Angiogenesis (2017) 20:163–173
DOI 10.1007/s10456-016-9537-2

ORIGINAL PAPER

Angiopoietins as serum biomarkers for lymphatic anomalies

Timothy D. Le Cras[1] · Paula S. Mobberley-Schuman[2] · Mary Broering[1] · Lin Fei[3] · Cameron C. Trenor III[4] · Denise M. Adams[4]

Received: 9 February 2016 / Accepted: 3 December 2016 / Published online: 18 December 2016

Abstract Vascular anomalies can cause significant morbidity and mortality. Advances in diagnosis will be improved if noninvasive biomarkers can be identified, as obtaining a tissue biopsy can worsen the disease and precipitate complications. The goal of this study was to identify biomarkers for vascular anomaly patients to aid diagnosis and potentially give insights into pathogenesis. Blood was collected at baseline and then 6 and 12 months after treatment with the mTOR inhibitor sirolimus. Patients groups included generalized lymphatic anomaly (GLA), kaposiform lymphangiomatosis (KLA) and kaposiform hemangioendothelioma (KHE) with or without the Kasabach–Merritt phenomenon (KMP) coagulopathy. Serum was obtained from healthy controls selected to match the age and sex of the patients (21 days–28.5 years; 42% males; 58% females). Angiogenic and lymphangiogenic factors (VEGF-A, C, D, Ang-1 and Ang-2) were measured in serum using ELISA. In lymphatic anomaly patients, baseline levels of VEGF-A and VEGF-D were not different compared to controls. Angiopoietin-2 (Ang-2) levels were near controls levels in GLA patients but 10-fold greater in KLA patients and 14-fold greater in KHE patients when the KMP coagulopathy was present but not when it was absent. VEGF-C and angiopoietin-1 (Ang-1) levels were lower in KHE patients with KMP. Our analyses suggest that Ang-2 and Ang-1 can be used as biomarkers to help identify KLA and KHE patients with KMP coagulopathy with high sensitivity and specificity. After 12 months of sirolimus treatment, Ang-2 levels were lower in KLA and KHE with KMP patients compared to baseline levels and with most patients showing a clinical response. Hence, serum Ang-2 and Ang-1 levels may help in the diagnosis of patients with lymphatic anomalies and are concordant to sirolimus response.

Keywords Vascular anomalies · Generalized lymphatic anomaly · Kaposiform lymphangiomatosis · Kaposiform hemangioendothelioma · Sirolimus · Kasabach–Merritt

Abbreviations

Ang-2	Angiopoietin-2
Ang-1	Angiopoietin-1
KLA	Kaposiform lymphangiomatosis
GLA	General lymphatic anomaly
KHE	Kaposiform hemangioendothelioma
KMP	Kasabach–Merritt phenomenon

Introduction

Vascular anomalies are a wide spectrum of diseases that include vascular tumors and malformations and are classified based on clinical, genetic and pathologic characteristics [1]. Lesions may be principally arterial, venous,

Electronic supplementary material The online version of this article (doi:10.1007/s10456-016-9537-2) contains supplementary material, which is available to authorized users.

✉ Timothy D. Le Cras
tim.lecras@cchmc.org

[1] Division of Pulmonary Biology, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati, 3333 Burnet Avenue, Cincinnati, OH 45229-3039, USA

[2] Cancer and Blood Disease Institute, Cincinnati Children's Hospital Medical Center, University of Cincinnati, 3333 Burnet Avenue, Cincinnati, OH 45229, USA

[3] Biostatistics and Epidemiology, Cincinnati Children's Hospital Medical Center, University of Cincinnati, 3333 Burnet Avenue, Cincinnati, OH 45229, USA

[4] Division of Hematology/Oncology, Vascular Anomalies Center, Boston Children's Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115, USA

lymphatic or capillary in nature or occur in various combinations and proportions. Vascular anomalies most often occur in the skin and soft tissue but can also be found in any organ and present with a wide spectrum of symptoms and complications depending on the type and location. Growth or expansion of these anomalies can occur with age, trauma or hormonal stimulation worsening symptoms, quality of life and in some cases can be fatal. The diagnosis of these rare diseases in infants and children can be a challenge, and obtaining a tissue biopsy can lead to severe complications such as effusions, lymphatic leak and/or worsened coagulopathy. Development of noninvasive diagnostic methods including biomarkers may provide a less risky, more accurate diagnosis, so clinical decisions and treatment can begin in a timely manner. Biomarker studies in patients with vascular anomalies are limited. One study examined levels of matrix metalloproteinases, basic fibroblast growth factor and vascular endothelial growth factor (VEGF) in the urine of patients with vascular tumors and malformations [2], but while this study reported increases in high molecular weight matrix metalloproteinases and basic fibroblast growth factor in vascular tumor and malformation patients, no changes in VEGF-A were detected and other well-known angiogenic factors were not assessed. The primary goal of our study was to evaluate differences in biomarkers between patient groups and controls, with the long-term goal being that these biomarker(s) might be used in the future to discriminate between different patient groups. In addition, we wanted to determine whether these biomarkers changed when patients were placed on therapy.

Until recently, there were limited treatment options for vascular anomalies apart from surgery and sclerotherapy. However, recently, a phase II clinical trial investigating the use of the mTOR inhibitor sirolimus in patients with complicated vascular anomalies found sirolimus efficacious and safe for the majority of enrolled patients [3]. Clinical and biomarker assessments were made after 6 and 12 months of sirolimus treatment. High-risk phenotypes assessed in the trial included generalized lymphatic anomaly (GLA), kaposiform lymphangiomatosis (KLA) and kaposiform hemangioendothelioma (KHE) with and without Kasabach–Merritt phenomenon (KMP) (KHE ± KMP). GLA is a disease characterized by abnormally proliferating lymphatic vessels in multiple organs. Complications include pleural and pericardial effusions, bone involvement, soft tissue masses and visceral disease. KLA is similar to GLA but is characterized by a distinctive histopathology of "kaposiform"-like clusters of spindled lymphatic endothelial cells accompanying malformed lymphatic vessels [4–8]. KLA is more aggressive and diffuse compared to GLA. Pulmonary involvement is frequently associated with KLA as well as coagulopathy causing significant hemorrhagic complications. KLA has a high morbidity and mortality, and so early diagnosis and treatment is preferable. Definitive diagnosis of KLA is often delayed due to complex constellation of symptoms. Currently, histopathology is considered crucial in making the diagnosis, but biopsy can worsen disease severity. As with most complicated vascular anomalies, this high-risk diagnosis has much heterogeneity with no risk stratification. Steroids, vincristine and sirolimus are agents used singly or in combination for the treatment of these patients [9]. KHE, usually presents at birth or in the first 3 years of age, is a locally aggressive vascular tumor with a pathology that also includes spindle cells but with a diffuse presentation [10]. KMP, a coagulopathy associated with KHE, has a mortality rate of 20–30% [4–7] and is characterized by profound thrombocytopenia and hypofibrinogenemia. Risk stratification has not been formally studied, but location, size of the tumor and degree of coagulopathy were found significant in a retrospective report [11]. A number of treatment regimens have been used, and recently, sirolimus has been effective in the treatment of these tumors [12–16]. Other vascular anomalies were evaluated, but numbers were generally small and will not be focused on in this paper. As expected, the majority of patients in the sirolimus trial conducted by our group [3] had a partial response (83% after course 6 and 85% after course 12). The results were significant and greatly improved the quality of life for these patients. As part of this clinical trial, we sought to identify biomarkers in the blood of the patients and whether these correlated with response to sirolimus.

Hence, the goal of this study was to identify biomarkers that might prove useful in the diagnosis of patients with GLA, KLA and KHE and that showed a concordant response to therapy. We chose to measure secreted angiogenic factors that are known to regulate vessel development, maturation and stability based on the hypothesis that these factors would be increased and playing a role in the pathogenesis of these vascular anomalies. We measured VEGF family members and the angiopoietins. VEGF-A is known for its potent pro-angiogenic activity stimulating endothelial cell proliferation through the VEGF receptor-2 (VEGFR-2 or Flk-1) [17]. VEGF-A also enhances vessel permeability, originally being known as vascular permeability factor. Interestingly, VEGF-A has also been reported to increase pulmonary lymphangiogenesis in transgenic mice although whether this is the case in humans is unclear [18]. VEGF-C and VEGF-D are well-known pro-lymphangiogenic factors and activate VEGFR-2 and VEGF receptor-3 (VEGFR-3) [19–21]. VEGFR-3 while initially expressed on embryonic blood endothelial cells becomes restricted to lymphatic endothelial cells around midgestation [22]. VEGF-D has been identified as a blood biomarker for lymphangioleiomyomatosis (LAM), a destructive cystic lung disease that occurs in women [23, 24]. VEGF-D levels can be used

to discriminate LAM from other cystic lung diseases as well as from healthy controls. Measurement of serum VEGF-D levels now represents a noninvasive alternative to open lung biopsy to help diagnose LAM. In addition, LAM cells have been shown to express VEGF-D and so identification of this biomarker has also opened up the possibility of VEGF-D playing a role in the pathogenesis of LAM. Angiopoietins-1 (Ang-1) and 2 (Ang-2) are ligands for the endothelial-specific receptor tyrosine kinase Tie2 (TEK) [17]. Ang-1 promotes blood vessel maturation and stability through activation of Tie2 on endothelial cells. While Ang-2 is also a ligand for Tie2, it is generally thought to be antagonistic to Ang-1 resulting in destabilization of vessels. Under certain conditions, Ang-2 may act as an agonist and so its effects appear to be context dependent. Ang-1 is generally thought to limit angiogenesis, whereas Ang-2 can facilitate VEGF-induced angiogenesis. The angiopoietins and Tie2 may play a role in lymphangiogenesis [25] and are now implicated in a number of human diseases [26]. Levels of these factors were measured in the serum from the patient groups and an age- and sex-matched control group. These angiogenic factors were measured in the blood of patients at baseline and also after 6 and 12 months of sirolimus therapy.

Methods

Patients with vascular anomalies and controls

All protocols were approved by the Cincinnati Children's Hospital Medical Center (CCHMC) and Boston Children's Hospital (BCH) Institutional Review Boards. Serum samples for biomarker studies were collected over a 5-year period from CCHMC and BCH clinics and were part of the phase II trial of sirolimus [3] in patients with complicated vascular anomalies (sponsored by the FDA Office of Orphan Products (R01FD003712; clinicaltrials.gov; NCT#00975819). Serum samples were obtained from the following patient groups: GLA, KLA, KHE + KMP and KHE − KMP. Table 1 shows the numbers of patients in each group, age and sex distribution, and clinical response to sirolimus at 6 and 12 months. Serum samples were also analyzed from healthy control children from the CCHMC Genomic Control Cohort (GCC). Children were excluded if they had acute or chronic illness. Samples for the control group were selected to closely match the age and gender of the patients with vascular anomalies (Table 1).

Analysis of serum biomarkers

Serum was collected from patients with vascular anomalies at baseline before treatment and then after 6 and 12 months of sirolimus treatment. Serum from patients at 6 and 12 months was available in most cases except for a few patients who did not respond to therapy and were removed from the study. Serum levels of VEGF-A, C, D, Ang-1 and Ang-2 were measured using ELISA in duplicate (R&D Systems: VEGF-A DVE00; VEGF-C DVEC00; VEGF-D DVED00; Ang-1 DANG10; Ang-2 DANG20). The average percent coefficient of variance (CV) of Ang-2 levels in the duplicate samples was 6.09 ± 0.35% (mean ± standard error). If samples had a CV of 20% or greater, then the ELISA was repeated.

Statistical analysis

For comparisons of biomarkers between disease groups and the control group, an ANOVA was applied, and Dunnett's test was used for multiple comparisons to mitigate the false positive rate when comparing levels in multiple patient groups to the control. The 95% confidence intervals (CI) for mean difference (as percentage) were obtained from Dunnett's test. Correlation coefficients among biomarkers

Table 1 Numbers of patients, age and sex distribution, and clinical response to sirolimus at 6 and 12 months

Group	Patient number	Age, years median (range in years)	Male %	Female %	Response to sirolimus at 6 months	Response to sirolimus at 12 months
Controls	55	8 (0.1–28)	40	60	N/A	N/A
Generalized lymphatic anomaly (GLA)	7	12 (2–18)	43	57	PR 7 (100%)	PR 7 (100%)
Kaposiform lymphangiomatosis (KLA)	7	12 (3–24)	43	57	PR 5 (71%), SD 1 (14%), PD 1 (14%)	PR 6 (86%), PD 1 (14%)
Kaposiform hemangioendothelioma (KHE) with Kasabach–Merritt phenomenon (+KMP)	8	0.6 (0.1–3)	25	75	PR 8 (100%)	PR 8 (100%)
Kaposiform hemangioendothelioma (KHE) without KMP (−KMP)	3	1.5 (0.2–2)	100	0	PR 1 (33%), SD 1 (33%), PD 1 (33%)	PR 2 (66%), PD 1 (33%)

PR partial response, *SD* stable disease, *PD* progressive disease

were analyzed using Pearson's method, with z test for significance. Receiver operating characteristic (ROC) curves were generated for Ang-2 and Ang-1 in the VA patient groups that were significantly different from healthy controls. ROC is an important tool for evaluating the accuracy of diagnostic tests [28]. The ROC represents the trade-off between the sensitivity (true positive rate) and the 1-specificity (false positive rate). The area under the curve (AUC) of a ROC provides a measure of the ability of the diagnostic test to discriminate between cases and controls, or between any two groups. A test that has perfect discrimination passes through the upper left corner of the ROC graph and has an AUC of 1.0. Receiver operating curves (ROC) and Pearson's correlation analysis were performed using the Prism software. Multivariable multinomial logistic regression analysis of Ang-1 and Ang-2 levels was also carried out to assess the joint impact of both markers on the three disease statuses under consideration. Due to the skewness of Ang-1 and Ang-2 measurements, they are analyzed on log scale in the model. The model terms are Ang-1 and Ang-2, with three disease statuses as responses. General logits is used for three categories. SAS®, Prism software and R nnet package [27] were used to analyze the data.

Results

Serum levels of factors in healthy controls

In samples from healthy controls, levels of VEGF-A, VEGF-C, and VEGF-D did not change significantly with age. When we combined all age groups together, VEGF-A levels were 62% (CI 13–110%) higher in males versus females (males 403 pg/ml vs females 250 pg/ml; $P = 0.0135$). Since normal levels of these factors in controls have not been previously reported, these data are included as supplemental figures (Supplemental Figures 1–3). Ang-2 and Ang-1 levels were similar over the age range of the controls, and no significant differences were observed between levels in the serum of males compared to females (Fig. 1). Overall, there were no major changes in the levels of VEGF-C, VEGF-D, Ang-1, or Ang-2 with age or gender.

Baseline serum levels of factors in patients with vascular anomalies

Baseline levels of VEGF-A and VEGF-D were not different in all the groups of patients compared to controls (Supplemental Figures 4 and 6). VEGF-C levels were 63% (CI 41–85%) lower in KHE with KMP (KHE + KMP) patients (Supplemental Figure 5) compared to healthy controls. Serum Ang-2 levels were elevated in KLA, KHE + KMP, and KHE without KMP (KHE − KMP) patients compared to controls (Table 2; Fig. 2a) when Dunnett's method was used as a post hoc test to ANOVA. Ang-2 levels in the GLA patient group were not significantly elevated compared to controls (Table 2). Ang-1 levels in the GLA and KLA patients were similar to controls (Table 2). Serum Ang-1 levels were lower in KHE + KMP patients compared to controls (Table 2; Fig. 2a). Ang-1 was not lower in KHE without KMP patients (Table 2). Platelet counts in the GLA (340 ± 41 k/mcl) and KLA (311 ± 71 k/mcl) patient groups were in the normal range (135–466 k/mcl), but those in KHE + KMP patients (78 ± 30 k/mcl) were below normal. We performed Pearson's correlation analysis and found that Ang-1 levels and VEGF-C levels were strongly correlated with platelet counts ($R = 0.93$ and $R = 0.87$, respectively, $P < 0.0001$) (Supplemental Figure 7).

Biomarker specificity and sensitivity

ROC for Ang-2 and Ang-1 was also used to compare the utility of these tests in patient groups compared to controls. For Ang-1, ROC analysis was also performed between KLA and KHE + KMP. Serum Ang-2 concentrations in the KLA patient group were higher than in healthy controls (Table 2; Fig. 3). AUC was 1.000 (CI 1.000–1.000) and this analysis showed that Ang-2 levels >5825 pg/ml differentiate between KLA and healthy controls. All of the KLA patients in the study were above this level. Serum Ang-2 concentrations in the KHE + KMP patient group were higher than in healthy controls (Table 2; Fig. 3). AUC was 1.000 (CI 1.000–1.000) and this analysis showed that Ang-2 levels >4200 pg/ml differentiate between KHE + KMP and healthy controls. All of the KHE + KMP patients in the study were above this level. Ang-1 levels were lower in the KHE + KMP patients compared to controls (Table 2; Fig. 4). The ROC curve for Ang-1 in KHE + KMP patients versus controls had an AUC of 0.917 (CI 0.7631–1.000). AUC for Ang-1 in KHE + KMP patients versus KLA patients was 0.889 (CI 0.705–1.000; Fig. 4c). The ratio of Ang-2 to Ang-1 (Ang-2/Ang-1) was higher in all disease groups compared to controls (Table 2; Fig. 4d).

Multivariable analysis of Ang-2 and Ang-1

A multinomial logistic regression model of baseline Ang-1 and Ang-2 in classifying three GLA, KLA and KHE + KMP patient groups was applied. The three-category analysis models generalized logits with GLA as the reference group. The analysis comprises the following model. The probability of a patient (from potentially three categories GLA, KLA and KHE + KMP) belonging to one category (as opposed to the other two) is jointly determined by his/her Ang-1 and Ang-2 levels. This function assumes

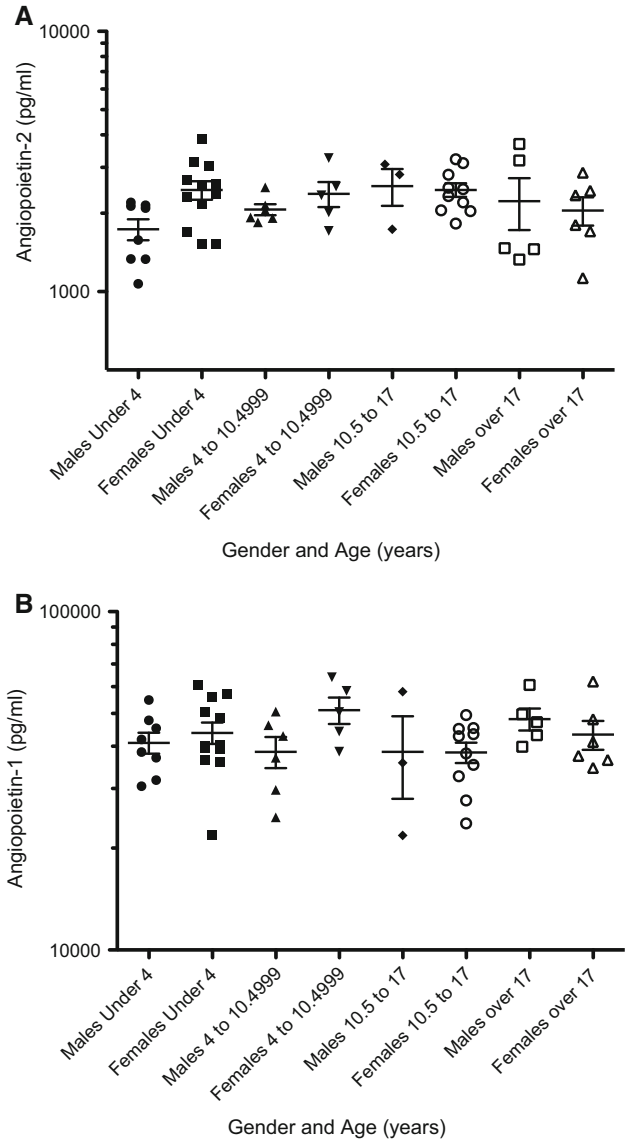

Fig. 1 Levels of angiopoietin-2 (Ang-2) and angiopoietin-1 (Ang-1) in the serum of healthy controls. Ang-2 (Panel **a**) and Ang-1 (Panel **b**) levels were measured by ELISA in the serum from controls to determine whether levels were different between males and females and changed with age. No significant differences in Ang-2 or Ang-1 were detected between the age groups and males versus females in each age group

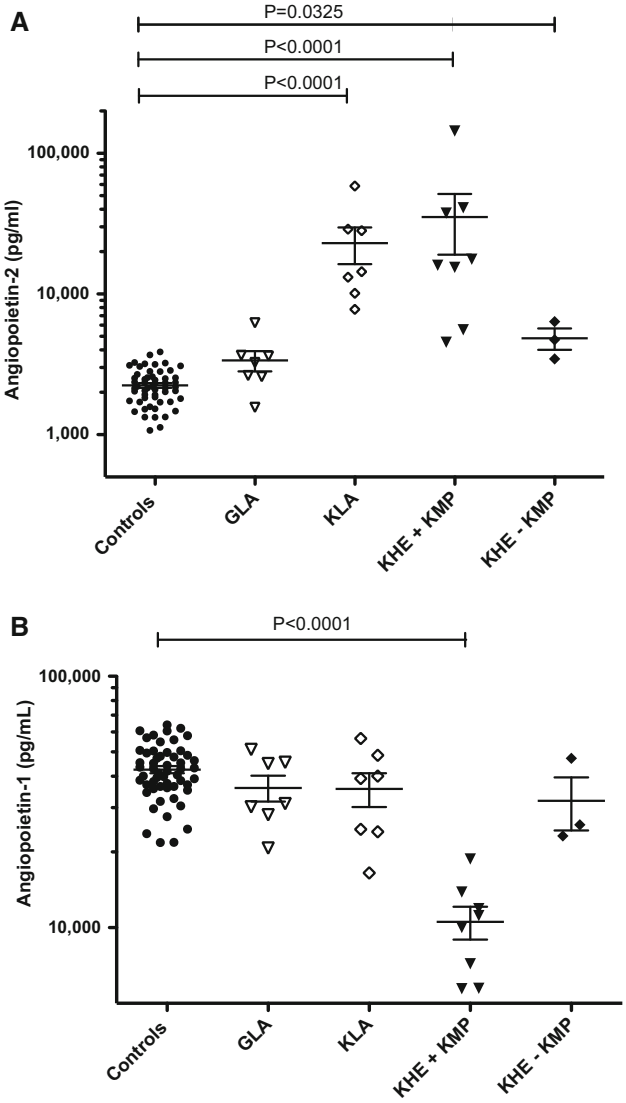

Fig. 2 Levels of angiopoietin-2 (Ang-2) and angiopoietin-1 (Ang-1) in the serum of patients. Ang-2 and Ang-1 levels were measured by ELISA in the serum of GLA, KLA and KHE patients prior to treatment with sirolimus and compared to levels in sex- and age-matched controls. Ang-2 levels were higher in KLA and KHE + KMP patients but not in GLA or KHE patients that did not have KMP (KHE − KMP). Ang-1 levels were lower in KHE + KMP but not in GLA, KLA or KHE − KMP patients compared to controls

Table 2 Ratios and *P* values of Ang-2 and Ang-1 levels in patient groups compared to control group

Group comparison to control	Ang-2		Ang-1		Ang-2/Ang-1	
	Ratio (95% CI)	*P* value*	Ratio (95% CI)	*P* value*	Ratio (95% CI)	*P* value*
GLA	1.45 (0.88–2.39)	0.2155	0.83 (0.61–1.14)	0.45	1.74 (1.01–3.01)	0.0463
KLA	8.56 (5.20–14.07)	<0.0001	0.80 (0.58–1.09)	0.24	10.71 (6.20–18.52)	<0.0001
KHE + KMP	9.26 (5.79–14.79)	<0.0001	0.24 (0.18–0.32)	<0.0001	39.18 (23.39–65.62)	<0.0001
KHE − KMP	2.19 (1.04–4.56)	0.0325	0.74 (0.47–1.16)	0.32	2.97 (1.32–6.66)	0.0039

P values from Dunnett's test

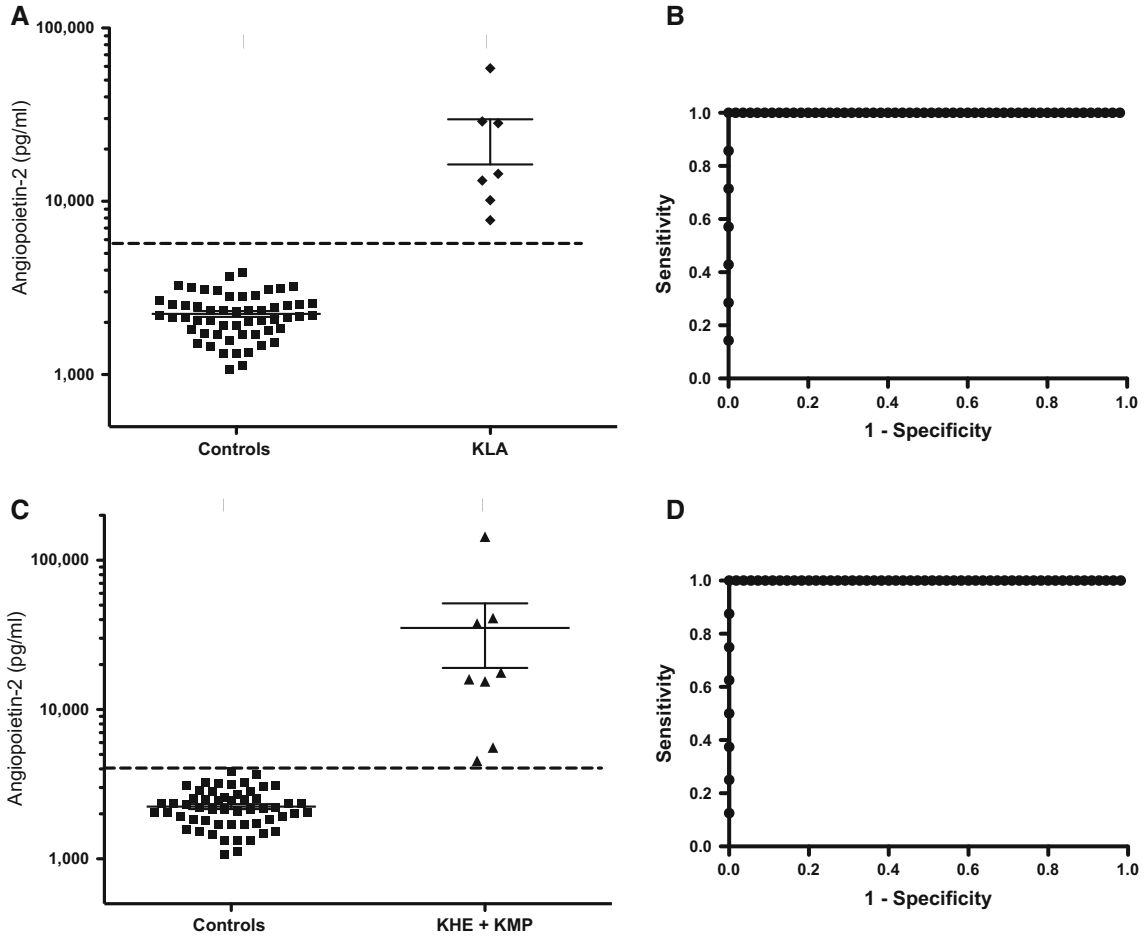

Fig. 3 Evaluation of Ang-2 as a serum biomarker in KLA and KHE + KMP patients. Ang-2 levels were measured by ELISA in the serum of patients and controls. **a** Ang-2 levels in the serum of KLA patients were higher than of healthy controls. The level of Ang-2 that differentiates between KLA patients and healthy controls is shown as a *dotted line*. **b** Receiver operating characteristic (ROC) curve for Ang-2 in KLA patients versus controls. **c** Ang-2 levels in the serum of KHE + KMP patients are higher than of healthy controls. The level of Ang-2 that differentiates between KHE + KMP patients and healthy controls is shown as a dotted line. **d** Receiver operating characteristic (ROC) curve for Ang-2 in KHE + KMP patients versus controls

logistic forms for three categories, respectively. Figure 5 shows Log Ang-1 and Log Ang-2 values for GLA, KLA and KHE + KMP patients. The contour lines separate disease conditions into two-dimensional regions in Ang-1 and Ang-2 defined by the above model. The two prediction functions jointly discriminate three groups with current patient data set. The misclassification rate is below 5%.

Blood measurements in patients receiving sirolimus therapy

Ang-2 levels were measured in the serum from patients after 6 and 12 months on sirolimus therapy as well as baseline levels from the same patients. Figure 6a shows that the levels of Ang-2 were lower in KLA patients ($n = 5$; patients for whom samples were available at all three times points) after both 6 and 12 months of sirolimus ($P < 0.0001$) which is consistent with these patients showing a partial clinical response [3]. While still higher than levels in healthy controls, the Ang-2 levels in the serum of KLA patients decreased to 23% (CI 0–69%) and 26% (CI 5–47%) of pre-treatment levels after 6 and 12 months on sirolimus. Serum levels of Ang-2 decreased to 11% (CI 7–16%) and 9% (CI 5–14%) of pre-treatment levels in the KHE + KMP patients ($n = 6$; patients for whom samples were available at all three times points) at 6 and 12 months ($P < 0.0001$; Fig. 6b) which is consistent with all of these patients showing a partial clinical response [3].

Discussion

We have identified Ang-2 and Ang-1 as novel biomarkers in patients with KLA and KHE + KMP. Ang-2 levels in KLA and KHE + KMP patients were highly and

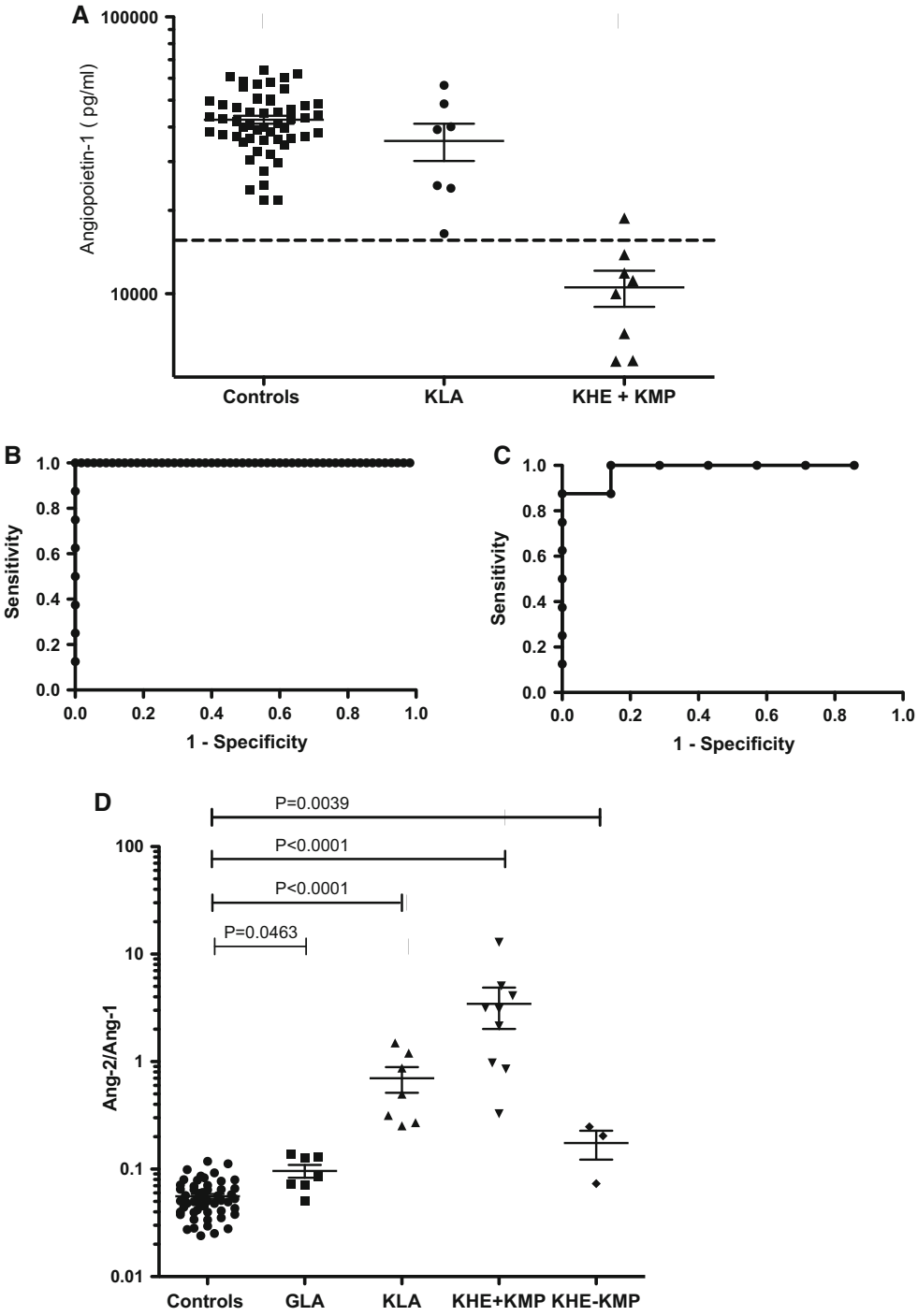

Fig. 4 Evaluation of Ang-1 and the ratio of Ang-2/Ang-1 as serum biomarkers in GLA, KLA and KHE + KMP patients. Ang-1 and Ang-2 levels were measured by ELISA in the serum of patients and controls. **a** Ang-1 levels in the serum of KLA and KHE + KMP patients compared to healthy controls. Ang-1 levels in the serum of KHE + KMP patients were lower than of controls. The level of Ang-1 that differentiates between KHE + KMP patients and controls is shown as a dotted line. **b** Receiver operating characteristic (ROC) curve for Ang-1 in KHE + KMP patients versus controls. **c** Receiver operating characteristic (ROC) curve for Ang-1 in KHE + KMP patients versus KLA patients. **d** Ratio of Ang-2 to Ang-1 levels is shown for GLA, KLA and KHE + KMP patients compared to controls. The Ang-2/Ang-1 ratio was highest in KHE + KMP patients and significantly different from KLA, GLA and controls

consistently elevated compared to the controls. The area under the curve (AUC) in the receiver operating curve analysis of Ang-2 levels in the KLA and KHE + KMP patients was 1.0, indicating that Ang-2 has strong discriminating power as a biomarker for these patients compared to controls [28]. While Ang-2 levels were elevated, serum Ang-1 levels were lower in KHE + KMP patients compared to healthy controls. To the best of our knowledge, no diagnostic data for these biomarkers have been reported before in these patient groups.

Ang-1 is produced by perivascular cells that surround vessels. Ang-2 is produced and stored by endothelial cells and then released in response to inflammatory stimuli. While Ang-2 is a weaker activator of Tie2 receptor signaling than Ang-1 and so may attenuate stronger activation by Ang-1, it can also act as a Tie2 agonist on lymphatic and

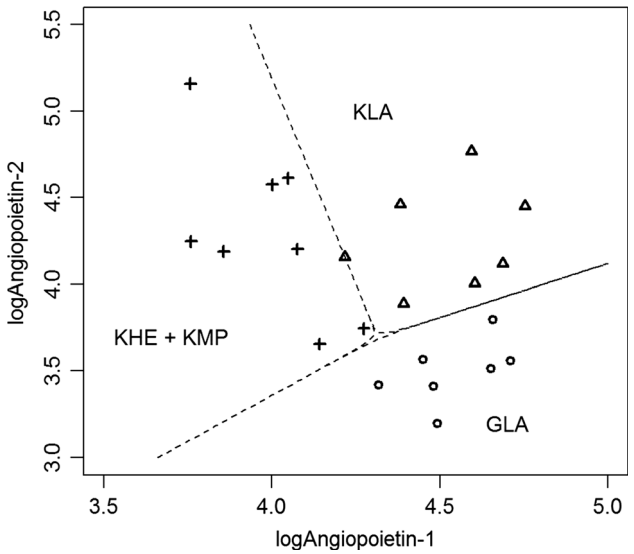

Fig. 5 Multivariable multinomial logistic analysis of Ang-1 and Ang-2 levels in GLA, KLA and KHE + KMP patients. Multivariable multinomial logistic analysis was carried out to assess the joint impact of Ang-2 and Ang-1 as biomarkers on disease status. Due to the skewness of Ang-1 and Ang-2 measurements, they are analyzed on log scale in the model. The multivariable logistic regression analysis model discriminates among three conditions, GLA, KLA and KHE + KMP, based on Log of baseline Ang-2 and Ang-1 levels. The contour lines shown are 50% predicted probability lines among three groups

tumor vessels stimulating lymphangiogenesis/angiogenesis. The role of Ang-2 and Ang-1 in the pathogenesis of these diseases is unclear; however, there is increasing interest in Ang-2 in a number of diseases, including cancers, inflammation and vascular pathologies [26]. While there are clinical, imaging and histologic distinguishing features between GLA, KLA and KHE that contribute to diagnosis, the abnormal vasculature in each diagnosis is lymphatic and angiopoietins are known to regulate lymphatic development and function [29–31]. Lymphatic development and function is abnormal in Ang-2 null mice, including lymphatic patterning and ectopic association of lymphatic vessels with smooth muscle cells, chylous ascites and frequently death before 2 weeks of age [30, 31]. Interestingly, insertion of the Ang-1 gene into the Ang-2 gene locus rescued the lymphatic but not the blood vascular abnormalities of Ang-2 null mice, suggesting that Ang-1 and Ang-2 have redundant roles in lymphatic but not blood vessels. Ang-1 can inhibit the permeability-enhancing effects and inflammation induced by a number of stimuli including VEGF and so has been shown to inhibit vascular leak in a number of in vivo models [29]. Ang-2 can regulate acute endothelial cell responses and activation by competing with Ang-1 and may counteract Ang-1-mediated endothelial stabilization. Ang-1 and Ang-2 likely have opposing effects, with Ang-2 promoting vascular leakiness in inflammation and Ang-1 anti-inflammatory suppressing vascular permeability and promoting normal function [26]. Ang-2 levels are elevated in a number of diseases characterized by increased vascular permeability/edema, and the ratio of Ang-2 to Ang-1 has been reported to be a predictor of acute lung injury in patients [32] and may contribute to pulmonary vascular leak in sepsis [33]. These concepts are supported by studies of heterozygous Ang-2 mice that are protected against vascular leak and in acute lung and kidney injury sepsis models and increased survival compared to wild types [29]. In contrast, Ang-1 administration reduced lung injury and increased survival in these models suggesting it has a complementary role and potential as a

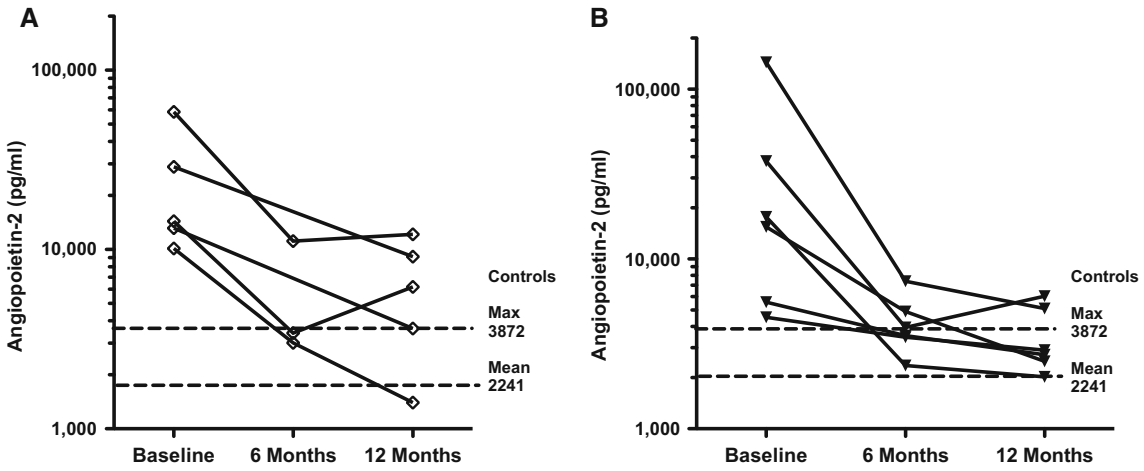

Fig. 6 Levels of angiopoietin-2 (Ang-2) and angiopoietin-1 (Ang-1) in the serum of KLA and KHE + KMP patients prior to and with sirolimus treatment. Ang-2 and Ang-1 levels were measured in the serum of patient groups before treatment (baseline) and then after 6 and 12 months on sirolimus. **a** Ang-2 levels in the serum of KLA patients compared to the maximum and mean level of Ang-2 in controls. **b** Ang-2 levels in the serum of KHE + KMP patients (KHE + KMP) compared to the maximum and mean level of Ang-2 in controls

therapeutic. Similarly, in diabetic retinopathy models in mice, loss of function of Ang-2 improved and increases in Ang-2 worsened vascular disease. Ang-1 also protected diabetic mice, and conditional loss of function led to more severe nephropathy. In KLA and KHE + KMP patients, the serum Ang-2 levels were 5-fold to 8-fold higher than in patients with Clarkson disease, a systemic capillary leak syndrome [34]. Ang-2 levels in the Clarkson patients correlated with episodic exacerbations, and serum from these patients caused endothelial leak in vitro that was attenuated by inhibiting angiopoietins but not VEGF.

Ang-2 levels are also elevated in many human cancers and correlate with increased tumor vascularization, metastasis and poor patient survival [reviewed in 29]. Blocking Ang-2 in tumor models in mice reduced angiogenesis, lymphangiogenesis, metastasis and overall tumor growth. In addition, a combination of Ang-2 and VEGF blocking strategies increased efficacy in preclinical models compared to one therapy alone. A study by Yu et al. [35] implicated Tie2 and the angiopoietins in the pathogenesis of infantile hemangiomas. In addition to VEGF receptors (Flk-1 and Flt-1), Tie1 and Tie2 as well as Ang-2 were strongly expressed in hemangioma-derived endothelial cells and hemangioma tissue. Tie2 expression and cellular responsiveness to Ang-1 were enhanced in most of the hemangioma-derived endothelial cells, whereas Ang-2 expression appeared to be dysregulated. At present, it is unclear what role Ang-2 is playing in the pathogenesis of KLA or KHE + KMP patients. However, given the very high levels in the blood of these patients and the emerging body of information supporting a role for dysregulated angiopoietins in a number of other diseases with vascular pathology it seems reasonable to speculate that increased Ang-2 from endothelial cells in lymphatic anomalies could have paracrine effects in the lesions, while the contribution of Ang-2 in blood is unclear. It is possible that increases in circulating Ang-2 in the KHE + KMP patients might be related to the coagulopathy or the causes of the coagulopathy although the exact underlying mechanism is unclear. KHE patients with KMP have a much higher risk of morbidity and mortality than those without KMP and the tumor becomes progressively infiltrative leading to multiorgan system failure if the KMP does not resolve. High-risk KLA patients usually have a significant coagulopathy similar to KMP; thus, it is not surprising there is an increase in circulating Ang-2 in these patients.

Our study did not find any differences in VEGF-A and VEGF-D in any of the patient groups. However, VEGF-C levels were lower in KHE + KMP patients. Ang-1 levels were also reduced in the KHE + KMP patients (but not KHE − KMP), and since these factors are released by platelets when they are activated it is possible that these lower levels may be due to platelet effects. Platelet counts were significantly lower in the KHE + KMP patients, likely due to platelet trapping in their lesions. Analysis of VEGF-C and Ang-1 levels did show significant correlation with platelet counts in GLA, KLA and KHE + KMP patients. Therefore, it is possible that reductions in serum Ang-1 and VEGF-C levels in KHE + KMP patients could be due to the coagulopathy although we cannot rule out other contributing pathologies.

Platelet counts and other blood levels were measured in the blood of patients at baseline and then after 6 and 12 months of sirolimus therapy and have been reported [3]. Platelet counts were previously reported to be below normal range in the KHE + KMP patients at baseline but increased after 6 and 12 months on sirolimus [3]. Ang-2 levels decreased with sirolimus treatment in most KLA and all KHE + KMP patients showing a clinical response to sirolimus [3]. Whether Ang-2 and Ang-1 predict response to sirolimus is unclear as all of the patients in our study showed a significant partial response to sirolimus [3]. In addition, whether Ang-2 and Ang-1 levels correlate with the disease course, exacerbations or disease severity is unclear from our data. A rigorous disease severity scoring system needs to be developed for these complicated vascular anomalies. Further study will be needed to assess the correlation of these biomarkers with disease severity to determine whether these might be useful to assess disease status, disease exacerbation and response to therapy.

Our study suggests that Ang-2 and Ang-1 may be biomarkers for KLA and KHE + KMP patients; however, there are limitations to this study. Most notably is the small patient cohort size. Some of the nonsignificant comparisons may be the results of low statistical power. In addition, we restricted comparisons of biomarker levels in the disease groups mostly to comparisons with control levels. In clinical practice, diagnostic tests are usually used to discriminate between different diseases, not to discriminate between patients that are ill and healthy controls and so the diagnostic utility of Ang-2 and Ang-1 may be overestimated. Some high values of AUC in ROC may also be susceptible to fluctuation when more patient data are introduced.

In the results, we report a multinomial logistic regression model using the Log of Ang-2 and Ang-1 levels was able to classify GLA, KLA and KHE + KMP patients. However, it is important to note that this applies only to these three patients groups and that our analysis did not include patients with other types of lymphatic anomalies, and so if this analysis was to be applied to all patients, there is a risk for misclassifying patients belonging to other disease groups. Therefore, the utility of this model is limited and needs to interpreted with caution. Further analysis of Ang-2 and Ang-1 levels in the blood from more patients is needed to verify whether this model and the ROC data

hold true. In the future, studies are also needed to discern whether these angiopoietins are purely biomarkers or are involved in the pathogenesis of these diseases. This latter possibility is especially exciting as it might lead to the development of new therapeutic targets for these patients.

Acknowledgements The authors would like to thank Tricia Pastura for assistance with the ELISA.

Funding Grant funding support was received from the Federal Drug Administration (5RO1FD003712-04) and the Lymphatic Malformation Institute.

References

1. Wassef M, Blei F, Adams D, Alomari A, Baselga E, Berenstein A, Burrows P, Frieden IJ, Garzon MC, Lopez-Gutierrez JC, Lord DJ, Mitchel S, Powell J, Prendiville J, Vikkula M, Board I, Scientific C (2015) Vascular anomalies classification: recommendations from the international society for the study of vascular anomalies. Pediatrics 136(1):e203–e214. doi:10.1542/peds.2014-3673
2. Marler JJ, Fishman SJ, Kilroy SM, Fang J, Upton J, Mulliken JB, Burrows PE, Zurakowski D, Folkman J, Moses MA (2005) Increased expression of urinary matrix metalloproteinases parallels the extent and activity of vascular anomalies. Pediatrics 116(1):38–45. doi:10.1542/peds.2004-1518
3. Adams DM, Trenor CC III, Hammill AM, Vinks AA, Patel MN, Chaudry G, Wentzel MS, Mobberley-Schuman PS, Campbell LM, Brookbank C, Gupta A, Chute C, Eile J, McKenna J, Merrow AC, Fei L, Hornung L, Seid M, Dasgupta AR, Dickie BH, Elluru RG, Lucky AW, Weiss B, Azizkhan RG (2016) Efficacy and safety of sirolimus in the treatment of complicated vascular anomalies. Pediatrics 137(2):1–10. doi:10.1542/peds.2015-3257
4. Croteau SE, Kozakewich HP, Perez-Atayde AR, Fishman SJ, Alomari AI, Chaudry G, Mulliken JB, Trenor CC III (2014) Kaposiform lymphangiomatosis: a distinct aggressive lymphatic anomaly. J Pediatr 164(2):383–388. doi:10.1016/j.jpeds.2013.10.013
5. Fernandes VM, Fargo JH, Saini S, Guerrera MF, Marcus L, Luchtman-Jones L, Adams D, Meier ER (2015) Kaposiform lymphangiomatosis: unifying features of a heterogeneous disorder. Pediatr Blood Cancer 62(5):901–904. doi:10.1002/pbc.25278
6. Safi F, Gupta A, Adams D, Anandan V, McCormack FX, Assaly R (2014) Kaposiform lymphangiomatosis, a newly characterized vascular anomaly presenting with hemoptysis in an adult woman. Ann Am Thorac Soc 11(1):92–95. doi:10.1513/AnnalsATS.201308-287BC
7. Trenor CC III, Chaudry G (2014) Complex lymphatic anomalies. Semin Pediatr Surg 23(4):186–190. doi:10.1053/j.sempedsurg.2014.07.006
8. ISSVA Classification of Vascular Anomalies ©2014 International Society for the Study of Vascular Anomalies Available at http://www.issva.org/classification. Accessed April 2014
9. Hammill AM, Wentzel M, Gupta A, Nelson S, Lucky A, Elluru R, Dasgupta R, Azizkhan RG, Adams DM (2011) Sirolimus for the treatment of complicated vascular anomalies in children. Pediatr Blood Cancer 57(6):1018–1024. doi:10.1002/pbc.23124
10. Requena L, Kutzner H (2013) Hemangioendothelioma. Semin Diagn Pathol 30(1):29–44. doi:10.1053/j.semdp.2012.01.003
11. Croteau SE, Liang MG, Kozakewich HP, Alomari AI, Fishman SJ, Mulliken JB, Trenor CC III (2013) Kaposiform hemangioendothelioma: atypical features and risks of Kasabach-Merritt phenomenon in 107 referrals. J Pediatr 162(1):142–147. doi:10.1016/j.jpeds.2012.06.044
12. Wang Z, Li K, Dong K, Xiao X, Zheng S (2015) Successful treatment of Kasabach-Merritt phenomenon arising from Kaposiform hemangioendothelioma by sirolimus. J Pediatr Hematol Oncol 37(1):72–73. doi:10.1097/MPH.0000000000000068
13. Lackner H, Karastaneva A, Schwinger W, Benesch M, Sovinz P, Seidel M, Sperl D, Lanz S, Haxhija E, Reiterer F, Sorantin E, Urban CE (2015) Sirolimus for the treatment of children with various complicated vascular anomalies. Eur J Pediatr 174(12):1579–1584. doi:10.1007/s00431-015-2572-y
14. Jahnel J, Lackner H, Reiterer F, Urlesberger B, Urban C (2012) Kaposiform hemangioendothelioma with Kasabach-Merritt phenomenon: from vincristine to sirolimus. Klin Padiatr 224(6):395–397. doi:10.1055/s-0032-1323823
15. Kai L, Wang Z, Yao W, Dong K, Xiao X (2014) Sirolimus, a promising treatment for refractory Kaposiform hemangioendothelioma. J Cancer Res Clin Oncol 140(3):471–476. doi:10.1007/s00432-013-1549-3
16. Blatt J, Stavas J, Moats-Staats B, Woosley J, Morrell DS (2010) Treatment of childhood kaposiform hemangioendothelioma with sirolimus. Pediatr Blood Cancer 55(7):1396–1398. doi:10.1002/pbc.22766
17. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000) Vascular-specific growth factors and blood vessel formation. Nature 407(6801):242–248. doi:10.1038/35025215
18. Mallory BP, Mead TJ, Wiginton DA, Kulkarni RM, Greenberg JM, Akeson AL (2006) Lymphangiogenesis in the developing lung promoted by VEGF-A. Microvasc Res 72(1–2):62–73. doi:10.1016/j.mvr.2006.05.002
19. Secker GA, Harvey NL (2015) VEGFR signaling during lymphatic vascular development: from progenitor cells to functional vessels. Dev Dyn 244(3):323–331. doi:10.1002/dvdy.24227
20. Karpanen T, Alitalo K (2008) VEGF-D: a modifier of embryonic lymphangiogenesis. Blood 112(5):1547–1548. doi:10.1182/blood-2008-06-159343
21. Enholm B, Karpanen T, Jeltsch M, Kubo H, Stenback F, Prevo R, Jackson DG, Yla-Herttuala S, Alitalo K (2001) Adenoviral expression of vascular endothelial growth factor-C induces lymphangiogenesis in the skin. Circ Res 88(6):623–629
22. Coso S, Bovay E, Petrova TV (2014) Pressing the right buttons: signaling in lymphangiogenesis. Blood 123(17):2614–2624. doi:10.1182/blood-2013-12-297317
23. Young LR, Inoue Y, McCormack FX (2008) Diagnostic potential of serum VEGF-D for lymphangioleiomyomatosis. N Engl J Med 358(2):199–200. doi:10.1056/NEJMc0707517
24. Young LR, Vandyke R, Gulleman PM, Inoue Y, Brown KK, Schmidt LS, Linehan WM, Hajjar F, Kinder BW, Trapnell BC, Bissler JJ, Franz DN, McCormack FX (2010) Serum vascular endothelial growth factor-D prospectively distinguishes lymphangioleiomyomatosis from other diseases. Chest 138(3):674–681. doi:10.1378/chest.10-0573
25. Karpanen T, Makinen T (2006) Regulation of lymphangiogenesis-from cell fate determination to vessel remodeling. Exp Cell Res 312(5):575–583. doi:10.1016/j.yexcr.2005.10.034
26. Thurston G, Daly C (2012) The complex role of angiopoietin-2 in the angiopoietin-tie signaling pathway. Cold Spring Harb Perspect Med 2:a006550. doi:10.1101/cshperspect.a006650
27. Venables WN, Ripley BD (2002) Modern applied statistics with S, 4th edn. Springer, New York, ISBN:0-387-95457-0
28. Zweig MH, Campbell G (1993) Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 39(4):561–577

29. Eklund L, Saharinen P (2013) Angiopoietin signaling in the vasculature. Exp Cell Res 319(9):1271–1280. doi:10.1016/j.yexcr.2013.03.011
30. Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D, Suri C, Campochiaro PA, Wiegand SJ, Yancopoulos GD (2002) Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. Dev Cell 3(3):411–423
31. Dellinger M, Hunter R, Bernas M, Gale N, Yancopoulos G, Erickson R, Witte M (2008) Defective remodeling and maturation of the lymphatic vasculature in Angiopoietin-2 deficient mice. Dev Biol 319(2):309–320. doi:10.1016/j.ydbio.2008.04.024
32. Ong T, McClintock DE, Kallet RH, Ware LB, Matthay MA, Liu KD (2010) Ratio of angiopoietin-2 to angiopoietin-1 as a predictor of mortality in acute lung injury patients. Crit Care Med 38(9):1845–1851. doi:10.1097/CCM.0b013e3181eaa5bf
33. Parikh SM, Mammoto T, Schultz A, Yuan HT, Christiani D, Karumanchi SA, Sukhatme VP (2006) Excess circulating angiopoietin-2 may contribute to pulmonary vascular leak in sepsis in humans. PLoS Med 3(3):e46. doi:10.1371/journal.pmed.0030046
34. Xie Z, Ghosh CC, Patel R, Iwaki S, Gaskins D, Nelson C, Jones N, Greipp PR, Parikh SM, Druey KM (2012) Vascular endothelial hyperpermeability induces the clinical symptoms of Clarkson disease (the systemic capillary leak syndrome). Blood 119(18):4321–4332. doi:10.1182/blood-2011-08-375816
35. Yu Y, Varughese J, Brown LF, Mulliken JB, Bischoff J (2001) Increased Tie2 expression, enhanced response to angiopoietin-1, and dysregulated angiopoietin-2 expression in hemangioma-derived endothelial cells. Am J Pathol 159(6):2271–2280. doi:10.1016/S0002-9440(10)63077-5