nitrogen. Clinical data in regard to the phenotype (Table 1), and localization (head & neck, trunk and extremities), size (smaller or larger than 10×10 cm) and cystic structure (micro, macro, mixed) of the lesions were collected (Table 8). Nine of the patients have been included in earlier reports: one KTS as patient #5 in (9) and the associated E545K mutation in (11); five common LMs with H1047R with histology as patients #1–5 in (22), and two LMs (patients #2 and #6) as well as one KTS (patient #18) in a clinical trial (25).

Targeted next-generation sequencing (NGS) or ddPCR to screen for somatic PIK3CA mutations on DNA extracted from resected lesional tissue or lymphatic endothelial cells (LECs) isolated from lesions.

DNA extraction

DNA extraction from frozen tissues as previously described [9]. DNA was quantified using NanoDrop 8000

Table 8 Summary of samples per cystic structure, size and localisation

Cystic structure	Mutation	Hotspot				Non-hotspot	No mutation	Total	% mutation
Size*			542K	545K	1047				
Microcystic	**48**	***43***	14	15	14	***5***	**14**	**62**	**77%**
< 10 × 10 cm	20 (7/4/9)	19 (7/3/9)	10	4	5	1 (0/1/0)	9 (3/3/3)	29	69%
> 10 × 10 cm	27 (6/10/11)	23 (5/9/9)	4	11	8	4 (1/1/2)	5 (0/2/3)	32	84%
Unknown	1 (0/1/0)	1 (0/1/0)	0	0	1	0	0	1	100%
Macrocystic	**21**	***21***	9	10	2	***0***	**13**	**34**	**62%**
< 10 × 10 cm	16 (7/2/7)	16 (7/2/7)	6	9	1	0	12 (5/6/1)	28	57%
> 10 × 10 cm	5 (2/1/2)	5 (2/1/2)	3	1	1	0	1 (1/0/0)	6	83%
Unknown	0	0	0	0	0	0	0	0	–
Mixed	**28**	***26***	8	9	9	***2***	**4**	**32**	**88%**
< 10 × 10 cm	10 (4/3/3)	10 (4/3/3)	4	4	2	0	2 (2/0/0)	12	83%
> 10 × 10 cm	18 (6/4/8)	16 (6/4/6)	4	5	7	2 (0/0/2)	2 (1/0/1)	20	90%
Unknown	0	0	0	0	0	0	0	0	–
Unknown	**11**	***2***	0	1	1	***9***	**4**	**15**	**73%**
Total	***108***	***92***	***31***	***35***	***26***	***16***	***35***	***143***	***76%***

*Numbers per localisation given between parenthesis: (Head&Neck/Trunk/Extremities). See Tables 6 and 7 for globalized numbers per size and localization. Mutation numbers are subdivided in Hotspot and Non-hotspot, with Hotspot numbers further subdivided per position

(Thermo Fisher Scientific) and Qubit 2.0 (Thermo Fisher Scientific).

Isolation and culturing of primary endothelial cells (EC) and primary lymphatic ECs

Single-cell solutions were obtained from CLVM and LM tissues by digesting with 0.04% dispase (Gibco), 0.25% collagenase II (Roche) and 0.01% DNAse I (Roche) for 1 h at 37 °C. Separated cells (= mixed cells) were seeded on fibronectin-coated flasks (2 μg/cm^2) (Millipore) and grown in ECGM2 (Bio-Connect life sciences) for CLVM isolated cells and ECGM-MV2 (Bio-Connect life sciences) for LM isolated cells, both supplemented with penicillin–streptomycin. When mixed cells reached a confluency of 80%, they were detached using Accutase (Sigma). Mixed CLVM cells were sorted for CD31-positive cells using Anti-CD31 MicroBeads (Miltenyi). To obtain LECs, we performed a fibroblast depletion using Anti-fibroblast MicroBeads (Miltenyi) as well as CD34-positive blood EC depletion using Anti-CD34 MicroBeads (Miltenyi), followed by a CD31-positive selection using Anti-CD31 MicroBeads. All different selected cells were plated on fibronectin-coated flasks (2 μg/cm^2). Cell morphology was confirmed using a bright field microscope (Zeiss).

PIK3CA sequencing

We designed an Ion AmpliSeq panel for targeted sequencing of the 21 coding exons of *PIK3CA* and ten nucleotides of all flanking introns (NM_006218.2) (http://www.ampliseq.com). Theoretical horizontal coverage was 96.75%, with 16 bp in the 5'UTR (exon 1) and 32 bp in exon 20 not covered. The panel consisted of 2 pools of primers for multiplexed PCR-amplification with Ion Ampliseq Library kit, and sequencing on an Ion Personal Genome Machine (PGM) or an ion Proton (Thermo Fisher Scientific). Reads were aligned to the human reference sequence hg19, using the Torrent Suite Server. Bam files were imported into Highlander software package (https://sites.uclouvain.be/highlander/) for analysis. We selected variants with at least 5 mutant reads representing at minimum 1% of all alleles by interrogating all positions reported with at least 4 changes in the COSMIC database (https://cancer.sanger.ac.uk/cosmic). Samples needed to have an average coverage above 500 × to be considered not to contain a mutation. Mutations with a VAF below 1% but confirmed by ddPCR when DNA was available, were considered to contain a mutation.

Digital droplet PCR (ddPCR)

Digital droplet PCR (ddPCR) was used to study four known PIK3CA hotspot mutations: *c.1624G > A* (p.E542K), *c.1633G > A* (p.E545K), *c.3140A > T* (p.H1047L) and *c.3140A > G* (p.H1047R) (NM_006218.2). Probes were designed by Bio-Rad (Bio-Rad Laboratories). The ddPCR experiments were performed, as described by Hindson et al. [28]. DNA input was 30 ng. At most, 93 samples were run in parallel. For the analysis, we used QuantaSoft software (Version 1.7). Samples had to have at least 5 mutant single positive droplets (SPD) among a minimum of 10,000 droplets to be considered mutated.

Nine patients with detection of mutation by ddPCR could not be tested by NGS. Their variant allele frequencies ranged from 1.6 to 15.6%.

Statistics

Data were analyzed to detect differences between groups using Fisher's exact test for two categorical variables, Pairwise Wilcoxon's rank-sum test for group comparisons of continuous variables, and the Kruskal–Wallis test for > 2 group comparisons of continuous variables. Nonparametric tests were used due to small sample size and non-normal distributions (tested with Shapiro test). Significance of p values: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. In order to control type I errors, multiple testing corrections were performed using Benjamini–Hochberg procedure. All analyses were performed using R and graphs were generated with ggplot2. Descriptive statistics were computed using groupedstats R package. VAF values for statistics were based on NGS results (average VAF values for patients with multiple sequencing, Table 4) or ddPCR (for 10 samples tested only by ddPCR).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13023-021-01898-y.

Additional file 1. Statistical comparisons of data shown in Fig. 4 d,f, g, h, i.

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Authors' contributions

MJS, NHS and EF carried out the DNA extractions, ddPCR and NGS panel experiments. PB and MJS analysed the data. RH developed bioinformatic tools for the analysis of somatic mutations within Highlander. AQ carried out the cell biology experiments. SB performed the statistical analyses. SS, PC, FH, AD, AWT, JL, LP, CV and LMB collected medical information and blood samples. MV conceived and coordinated the study. MJS and PB drafted the manuscript. PB and MV helped to develop the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Informed consent was obtained for all patients, as approved by the ethical committee of the Medical Faculty at the University of Louvain, Brussels, Belgium (ref B403201629786) and the local committees of collaborators.

Consent for publication

All patients gave consent to publish the pictures shown in Fig. 1.

Competing interest

The authors declare no conflict of interest.

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