Protocol

A prospective international observational study on Epithelioid Haemangioendothelioma 1/describing the clinical presentation, natural history, and treatment outcomes, 2/evaluating cytokines and hormones as biomarkers and 3/generating patient-derived preclinical models as a tool to assess the activity of anticancer agents and validate novel therapeutic targets

NCT 06680401

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A PROSPECTIVE INTERNATIONAL OBSERVATIONAL STUDY ON EPITHELIOID HEMANGIOENDOTHELIOMA: 1/DESCRIBING THE CLINICAL PRESENTATION, NATURAL HISTORY, AND TREATMENT OUTCOMES; 2/EVALUATING CYTOKINES AND HORMONES AS BIOMARKERS; 3/GENERATING PATIENT-DERIVED PRECLINICAL MODELS AS A TOOL TO ASSESS THE ACTIVITY OF ANTICANCER AGENTS AND VALIDATE NOVEL THERAPEUTIC TARGETS (POEM).

Short Title	POEM
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Methodology	Prospective, international, observational study
Type	No Profit
Laboratory sites	Molecular Pharmacology Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Via G A Amadeo, 42 20133 Milan, ITALY The Institute of Cancer Research, 123 Old Brompton Road, London, UK
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1. RATIONALE AND BACKGROUND

Epithelioid hemangioendothelioma (EHE) is an ultra-rare (incidence rate < 1/1,000,000 of population), translocated, vascular soft tissue sarcoma. It shows a peak of incidence in the 4th decade of life, and it is more commonly diagnosed in females, with reported disease onset during pregnancy[1,2].

Two specific translocations have been identified in EHE, representing an hallmark in diagnosis today: the fusion of Transcriptional Co-activator with a PDZ-motif (*TAZ*) with Calmodulin Binding Transcription Activator 1 (*CAMTA1*) genes (*TAZ-CAMTA1*) which is present in almost 90% of cases, and the fusion of Yes-associated Protein (*YAP*) and Transcription Factor E3 (*TFE3*) (*YAP-TFE3*), which can be found in around 10% of the patients[3,4]. YAP and TAZ are well-defined downstream effectors in the Hippo pathway. Forced activation of YAP/TAZ is thought to drive EHE and contribute to key aspects of the cancer phenotype, including metastasis and fibrosis[5].

EHE most often presents as multifocal or metastatic at diagnosis, with lung, liver and bone being the most commonly involved sites. The clinical course ranges from cases naturally stable over time to those highly aggressive and rapidly fatal. Pleural effusion, lymph node metastases and pathologic features (nuclear pleomorphism, mitotic figures and presence of necrosis) have been reported to be associated with a worse outcome, but biological and molecular predictors are still lacking[6]. In particular, there is a subgroup of EHE presenting with serosal involvement, typically associated with chronic mild fever, weight loss, asthenia, anorexia, severe disease- related pain, (more responsive to anti- inflammatory pain killers than morphine), and dyspnoea which seems to perform very poorly. The biological basis sustaining this presentation is completely unknown.

As of today, there are no reports available in the literature providing a comprehensive description of the specific radiological features of EHE, both for primary and metastatic disease at different sites, and their potential prognostic role has not been explored. In addition,

there are no published data to indicate the optimal routine follow-up policy of surgically treated EHE patients with localised disease and the routine follow-up schedules differ across institutions. The appropriate frequency of imaging in cases suffering from distant metastases is also left to be determined.

Also, the definition of radiological progression and the assessment of treatment responsein EHE remain a major challenge. The appearance or worsening of serosal effusion, the changes in serosal involvement and the limited increase in size over a short-time interval in slow-growing variants are not properly captured by Response Evaluation Criteria for Solid Tumor (RECIST) definitions for disease progression. This makes the use of such criteria unsatisfactory in this complex disease and could potentially lead to a delay in progression recognition and treatment start. Similarly, being frequently observed in EHE under treatment, improvement of serosal effusion, reduction in size <30%, and correlation between radiology and symptoms should be considered when assessing treatment response.

Surgery is the mainstay of care in the local setting. Active surveillance can be a reasonable strategy for patients with naturally stable or asymptomatic, slowly progressive disease, reserving medical treatment to symptomatic or progressive cases[7].

Data on conventional chemotherapy in advanced EHE are limited to case reports and single-institution experiences and suggest a limited role for the drugs commonly used in adult-type soft tissue sarcomas[8,10]. Signs of activity have been reported with the use of anti-angiogenics, including pazopanib, sorafenib, bevacizumab, alone or in combination with chemotherapy, and apatinib[11-14]. Due to the unique natural history of the disease, the value of antiangiogenics and/or immunomodulatory agents has also been explored, with responses described with sirolimus, thalidomide, interferon, and celecoxib[15-18].

In absence of any available active treatment, EHE is a neglected disease, and the identification of new potentially active compounds, especially for patients affected by the more aggressive EHE variant is critically important. To this end, it is of major importance to identify

the mechanism of disease progression, the “inflammatory-like” disease presentation, and the prevalence of the disease in young females.

Several lines of evidence have highlighted the significance of inflammation at the local and/or systemic level in human tumor pathobiology. Indeed, inflammation can influence tumor progression, metastasis and therapeutic outcome by establishing a tumor supportive immune microenvironment. These processes are mediated through a variety of cytokines and hormones that exert their biological actions either locally or distantly via systemic circulation. Estrogen signaling is mediated *via* several receptor proteins. In addition to the classical ERα and ERβ[19], the membrane-bound G-protein coupled estrogen receptor (GPER) mediates both the genomic and non-genomic effects of estrogen and has been implicated in the development of other tumors such as breast cancer[20]. Interestingly, GPER stimulation activates YAP and TAZ as key effectors of the Hippo pathway[21]. Insulin-like growth factor-1(IGF-1) has also been shown to regulate GPER expression and function, suggesting a crosstalk between growth factors and ERs[22].

The availability of translatable preclinical models of human EHE, able to properly recapitulate tumor biology and response to treatment of the clinical tumors, appears instrumental for the development of innovative and effective treatments. Patient-derived xenograft (PDX) models preserve the original histomorphological and molecular characteristics of the originating clinical tumors. We previously demonstrated the consistency between preclinical data obtained on PDXs of different soft-tissue sarcoma histotypes (solitary fibrous tumor, epithelioid sarcoma and dedifferentiated liposarcoma) and clinical results concerning the activity of several cytotoxic and molecularly targeted drugs, providing novel insight into the antitumor effect of different combinations that was instrumental to design novel clinical trials[23-27].

MicroRNAs (miRNAs) are small non-coding RNAs that negatively regulate gene expression at the post-transcriptional level. Their proven deregulation in several types of human cancer,

and the possibility to be reliably detected in both tissue and bloodspecimens, have prompted the assessment of miRNAs as novel cancer biomarkers[21]. Noinformation is currently available on miRNA expression and function in EHE.

2. RESEARCH OBJECTIVES

2.1. Clinical research objectives

1. To provide a demographic description of the population affected by advanced EHE
2. To provide a description of clinical presentation, natural history, and treatment pattern in patients with advanced EHE.
3. To provide a description of tumour-related symptoms and their changes over time
4. To provide a tumor-related pain assessment
5. To prospectively identify clinical prognostic factors
6. To describe the radiological features of the disease (group A, B, C, D, E)
7. To correlate radiologic features with the outcome and the tested plasma levels of the cytokines and hormones (group A, B, C, D, E)
8. To assess radiologic response to systemic treatments by comparing different assessment criteria and, in particular, antitumor activity of sirolimus in patients affected by progressing EHE (group B, D)
9. To assess the proportion of patients in groups B and D who experience disease progression after discontinuing sirolimus, as well as their outcomes following sirolimus discontinuation or potential rechallenge.

2.2. *Translational research objectives*

1. To assess *i)* the longitudinal profiles of circulating cytokines, hormones, and miRNAs, and *ii)* the ERα, Erβ and GPER expression and the YAP/TAZ activation in tumor tissues, according to the clinical course of the disease. To this end the analysis will be conducted on the whole study patient population compared to healthy controls, and by stratifying EHE patients who will enter the study in 3 subgroups according to disease behavior (non- growing disease, slow- growing disease, highly- aggressive disease)

2. To generate preclinical tumor models (PDXs and cell lines) for comparatively assessing the activity of anticancer agents and inform the design of new clinical trials. EHE preclinical models will be also used for validating novel therapeutic targets.
3. To identify and validate novel biomarkers to inform patient management (prognosticators and predictors of response to medical agents) as well as potential therapeutic targets.

3. STUDY DESIGN

In this observational, prospective study all consecutive patients diagnosed with EHE treated at participating centers will be included. We will enroll a minimum number of 150 EHE patients, including:

- Group A: newly diagnosed, advanced (i.e., locally advanced and or metastatic), EHE, naturally stable disease on follow-up
- Group B: newly diagnosed, advanced, EHE with progressive disease requiring systemic treatment
- Group C: previously diagnosed, advanced, EHE already on active surveillance
- Group D: previously diagnosed, advanced, EHE already on systemic treatment
- Group E: localized EHE.

The details of the sub-studies designed to assess clinical research objectives 8 and 9, specifically the evaluation of sirolimus's antitumor activity in patients with progressing EHE and the outcomes following sirolimus discontinuation or potential rechallenge, are provided in Appendix 1 and 2, respectively.

The stratification according to disease behavior (non-growing disease, slow-growing disease, highly- aggressive disease) foreseen by the translational study will be done according to the following criteria:

a) “Non-growing disease”: absence of progressive disease over 12 months.

b) “Slow-growing disease”: evidence of progressive disease between 6 and 12 months and patient belonging to group D (provided that treatment was started in evidence on progressive disease).

c) “Highly-aggressive disease”: evidence of progressive disease within 6 months.

Progressive disease will be defined as:

c.1. Evidence of RECIST 1.1 progression

c.2. Any increase in tumor size of the known lesions (even not meeting RECIST 1.1 definition of progression) in association with worsening of at least two tumour related symptoms (tumour-related pain*, fever, weight loss, asthenia)

c.3. Evidence of progression evaluated by Serosal Changes and Outcomes Reporting (RESCORe) criteria and worsening of at least two tumour-related symptoms (tumour-related pain*, fever, weight loss, asthenia)

* Worsening of pain is defined accordingly to Pain Assessment Criteria (Appendix 3) as: at least a 30% increase in worst pain intensity (WPI) within the last week with no more than a 10% reduction in opioid/NSAID daily dose, and/or at least a 30% increase in opioid/NSAID daily dose with no more than a 1-point reduction in WPI and/or the development of new disease related pain with a WPI more than 4.

3.1. Pathological review

A centralized review of the pathological diagnosis will be required for all patients (group A-E) entering the study.

The following data will be recorded:

- Evidence of nuclear pleomorphism, mitotic figures and presence of necrosis
- Immunohistochemical staining for CAMTA1

- Molecular testing for WWTR1-CAMTA1 and / or YAP-TFE3
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3.2. Clinical assessments and data collection

3.2.1. Baseline

The following information will be recorded for all patients (group A-E) entering the study atthe baseline:

- Demographics – patient ID, DOB, gender, data of diagnosis; childbearing potential, use of oral contraception or post-menopausal hormonal therapies for female; date of last menstrual cycle; history of autoimmune disease, malignancies, allergy, prolonged immunosuppressive treatment
- Data on disease extent – single lesion, metastatic multifocal; metastatic multicentric
- Data on staging – evidence of primary disease, lymph nodal metastases, liver involvement, lung involvement, bone involvement, other sites of metastatic disease, evidence of serosal involvement, evidence of serosal effusion
- Data on treatment – surgery, active surveillance, radiation therapy (site, dose), medical treatment (type), other
- Data on symptoms – pain (NRS), weight loss in the last 3 months (%), episodes of temperature (>37.5°) in the last 4 weeks, presence of asthenia in the last 4 weeks, anorexia in the last 4 weeks, night sweat in the last 4 weeks, and dyspnoea in the last 4 weeks.
- Pain assessment visit will be performed if a WPI >4 on a scale from 0 to 10 is reported, as indicated in “Pain Assessment” (Section 3.2.4). If WPI is less than 4, data regarding pain using BPI will be recorded
- Concomitant medications
- Physical examination (including BMI)
- Quality of life assessment by the ESAS-r questionnaire

The following tests will be performed for all patients (group A-E) at baseline according to standard of care:

- Blood tests (complete blood count, AST, ALT, GGT, ALP, total bilirubin, creatinine, BUN, sodium, potassium, calcium, PT, PTT, fibrinogen, glycaemia, total cholesterol, LDL, HDL, triglycerides, screening for HBV and HCV); plasmatic B- HCG (for female); GDF-15 assay
- Echocardiogram and ECG
- Radiological assessment: CT scan, MRI, bone scan (as clinically indicated)
- Gynaecological assessment: blood tests (FSH, LH, progesterone, estradiol, prolactin), clinical assessment, US

3.2.2. Clinical monitoring

Patient on active surveillance (group A and C) will be monitored every 3-4 months for the first 2 years and every 6 months thereafter as follows:

- Data on symptoms – pain (NRS), weigh loss in the last 3 months (%), episodes of temperature (>37.5°) in the last 4 weeks, presence of asthenia in the last 4 weeks, anorexia in the last 4 weeks, night sweat in the last 4 weeks, dyspnoea in the last 4weeks. Following Amendment 6 (22/05/2025), pain will be also assessed by disease-specific criteria, as described in Appendix 3.
- Pain assessment visit will be performed if a WPI >4 on a scale from 0 to 10 is reported, as indicated in “Pain Assessment” paragraph. If WPI is less than 4, data regarding pain using BPI will be recorded
- Total daily dosages of pain medications, including the cumulative dose of rescue pain medication used.
- Physical examination – BMI
- Quality of life assessment by the ESAS-r questionnaire

- Blood tests (complete blood count, AST, ALT, GGT, ALP, total bilirubin, creatinine, BUN, sodium, potassium, calcium, PT, PTT, fibrinogen, glycaemia, total cholesterol, LDL, HDL, triglyceride); plasmatic B-HCG (for female); GDF-15 assay (as clinically indicated)
- Radiological assessment: CT scan, MRI, bone scan (as clinically indicated)

Patient on active treatment (group B and D), including patients receiving sirolimus, will be monitored clinically after 2 weeks from treatment start, then every 4 weeks (for the first 3 months) and every 8 weeks thereafter as follows:

- Data on symptoms — pain (NRS), weight loss in the last 3 months (%), episodes of temperature (>37.5°) in the last 4 weeks, presence of asthenia in the last 4 weeks, anorexia in the last 4 weeks, night sweat in the last 4 weeks, dyspnoea in the last 4 weeks. Following Amendment 6 (22/05/2025), pain will be also assessed by disease-specific criteria, as described in Appendix 3.
- Pain assessment visit will be performed if a WPI >4 on a scale from 0 to 10 is reported, as indicated in “Pain Assessment” paragraph. If WPI is less than 4, data regarding pain using BPI will be recorded
- Total daily dosages of pain medications, including the cumulative dose of rescue pain medication used.
- Childbearing potential (females) and length of menstrual cycle (fertile females)
- Physical examination – BMI
- Quality of life assessment by the ESAS-r questionnaire
- Blood tests (complete blood count, AST, ALT, GGT, ALP, total bilirubin, creatinine, BUN, sodium, potassium, calcium, PT, PTT, fibrinogen, glycaemia, total cholesterol, LDL, HDL, triglycerides); plasmatic B-HCG (for female); GDF-15 assay (as clinically indicated)
- Radiological assessment (CT scan, MRI, bone scan as clinically indicated) will be performed every 3-4 months. For patients receiving sirolimus, radiological assessments

will be performed every 12 weeks (+/- 1 week).

- Gynecological assessment (FSH, LH, progesterone, estradiol, prolactin, clinical assessment, US) will be performed every 6-8 months.
- For patients on sirolimus, sirolimus plasma levels will be monitored after 2 weeks and 4 weeks from treatment start and every 4 weeks thereafter. Sirolimus plasma level target will be ≥ 15 ng/mL.
- Reason for oncologic treatment discontinuation (e.g. disease progression, unacceptable toxicity, patient's refusal to continue treatment, etc) will be collected.

Patients with localised disease (group E) will be monitored every 3-4 months for the first 2 years, every 6 months up to year 5 and yearly thereafter, as follows:

- Data on symptoms — pain (NRS), weight loss in the last 3 months (%), episodes of temperature (>37.5°) in the last 4 weeks, presence of asthenia in the last 4 weeks, anorexia in the last 4 weeks, night sweat in the last 4 weeks, dyspnoea in the last 4weeks. Following Amendment 6 (22/05/2025), pain will be also assessed by disease-specific criteria, as described in Appendix 3.
- Total daily dosages of pain medications, including the cumulative dose of rescue pain medication used.
- Physical examination – BMI
- Quality of life assessment by the ESAS-r questionnaire
- Blood tests (complete blood count, AST, ALT, GGT, ALP, total bilirubin, creatinine, BUN, sodium, potassium, calcium, PT, PTT, fibrinogen, glycaemia, total cholesterol, LDL, HDL, triglycerides); plasmatic B-HCG (for female); GDF-15 assay (as clinically indicated)
- Radiological assessment: CT scan, MRI, bone scan (as clinically indicated)

3.2.3. Radiologic assessment

Radiologic images (CT and/or MRI scans) will be uploaded on to the XNAT platform and centrally reviewed by a sarcoma dedicated radiologist.

XNAT is a cross-platform, open-source tool designed to support imaging research. Core functions include import, archiving, processing, annotation and secure distribution of imaging and related study data.

The centralized radiological review will 1) describe the radiological features of the disease (group A, B, C, D, E), 2) assess response to systemic treatments by RECIST, by CHOI criteria and by RESCORe criteria (group B, D), presented in Appendix 4.

3.2.4. Pain assessment

A pain assessment visit will be performed if a WPI >4 on a scale from 0 to 10 is reported. When possible, pain assessment will be conducted by a palliative care specialist or a pain therapist. Starting from Amendment 6 (22/05/2025), during this evaluation, the clinician will address the following aspects:

- relationship between pain and the disease. If uncertainty exists regarding the pain's origin, further investigation with appropriate radiological imaging will be considered;
- pain characteristics. The presence of nociceptive, neuropathic, and/or nociplastic pain will be determined using validated tools (i.e. EAPC/IASP algorithm for neuropathic pain[28]) in order to tailor appropriate therapy;
- pain intensity. The clinician will access current pain intensity, average pain intensity of the last week, and WPI of the last week, using a NRS ranging from 0 to 10 during the visit;
- presence of a pain syndrome. Syndromes will be evaluated according to established criteria, such as those defined by the IASP Task Force[29].

If disease-related WPI >4 is present, tumor response to pain treatment will also be evaluated

with Pain Assessment Criteria (Appendix 3).

During a pain assessment visit, the reported pain will serve as the reference for evaluating pain intensity and its link to the disease. If unavailable, follow-up questionnaire data will be used. For patients evaluated before amendment 6, data of pain will be retrospectively retrieved, whenever feasible. The primary source will be the pain assessment visit conducted by a palliative care specialist and/or a pain therapist that is closest to the oncological visit and/or radiological assessment, ensuring that the two assessments are no more than two weeks apart. If multiple pain assessment visits are available, priority will be given to the one closest to the corresponding oncological or radiological evaluation. If such a visit was not performed within the proposed time-frame, pain intensity and characteristics will be extracted from available follow-up questionnaires.

3.2.5. Progression

At the time of progression, the following data will be recorded, and the following tests performed for all patients (group A-D):

- Data on treatment choice – surgery, active surveillance, radiation therapy (site, dose), medical treatment (type), other
- Data on symptoms – pain (NRS), weight loss in the last 3 months (%), episodes of temperature (>37.5°) in the last 4 weeks, presence of asthenia in the last 4 weeks, anorexia in the last 4 weeks, night sweat in the last 4 weeks, dyspnoea in the last 4weeks.
- Pain assessment visit will be performed if a WPI >4 on a scale from 0 to 10 is reported, as indicated in “Pain Assessment” paragraph. If WPI is less than 4, data regarding pain using BPI will be recorded
- Total daily dosages of pain medications, including the cumulative dose of rescue pain medication used.
- Physical examination – BMI

- Quality of life assessment by the ESAS-r questionnaire
- Blood tests (complete blood count, AST, ALT, GGT, ALP, total bilirubin, creatinine, BUN, sodium, potassium, calcium, PT, PTT, fibrinogen, glycaemia, total cholesterol, LDL, HDL, triglycerides); plasmatic B-HCG (for female); GDF-15 assay
- Radiological assessment: CT scan, MRI, bone scan (as clinically indicated)
- Gynaecological assessment: blood tests (FSH, LH, progesterone, estradiol, prolactin), clinical assessment, US

4. STUDY POPULATION

We plan to include at least 150 patients (range: 150-200) in 72 months, followed by a follow-up time of 5 years.

4.1. Inclusion criteria

1) Histological diagnosis of EHE according to 2020 WHO classification, performed on biopsy or surgical specimen
2) Signed informed consent
3) Adequate patient compliance to treatment or follow up
4) No age limits

4.2. Exclusion criteria

1) Impossibility to ensure adequate compliance

5. TRANSLATIONAL STUDY

The translational part of the study will be carried out on independent case series, limited to INT, Milan, University of Torino (UNITO), and to Institute of Cancer Research (ICR)/Royal Marsden

Hospital (RMH), London:

INT-Milano and UNITO series: overall, blood (plasma and serum) samples from ≥ 50 molecularly confirmed EHE patients and FFPE tissues from at least 20 molecularly confirmed EHE patients among those in which the blood sample is taken will be collected. Blood samples will be collected from all consecutive molecularly confirmed EHE patients entering INT irrespective of the disease phase (group A-E). In addition to the baseline sample, for each patient longitudinal samples will be collected after 1 month from baseline, at 6 months and in case of evidence of progression. For patients starting a medical treatment, samples will be collected at baseline then after 2 weeks, 1 and 6 months of treatment and in case of disease progression.

Only patients with a pathologic diagnosis of EHE confirmed by the presence of either *WWTR1- CAMTA1* or *YAP-TAZ* fusion gene will be considered eligible for these analyses.

Blood samples from healthy individuals will be also collected for comparative purpose with EHE cases; gender and age will also be registered in order to perform adjusted comparative analyses.

For EHE patients undergoing surgical procedures, a tumor sample will be transplanted into immune-compromised mice to generate PDXs (Section 5.6). Corresponding cell lines will be then established following PDX disaggregation.

Clinical data of all EHE patients who will enter this study will be collected prospectively in a dedicated database, also recording samples for study-related translational research analysis, and progressively updated with patient outcome information.

ICR/ RMH - London series:

Similar to the INT series, blood and tissue samples will be collected from all patients who are treated at or are referred to the RMH. The expected number of patients diagnosed with

EHE at RMH is approximately 5-6 patients per year (approx. 18 over the 3-year period).

Mirroring the blood collection at INT, blood will be collected at the following time points:

Patients who undergo observation

- Diagnosis (Baseline Pre-treatment)
- Baseline +1month
- Baseline +6months
- Treatment Baseline

Patients who undergo a systemic therapy

- Treatment Baseline + 2 weeks
- Treatment Baseline + 1 month
- Treatment Baseline + 6 months
- Evidence of disease progression

RMH will also collect tissue alongside these patient groups including diagnostic FFPE andany excess surgical resection tissue (fresh frozen and FFPE). RMH will also collect samples from patients who are already being treated at RMH for EHE, to maximize sample numbers.

All blood and tissue will be stored in the UK's National EHE Biobank, the setup of which was funded by the EHE Rare Cancer Charity, and is situated at The Royal Marsden Hospital NHS Foundation Trust in line with HTA regulations and released for analysis when required.

ICR will also carry out CRISPR whole genome screen on PDX-derived cell lines generated at INT to identify new therapeutic targets for the disease.

The RMH will participate in the outlined research project as a hosted non-commercial study and will submit the protocol proposal to the UK regulatory authorities before commencing consent and tissue collection. Once the appropriate regulatory approvals are in place, the Human Tissue Manager based at RMH, who is part funded by the EHE Rare Cancer Charity, will consent any patients to this study and funding for this work is already covered within the percentage of funded whole time equivalent. In addition, samples collection can be undertaken by the unit's Biological Specimen Coordinator.

The study will be started on the INT case series (Training set, used for biomarkers discovery) and the circulating and/or tissue factors emerging as candidate biomarkers will be assessed on the UNITO (Training set) and ICR case series (Testing set) by using the same experimental approaches. If results generated in the Training set will identify specific biomarkers able to provide useful information for a specific subgroup of patients, the number of patients in that group will be enriched.

Based on an already established collaboration, the top candidates selected in the previous steps will be validated on an independent case series (Validation set) collected at MSKCC-New York.

5.1. Circulating cytokines (limited to INT, Milan, UNITO and ICR/ RMH, London)

Cytokines will be initially analysed in plasma samples from EHE patients by using a protein array able to simultaneously detect the expression of 105 different cytokines.

Successively, cytokines differentially expressed between patients and healthy donors, or between different groups of patients, will be assessed in the same plasma and serum samples usingspecific ELISA assays and the Elecsys test ® (vitro diagnostic immunoassay). Also, pleural and peritoneal effusion can be analyzed, when available. Following Amendment 6 (20/05/2025), this analysis will be performed only using Elecsys test ®

5.2. Circulating hormone profiles (limited to INT, Milan, UNITO, and ICR/RMH, London)

Serum concentrations of estradiol, estrone, estriol, progesterone, DHEAS, androstenedione, testosterone, dihydrotestosterone, luteinizing hormone, follicle- stimulating hormone, prolactin, sex hormone-binding globulin and IGF-1 will be determined by different kinds of immunoassays using commercial kits.

5.3. Circulating miRNA profiles (limited to INT, Milan, UNITO, and ICR/RMH, London)

The expression profiling of plasmatic miRNAs will be carried out by the qRT-PCR-based OpenArray Technology (which simultaneously evaluate the expression of 754 different miRNAs and 4 control RNAs in replicates).

5.4. Multi-omic analysis of EHE clinical samples (limited to INT, Milan, UNITO, and ICR/RMH, London)

Comprehensive genomic, transcriptomic and proteomic profiling of FFPE specimens WES analysis, RNA-seq analysis, metabolomics profile and mass spectrometry will be undertaken (30 samples combined from INT, UNITO and RMH). WES, RNA-seq and metabolomic profile will be performed at INT or UNITO. Mass spectrometry will be performed at the ICR using established protocols developed in the team. A small subset of tumors (N=4) from INT will undergo single-cell RNA-seq analysis and spatial transcriptomic analysis.

5.5. Immunohistochemistry

Immunostaining of ERα, phosphoERα, ERβ, GPER, YAP, AR, PR, phosphoYAP, TAZ, phosphoTAZ and aromatase will be performed on 5-μm thick FFPE sections using specific moAbs and standard immunohistochemical techniques.

Whenever possible, the expression of ERα, ERβ, AR and PR on PBMCs will be also detected by flow-cytometric analysis.

5.6. PDX model generation (limited to INT, Milan, and ICR/RMH, London)

Patients who will have surgery at INT will be considered for generation of a patient-derived xenograft. PDXs will be obtained by directly implanting freshly resected tumor pieces subcutaneously/orthotopically into immune-compromised (nude or SCID) mice and characterized for consistency with the originating clinical tumors in terms of histo-morphology, presence of specific translocations (WWTR1-CAMTA1 or YAP-TFE3), genomic

and transcriptomic profiles.

PDX will be then be mechanically or enzymatically disaggregated into single cells to establish cell lines.

5.7. CRISPR whole genome screen (limited to INT, Milan, and ICR/RMH, London)

A human genome-wide knockout CRIPSR library consisting of 90,709 single guide RNA (sgRNA) sequences to target 18,010 human genes, will be used to stably express a single sgRNA per cell in a cas9 expressing PDX-derived cell line.

6. STATISTICAL ANALYSIS

Descriptive statistics will be used to summarize patient and tumour characteristics. Contingency tables will be used to describe the associations between pairs of categorical variables. Multivariate association between clinical characteristics, such as symptoms at baseline and new symptoms during follow-up, baseline metastatic sites and sites of progressive disease, and treatments will be studied by applying cluster analysis, the results of which will be represented using heat map plots. The identified patient clusters will be compared with the 5 groups A-B-C-D-E defining the EHE diagnosis to better characterize disease heterogeneity.

Overall survival (OS) and progression-free survival (PFS) curves will be estimated with the Kaplan-Meier method in the 5 groups along with survival estimates at predefined endpoints (i.e., 6-, 12-, 18-, 24-month). We will also estimate the post- progression OS (ppOS) curves for patients who will develop progressive disease. Multivariable prognostic analyses will be performed using Cox models; due to the low number of cases and events the analyses will be performed by applying penalized likelihood methods. Variable selection will be performed beforehand by applying appropriate variable selection procedures within the framework of

Machine Learning algorithms for survival data (e.g. random forest) for inclusion in subsequent multivariable Cox models for OS and PFS.

Duration of response (DoR) will be computed with the Kaplan-Meier method as the difference between first assessment of response and disease progression or death whichever occurs first.

For DoR, OS, PFS, and ppOS, in the absence of the specific event of interest, the times will be censored at the date of last follow up where the patients were free from the event.

The details of the sub-studies designed to assess the evaluation of sirolimus's antitumor activity in patients with progressing EHE and the outcomes following sirolimus discontinuation or potential rechallenge are provided in Appendix 1 and 2, respectively.

For the translational study, the design and statistical analysis pipeline will be later established in detail. In general, the discovery phase will follow a case-control design for binary endpoints (e.g. response) and a nested-case control design for survival endpoints (e.g. progression-free survival or overall survival). Hence, statistical models taking into account the nature of paired data will be applied. To adjust for the sex and age heterogeneity, and other inhomogeneity factors between cases and controls we will estimate a propensity score (PS)[30], as a balancing score, a function of which will be used as weight in all the comparative analyses[31].

Unsupervised analysis of blood tests, cytokines, hormones, miRNA and immunohistochemistry data will be performed by applying appropriate methods of cluster analysis, the results of which will be represented using heatmap plots. We will also use appropriate Machine Learning algorithms for variable selection and model building to integrate multi-omics data into the aim of deriving prognostic signatures. To increase the statistical power, analysis of biomarkers will be performed comparing patients with higher risk tumors (in this protocol labelled as "Non-growing disease"):vs lower risk tumors (in this protocol labeled as "Slow-growing disease":plus "Highly- aggressive disease").

In the validation phases, appropriate analyses will be performed in coherence with the discovery phase design.

The analyses will be carried out using the SAS® and R software. We will consider a statistical test as significant when achieving a p value <0.05.

7. PROTECTION OF HUMAN SUBJECTS

This study will be conducted in accordance with the principle of World Medical Association Declaration of Helsinki, 2008.

7.1. Independent Ethics Committee (IEC) or Institutional Review Board (IRB)

Documented approval from the appropriate IEC or IRB will be obtained for all participating centers prior to study start. When necessary, an extension, amendment, or renewal of the IEC or IRB approval must be obtained and also forwarded to the study initiator, except for changes involving only logistical or administrative aspects of the study.

Before documentation of any patient data, informed consent should be given by the patient in writing. The investigator must explain to each subject the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits involved, and any potential discomfort. Each subject must be informed that participation in the study is voluntary and that he/she may withdraw from the study at any time and that withdrawal of consent will not affect his/her subsequent medical treatment or relationship with the treating physician. This informed consent should be given by means of a standard written statement, written in nontechnical language. The form must be signed and dated by the appropriate parties.

In countries where required by law or regulation, the investigator must have the IEC or IRB's written approval or favorable opinion of the written informed consent form(s) and any other written information to be provided to patients prior to the beginning of the observation.

The original, signed ICF for each subject will be verified by the investigators and kept on file, according to local procedure, at the study center.

7.2. Management of Patient Data

All investigators will ensure adherence to applicable data privacy protection regulation. All patient data will be captured and maintained in a study specific database with password-protected access. Data is entered using an assigned study subject identification number. Data or any research samples obtained in this study will only be transferred in encoded form and should not contain any data which, on its own account or in conjunction with other freely available data, can be used to re-identify natural persons. The investigators are obligated to ensure that no documents contain such data. The investigator will maintain a list to enable patients' records to be identified in case of queries. All documentation that contains personal health information that may include patient identifiable information will be maintained at the site to preserve patient confidentiality.

8. QUALITY CONTROL

The study will be organized, conducted, and reported in compliance with the International Council for Harmonisation (ICH) Good Clinical Practice (GCP) guidelines and all applicable regulations.

8.1. Data quality

Before study start at each site, the investigators and all study personnel will be sufficiently trained on the study. Investigators will have the chance to discuss and develop a common understanding

of the protocol and the eCRF through virtual meeting(s). All data will be recorded in a standardized eCRF. After data entry, missing or implausible data will be queried, and the data will be validated. A check for multiple documented patients will be done. National and international data protection laws will be followed.

8.2. Quality review

Before study start at each site, the investigators and all study personnel will be sufficiently trained on the study. Investigators will have the chance to discuss and develop a common understanding of the protocol and the eCRF through virtual meeting(s). The investigator has ultimate responsibility for the collection and reporting of all clinical, safety and laboratory data entered on the CRF. Quality review will be done initially during site training to review procedures to assess data accuracy, completeness, consistency, and reliability. During the study, quality reviews will be conducted to ensure completeness and plausibility of data, adherence to the protocol, and verification with source documents. Detailed measures for quality reviews will be described in the Quality Review Plan (QRP).

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APPENDIX 1

SUBSTUDY 1: PROSPECTIVE OBSERVATIONAL STUDY OF EPITHELIOID HEMANGIOENDOTHELIOMA PATIENTS TREATED WITH SIROLIMUS

1. RATIONALE AND BACKGROUND

Epithelioid hemangioendothelioma (EHE) is an ultra-rare and distinctive vascular sarcoma, with an incidence of 0.038 per 100,000 people per year, a prevalence of fewer than 1 per 1,000,000, and approximately 180 new cases annually in the European Union (EU) (de Pinieux et al., 2021; Lau et al., 2011; Silvia Stacchiotti et al., 2021a). Around 40% of patients are diagnosed with metastatic disease, while at least 20% of those initially presenting with localized disease experience distant metastasis upon relapse (Blay et al., 2023). Among patients with advanced disease, only those with evidence of disease progression and/or paraneoplastic symptoms are candidates for systemic therapy (S. Stacchiotti et al., 2021). Patients with serosal involvement may experience excruciating pain, often associated with common features as part of a complex pain syndrome (as described in Appendix 3). For patients with this disease presentation, progression and response to systemic treatments are challenging to assess, as they may involve disease modifications not well captured by the Response Evaluation Criteria in Solid Tumors (RECIST). These changes include worsening or appearance of serosal effusions, thickening of the pleura or peritoneum, and alterations in lung parenchyma with mediastinal shift, even in the absence of clear dimensional changes in target lesions or appearance of new ones (as described in Appendix 4) (Anna M. Frezza et al., 2021; Rosenbaum et al., 2020; Vanzulli,A, 2024).

For patients with EHE who need a systemic treatment, there are no active drugs among those currently approved and available for front-line therapy of soft tissue sarcomas. Indeed, doxorubicin, the standard first-line treatment for advanced soft tissue sarcoma – obtained a prolonged disease stabilization in less than 30% of advanced EHE patients with a median

progression free survival (PFS) of 5.5 months (Anna M. Frezza et al., 2021). Pazopanib, approved for treatment of soft tissue sarcoma from second-line has also showed marginal activity with a median PFS of 2.9 months (Anna M. Frezza et al., 2021). Among non-standard therapies, sirolimus is considered one of the most effective antitumor agents in the disease and is administered worldwide (Gronchi et al., 2021; S. Stacchiotti et al., 2021). Sirolimus is used off-label for this indication and current extensive evidence is highly supportive but it is limited to retrospective data (S. Stacchiotti et al., 2021). In 2012 a significant response lasting 16 months in a patient with EHE associated with Maffucci's syndrome was reported (Orbach et al., 2022). Another response to sirolimus was noted in a prospective phase 1 trial of this agent in advanced solid tumors, with a partial response (PR) observed in an EHE patient lasting more than 3 years (Cohen et al., 2012). Subsequently, Stacchiotti et al. reported a retrospective series in 2016, comprising 18 patients with advanced, molecularly confirmed, and progressive EHE treated with sirolimus within the Italian Rare Cancer Network (Stacchiotti et al., 2016). They observed 1 RECIST PR and 12 cases of stable disease (SD), with a median progression-free survival (m-PFS) of 12 months. These findings were updated in 2021 in a larger cohort (37 advanced and progressive EHE patients), with a RECIST overall response rate (ORR) of 4/37 (11%), a m-PFS of 13 months (range 3.7–not estimable), and a 12-month progression-free survival rate of 54%, with a median follow-up (m-FU) of 41.5 months (interquartile range [IQR] 23.9–56.8 months) (Silvia Stacchiotti et al., 2021b). These data also suggested that spontaneous regression and/or spontaneous stabilization are unlikely to occur in the advanced setting after evidence of disease progression, indicating that any observed stabilization is likely due to treatment. Thirteen patients with advanced EHE treated with sirolimus, who had previously shown disease stability but discontinued therapy for reasons other than progression, experienced poor outcomes after stopping sirolimus (Giani et al., 2023). Nine of the 13 patients progressed, 2 died, and only 2 remained progression-free, with a median post-progression PFS of 3.02 months (95% CI, 2.2-3.8). However, among six

patients who were rechallenged with sirolimus, one died 37 months later, while five remained alive; the best response was RECIST stable disease in five patients and progression in one. In these pts, at 81.3-months median follow up (IQR: 62.2-143.1), median post-rechallenging PFS was unreached. Although limited by small numbers, this study suggests that a subset of EHE patients can achieve durable benefit from sirolimus rechallenge, further supporting its antitumor effect in EHE (Giani et al., 2024, 2023). The activity of sirolimus in a pediatric population with EHE was reported by Engel, who identified retrospectively 4 partial responses out of 6 patients (Engel et al., 2020). Lastly, sirolimus's efficacy was confirmed in patients through a survey of the EHE global patient community conducted jointly by the EHE Rare Cancer Charity (UK) and the EHE Foundation (USA) patient advocacy groups (Robinson et al., 2024).

Since the activity of sirolimus was detected in the disease, sirolimus has only been investigated retrospectively in this tumour. This prospective observational study is aimed at prospectively confirming the role of sirolimus in advanced and progressive EHE.

2. OBJECTIVES

2.1. Primary objective.

The primary objective of this study is to provide prospective evidence of sirolimus activity in progressive EHE by assessing its ability to stabilize the disease and assess the interval without progression.

2.2. Secondary objectives

In addition, the activity of sirolimus in advanced progressive EHE will be evaluated looking at radiologic response. The overall response rate (ORR) and the duration of response (DoR) will be determined based on RECIST v1.1. Besides RECIST v1.1, treatment response

assessment according to additional radiological criteria [Response Evaluation by Serosal Changes and Outcomes Reporting (RESCORe)] is included as an exploratory objective (Appendix 4). Specifically, EHE can show specific patterns of response and disease progression that are not detectable by RECIST and are characterized by improvement / appearance of serosal effusion, changes in pleural thickening, and increase / reduction of lung parenchyma with mediastinal shift, in the absence of changes in target lesions or even appearance of new ones according to RECIST. RESCORe criteria (Appendix 4), are intended to take these parameters into account and complement RECIST v1.1 in the evaluation of progression and response.

Moreover, EHE can be characterized by tumor-related symptoms such as tumor-related pain which can interfere with everyday living and deeply impact quality of life. This is especially true for patients with serosal infiltration, which may experience excruciating pain associated with common features characterizing a complex pain syndrome (as described in Appendix 3). As such, pain evaluation is also incorporated as a secondary objective in the assessment of response/disease progression (Appendix 3). Quality of life assessed by EORTC QLQ-C30 and ESAS will also be evaluated.

As a further exploratory endpoint, the predictive value of GDF15 will be evaluated. GDF15 is a plasma cytokine produced by EHE cells and is detected in the plasma of patients with EHE (Stacchiotti et al., 2024). Indeed, plasma levels of GDF15 were recently shown to predict EHE aggressiveness in a retrospective and a prospective cohort, part of the main “Prospective International Observational Study on Epithelioid Hemangioendothelioma”. Indeed, patients with high-risk disease were characterized by high levels of GDF-15 which may grow at disease progression. Preliminary findings suggest that plasma concentration of GDF-15 can be modulated during treatment with sirolimus, decreasing even in patients who achieve disease stabilization and increasing in patients experiencing disease progression (Stacchiotti et al., 2024).

3. STUDY DESIGN

This is a prospective Sub-study embedded within the “PROSPECTIVE INTERNATIONAL OBSERVATIONAL STUDY ON EPITHELIOID HAEMANGIOENDOTHELIOMA: 1/DESCRIBING THE CLINICAL PRESENTATION, NATURAL HISTORY, AND TREATMENT OUTCOMES; 2/EVALUATING CYTOKINES AND HORMONES AS BIOMARKERS; 3/GENERATING PATIENT-DERIVED PRECLINICAL MODELS AS A TOOL TO ASSESS THE ACTIVITY OF ANTICANCER AGENTS AND VALIDATE NOVEL THERAPEUTIC TARGETS”. This study will analyze the data of patients with advanced and progressive EHE who will have started sirolimus after entering the main Prospective International Observational Study (i.e. patients in Groups B, D as described on Page 5 of the main protocol) and after the approval of Amendment 6. Patients who will have started sirolimus within the main study before the activation of Sub-study 1 and those included in the main protocol after reaching the target for Sub-study 1 will not be part of this analysis. Patients included in this study will be selected according to strict eligibility criteria and within an established study design.

The present study is designed as a one-arm prospective observational study, mirroring as much as possible a single-arm phase II trial in terms of objectives, eligibility criteria, endpoints, sample size calculation, treatment administration, follow-up monitoring, and ascertainment of endpoints, as discussed in Table 1.

Characteristics	Single Arm Efficacy Phase II Trial	Sub-study 1
Objective	Assess sirolimus activity and safety using pre-defined outcomes in a controlled clinical trial setting.	Assess sirolimus activity using pre-defined outcomes and performing direct comparison using historical controls (see “Statistical methods” section). As regards safety assessment, see comments in ”Safety Reporting”.

Study Design	Interventional. Patients receive sirolimus according to a specific protocol.	Non interventional. Sub-study 1 is a sub study embedded within the main Prospective Observational Study of EHE, which aims to describe all EHE patients regardless of the disease phase or the treatment they are receiving. In this context, it is not considered an interventional study. In the main Prospective Observational Study Protocol Sirolimus is administered as an off-label treatment according to institutional clinical practice. Sub-study 1, as a sub study of this main protocol, will focus on analyzing those patients who, among all those receiving sirolimus in the main study, meet the predefined eligibility criteria.
Ethics Committee/IRB Approval and informed consent	Required. Focus on explaining the experimental nature of the treatment.	Required. Focus on describing what real-world data will be collected and how, as well as what kind of translational research will be conducted.
Good Clinical Practice (GCP) Compliance	Strict adherence to GCP, including monitoring, documentation, and protocol adherence.	Must adhere to GCP for observational studies, focusing on accuracy of data collection.
Patient Eligibility Criteria	Defined a priori with strict inclusion/exclusion criteria.	Defined a priori with strict inclusion/exclusion criteria.
Sample Size Calculation	Required. Based on statistical power to detect a predefined sirolimus effect. Input from historical controls used for sample size calculation.	Required. Based on statistical power to detect a predefined sirolimus effect. Input from historical controls used for sample size calculation.
Treatment Administration	Specified by the protocol, including dosage, schedule, and any adjustments.	Reflects routine clinical practice, with data collected on how the treatment is administered.
Data Monitoring	Strict monitoring by an external Data Monitoring Committee to ensure safety and protocol adherence.	Data monitoring by internal committee, focusing on data quality, integrity, and completeness.
Endpoints / Outcomes	Focus on primary efficacy endpoints (i.e. Disease-control rate and progression-free survival within 12 months from initiation of sirolimus).	Focus on primary efficacy endpoints (i.e. Disease-control rate and progression-free survival within 12 months from initiation of sirolimus).

Safety Reporting	Mandatory adverse events (AE) reporting to regulatory authorities (e.g., serious adverse events, SUSARs).	Required to report serious adverse events, SUSARs, as per routine clinical practice.
Statistical Analysis Plan (SAP)	Predefined, detailing methods to assess efficacy, adjust for confounders, and handle missing data.	Predefined, detailing methods to assess efficacy, adjust for confounders (propensity score methods), and handle missing data.
Documentation and Audit Requirements	Detailed and comprehensive, with high levels of scrutiny during audits.	Detailed and comprehensive, with maintenance of accurate records and patient data.
Data Privacy and Confidentiality	High standards due to clinical trial regulations (e.g., GDPR, HIPAA).	High standards due to clinical trial regulations (e.g., GDPR, HIPAA).

4. ELIGIBILITY CRITERIA

These will consist of the criteria required for participation in the main protocol (i.e., the Prospective International Observational Study of Epithelioid Hemangioendothelioma) plus additional criteria specific for this sub-study (i.e., Sub-study 1):

4.1. General criteria required for participation in the main protocol, Prospective International Observational Study of Epithelioid Hemangioendothelioma:

4.1.1. Inclusion criteria

1. Histological and molecularly confirmed diagnosis of EHE according to 2020 WHO classification, performed on biopsy or surgical specimen
2. Adequate patient compliance to treatment or follow up
3. Signed informed consent

4.1.2. Exclusion criteria

1. Impossibility to ensure adequate compliance

4.2. Additional criteria specific for this study, Sub-study 1

4.2.1. Inclusion criteria

1. Age =/>18 years
2. Initiation of sirolimus following the completion of the consent process for enrollment in the observational study.
3. Advanced disease not amenable to curative resection
4. Documented baseline evidence of RECIST v1.1 progression within the 12 months prior to treatment initiation
5. Measurable disease by RECIST v1.1
6. No anticancer chemotherapy or immunotherapy, or any investigational drug therapy outside of this trial during or within 4 weeks of study entry.
7. No radiation therapy within 2 weeks prior to start of sirolimus. Prior palliative radiotherapy to non-target metastatic lesion(s) is permitted.
8. No major surgery within 4 weeks prior to the start of sirolimus.

4.2.2. Exclusion criteria

1. Anticancer chemotherapy or immunotherapy, any investigational drug therapy outside of this trial during or within 4 weeks (or 5 half-lives of the drug) of study entry.
2. Dementia or significantly altered mental status (*e.g.*, psychiatric disorder) that would prevent the understanding or rendering of informed consent and compliance with the requirements of this study.
3. Any severe and/or uncontrolled medical conditions that might jeopardize the interpretation of the study results.
4. Immunocompromised patients, e.g., patients who are known to be serologically positive for human immunodeficiency virus (HIV).
5. Active viral hepatitis (HBV or HCV infection). Active hepatitis B virus (HBV) is defined by a known positive HBV surface antigen (HBsAg) result. Patients with a past or resolved

HBV infection (defined as the presence of hepatitis B core antibody and absence of HBsAg) are eligible. Patients positive for hepatitis C virus (HCV) antibody are eligible only if polymerase chain reaction is negative for HCV RNA.

6. For female subjects of childbearing potential lack of a serum or urine beta-hCG pregnancy test within ≤ 7 days prior to initiating sirolimus therapy
7. Other malignancy unless curatively treated with no evidence of disease for ≥3 years except adequately treated non-melanoma skin cancer, curatively treated in situ cancer of the cervix, ductal carcinoma in situ (DCIS), stage 1, grade 1 endometrial carcinoma, or other malignancy considered as definitely cured by the investigator.
8. Any other severe and/or uncontrolled concurrent medical disease that in the opinion of the investigator could cause unacceptable safety risks or compromise compliance (e.g. impairment of gastrointestinal (GI) function, or GI disease that may significantly alter the absorption of the study drugs).
9. Radiation therapy within 2 weeks prior to sirolimus start. Prior palliative radiotherapy to metastatic lesion(s) is permitted, provided it has been completed 2 days prior to study enrollment and no clinically significant toxicities are expected (e.g., mucositis, esophagitis). Palliative radiation therapy is allowed during sirolimus treatment.
10. Major surgery within 4 weeks of start of sirolimus. Patients must have recovered from wound or directly surgical related complications at time of sirolimus start.

5. STUDY ENDPOINTS

5.1. Main end-points

- Disease Control Rate (DCR) according to RECIST 1.1 at 12 months from initiation of sirolimus.

- Progression-free Survival (PFS) according to RECIST1.1 within 12 months from initiation of sirolimus.

5.2. Secondary end-points

- Duration of stable disease according to RECIST 1.1 (DSD)
- Overall response rate (ORR) according to RECIST 1.1
- Duration of response according to RECIST 1.1 (DoR)
- Overall survival (OS)
- Post-progression OS (ppOS)
- Quality of life changes during treatment according to EORTC QLQ-C30 questionnaire and ESAS

5.3. Exploratory end-points

- PFS according to RESCORe (Appendix 4) or pain assessment (Appendix 3)
- Disease Control Rate (DCR) according to RESCORe (Appendix 4) or pain assessment criteria (Appendix 3)
- ORR according to RESCORe (Appendix 4) or pain assessment criteria (Appendix 3)
- Correlation between sirolimus plasma levels and PFS, DCR and ORR (sirolimus ≥20 ng/dL, between 15 and 20 ng/dL and < 15 ng/dL for at least 80% of the measures)
- Correlation between GDF-15 levels at the predefined timepoints (baseline, first disease radiological evaluation, disease progression) and PFS, DCR, and ORR.

5.4. Justification for the end-points choice

We choose to consider both DCR and PFS at the fixed time of 12 months as primary endpoints for Sub-study 1, as also suggested by EORTC for phase II studies (Van Glabbeke et al., 2002). For both the endpoints an observation period of 12 months was considered appropriate to

clearly determine the clinical relevance of disease stabilization in patients with progressive EHE. In fact, this timeframe would help to confirm that sirolimus induces clinically meaningful stabilization in progressive disease by:

- Distinguishing between temporary stabilization and more durable responses.
- Demonstrating that stabilization translates into meaningful outcomes, such as longer progression-free intervals.

In addition to RECIST v1.1, treatment response assessment according to RESCORe criteria (Appendix 4) and Pain Assessment Criteria (Appendix 3) is included as an exploratory end-point. RESCORe criteria are intended to take disease specific parameters into account and complement RECIST v1.1 in the evaluation of radiologic progression and response, while Pain Assessment Criteria intend to take pain evolution as reference for disease evolution.

In the retrospective study from the Italian Rare Cancer Network, 38 patients received sirolimus for advanced EHE (Silvia Stacchiotti et al., 2021b). In these patients, investigators observed a RECIST ORR of 10.8% (16% in patients without serosal effusion, 0% in patients with serosal effusion) and a median PFS of 13 months (47.8 and 4.8 months in patients without and with serosal effusion, respectively). Given the differences in outcomes observed in patients with serosal effusion, in an exploratory analysis, PFS, DCR and ORR will be evaluated both in the entire cohort as well as in the two subgroups of patients with and without serosal effusion (Silvia Stacchiotti et al., 2021b).

Pain evaluation is also incorporated as a secondary objective in the assessment of response (Appendix 3). Following the initial documentation of a Worst Pain Intensity (WPI) score exceeding 4 (on a 0 to 10 scale), a comprehensive assessment by a Palliative Care or Pain Specialist will be conducted. During this evaluation, the specialist will assess the pain characteristics, explore its association with the underlying disease, and implement a tailored analgesic regimen. A baseline Pain Assessment will be scheduled 3 to 7 days after the start of an adequate analgesic treatment. To ensure accurate pain evaluation, patients will be

instructed to document WPI, baseline analgesic regimen, and usage of rescue pain medication in a structured paper diary every evening. Palliative Care/Pain specialist follow up will be performed at least once a month for the first six months to monitor pain progression and optimize pain management. These FU may be conducted also via telemedicine, if clinically appropriate. After six months, the FU visit should be performed within one week from the oncological FU visit. Pain assessment-based evaluations of disease status will be performed during the visit nearest to each scheduled radiological assessment (refer to Appendix 4).

As an exploratory endpoint, GDF15 will also be evaluated. GDF15 is a plasma cytokine produced by EHE cells, present in the blood of patients with EHE (Stacchiotti et al., 2024). GDF-15 analysis will be descriptive and based on recent data showing that plasma levels of GDF15 can predict EHE aggressiveness. (Stacchiotti et al., 2024). Results of this study are expected to confirm the association between GDF-15 concentration levels and patient risk. Additionally, reduction in GDF-15 after treatment of patients with sirolimus will enhance the value of this cytokine as predictive of tumor response

6. TREATMENT

Patients eligible for Sub-study 1 analysis will be those treated with oral sirolimus, at a starting dose of 5 mg/day, in accordance with the instructions provided in the technical data sheet and consistent with what is presented in the main protocol. Sirolimus blood levels will be regularly monitored: at baseline, before starting treatment, 2 weeks after treatment initiation and every 4 weeks thereafter. Blood levels will also be checked in cases of severe toxicity. Sirolimus blood levels should be maintained between 15 and 40 ng/dL, and daily dosing will be adjusted accordingly to achieve this range. Treatment with sirolimus will be continued until disease

progression, unacceptable toxicity, or a patient's refusal to continue treatment.

7. CLINICAL ASSESSMENT AND DATA COLLECTION

In Sub-study 1 the protocol for patients on active treatment in the larger prospective study will be followed as described in chapter 3 (page 15 and following) of the main protocol. In particular, radiological assessment of all sites of disease by CT scan, and or MRI, and bone scan as clinically indicated, will be performed at baseline and every 3 months, thereafter. For patients entering this study and still on therapy, particular attention will be given to planning and conducting a radiologic disease reassessment at 12 months.

As for what is foreseen in the main protocol, radiologic images (CT and/or MRI scans) will be uploaded onto the centralized XNAT platform and centrally reviewed by a dedicated sarcoma radiologist. The XNAT Platform is a cross-platform, open-source tool designed to support imaging research with its core function to manage the import, archiving, processing, annotation and secure distribution of imaging and related study data. The radiological review will assess response to systemic treatments by RECIST, and by RESCORe (Appendix 4).

8. STATISTICAL CONSIDERATION

8.1. Sample size

Sample size considerations are based on DCR over a 12-month period. A single-group design will be used to test whether DCR proportion for patients receiving sirolimus differs from that of a historical control group. The control group comprises 33 patients analyzed in the study by Frezza et al. (Anna M. Frezza et al., 2021) who were treated with anthracyclines, formally approved as first-line therapy for patients with advanced STS. From this series we obtained

the estimate of DCR within 12 months of 33% (11 over 33 patients, unpublished data).

Under the following assumptions:

- 12-month DCR with anthracyclines: 35% (slightly higher than the historical pooled estimate of 33%).
- Increase in 12-month DCR from 35% to 60% with sirolimus, consistent with the DCR estimate obtained by Stacchiotti et al. (Stacchiotti et al., 2021), where among 38 patients with progressive EHE, the best RECIST response was a partial response in 4 patients and stable disease in 18 patients.
- Accrual 24 months, follow-up 12 months.

Based on these assumptions, we calculated that 35 patients would be needed to test the null hypothesis H0: DCR = 35% versus H1: DCR ≠ 35% versus H1: DCR ≠ 35%, using a two-sided, one-sample exact test for proportions with type I error alpha 5%, to detect a difference of 25% (from 35% to 60%) with 80% power.

Sample size and power calculations were performed using PASS 2024, version 24.0.2.

8.2. Statistical analyses

The statistical analyses will be carried out using SAS® Studio (version 5.2., SAS Institute, Cary, NC) and R software (last available version, R Foundation for Statistical Computing, Vienna, Austria).

Standard descriptive statistics will be calculated to describe the study patients. Means, medians, standard deviations and ranges will be computed, as appropriate, to describe the distribution of numerical variables; frequencies and percentages will be calculated for the categorical variables. Unless otherwise specified, missing data will not be replaced.

For each endpoint, the analyses will be done using the corresponding evaluable population.

8.2.1. Main endpoints

The RECIST 1.1 Disease Control Rate (DCR) point estimate will be calculated as the number of patients with CR+PR+SD divided by the total number of treated patients who will be evaluable for response by RECIST 1.1; the exact 95% confidence interval will also be estimated. A two-sided, one-sample exact test for proportions, with type I error alpha 5%, will be used to test the null hypothesis H0: DCR = 35% versus H1: DCR ≠ 35%).

PFS time will be calculated from the date of sirolimus start to the date of progression according to RECIST 1.1 or death from any cause, whichever will occur first; time will be censored at the date of the last follow-up for the patients alive and without progression before the study cut-off.

One sample log-rank test will be used to test the difference in 12-month PFS between sirolimus and the 35% hypothesized in the historical control. Potential historical control series may include patients with progressive disease (PD) from the series analyzed by Frezza et al. (Anna M. Frezza et al., 2021), and other series available at the centers participating in the PUSH consortium. We will strive to apply the same eligibility criteria as Sub-study 1 to these historical series of patients. Moreover, a Cox model will be fitted to estimate the hazard ratio (HR) of sirolimus vs other historical treatments. To adjust for the bias resulting from the non-random assignment of treatments, each patient will be weighted using the inverse of the propensity score (PS) for patients receiving sirolimus, or (1-PS) for those receiving other treatments. The weights will be stabilized by using the minimum value between PS and (1-PS). PS represents the likelihood of receiving sirolimus based on baseline characteristics, and it will be estimated beforehand using a multivariable logistic regression model with a binary response (sirolimus vs. other treatments), adjusting for the following covariates: patient's age, serosal effusion (present, absent), primary tumor site (visceral vs others), and number of metastatic sites (single vs multiple); possible additional covariates will be selected based on clinical considerations.

The PFS curve will also be estimated with the Kaplan-Meier method, and summaries of the curve will be estimated at specific timepoints of clinical interest, i.e. 6 and 12 months, together with the 95% confidence intervals, along with median times and corresponding interquartile ranges.

8.2.2. Secondary endpoints

- Duration of SD according to RECIST 1.1 (DSD) will be evaluated for subjects with SD as best response as the time between the date of treatment start to the date of RECIST 1.1 PD or death from any cause, whichever will occur first; time will be censored at the date of the last follow-up for the patients alive and without progression before the study cut-off.
- Overall response rate (ORR) according to RECIST 1.1: point estimate will be calculated as the number of patients with CR+PR divided by the total number of treated patients evaluable for RECIST 1.1 response; the exact 95% confidence interval will also be estimated.
- Duration of response according to RECIST 1.1 (DoR): evaluation will be based on responders only and will be assessed regardless of treatment modifications of any kind. DoR time will be calculated from the date of first disease response to the date of RECIST PD or death from any cause (in case of patients who die before evidence of disease progression); time will be censored at the date of the last follow-up for the patients alive before the study cut-off.
- Overall survival (OS): time will be calculated from the date of sirolimus start to the date of death for any cause; time will be censored at the date of the last follow-up for the patients alive before the study cut-off.

- Post-progression OS (ppOS): time will be calculated from the date of RECIST 1.1 progression to the date of death for any cause; time will be censored at the date of the last follow-up for the patients alive before the study cut-off.
- Quality of life changes during treatment according to EORTC QLQ-C30 questionnaire and ESAS: for both the questionnaires descriptive analyses will be performed: mean and standard deviation at each time will be estimated and, to represent trends over time, plots of mean scores will be depicted for patients with and without PFS events, and for patients exhibiting and not exhibiting DCR or ORR.

8.2.3. Exploratory end-points

- PFS according to RESCORe criteria (Appendix 4) or Pain Assessment Criteria (Appendix 3): time will be calculated from the date of sirolimus start to the date of progression or death from any cause, whichever will occur first; time will be censored at the date of the last follow-up for the patients alive and without progression before Sub-study 1 cut-off. The PFS curve will also be estimated with the Kaplan-Meier method, and summaries of the curve will be estimated at specific timepoints of clinical interest, i.e. 6 and 12 months, together with the 95% confidence intervals
- Disease Control Rate (DCR) according to RESCORe criteria (Appendix 4) or Pain Assessment Criteria (Appendix 3): point estimate will be calculated as the number of patients with CR+PR+SD by RESCORe and Pain Assessment Criteria, respectively, divided by the total number of treated patients who will be evaluable for RESCORe and Pain Assessment Criteria, respectively; the exact 95% confidence interval will also be estimated.
- ORR according to RESCORe (Appendix 4) or Pain Assessment Criteria (Appendix 3): point estimate will be calculated as the number of patients with CR+PR by RESCORe and Pain Assessment Criteria, respectively, divided by the total number of patients

treatet with sirolimus who will be evaluable for RESCORe and Pain Assessment Criteria, respectively; the exact 95% confidence interval will also be estimated

- Correlation between PFS, DCR or ORR by RECIST 1.1 and sirolimus plasma levels categorized in 3 subgroups (≥20 ng/dL, between 15 and 20 ng/dL and < 15 ng/dL for at least 80% of the measures): Kaplan-Meier PFS curves will be estimated in the 3 sirolimus plasma levels subgroups. A 3x2 contingency table will display the percentages of patients with DCR or ORR within each of the 3 sirolimus plasma levels subgroups. These analyses have exploratory purposes and no statistical testing will be performed.
- Correlation between PFS, DCR or ORR by RECIST 1.1, and GDF-15 levels at the predefined timepoints (baseline, first disease radiological evaluation, disease progression). Descriptive analyses of GDF-15 time profile using appropriate plots (such as line plots and boxplots to show the distribution of values each time point for the different stratification groups) and descriptive statistics (such as mean, standard deviation, median, interquartile range (IQR), minimum, and maximum values at each time point) will be performed stratifying for PFS status (progression or death present vs no events), for DCR (CR+PR+SD vs PD) and RECIST response (CR+PR vs SD+PD). Moreover, descriptive analyses will be performed to evaluate the changes in GDF-15 levels over time relative to baseline values. These analyses will be stratified by PFS status, DCR and ORR. For each time point, descriptive statistics will be calculated. The results will be visualized using line plots to display mean changes over time with their variability (e.g., mean ± standard deviation) and boxplots to show the distribution of changes at each time point for the different stratification groups. This approach will allow us to assess how GDF-15 levels change over time in relation to key clinical outcomes.

- Correlation between PFS, DCR or ORR by RECIST 1.1 and serosal effusion: Kaplan-Meier PFS curves will be estimated in the 2 subgroups serosal effusion present or absent. A 2x2 contingency table will display the percentages of patients with DCR or ORR within each of the 2 subgroups. These analyses have exploratory purposes and no statistical testing will be performed.

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APPENDIX 2

SUB-STUDY 2: A PROSPECTIVE OBSERVATIONAL STUDY OF EPITHELIOID HEMANGIOENDOTHELIOMA PATIENTS TREATED WITH SIROLIMUS AND DISCONTINUING SIROLIMUS FOR ANY REASON OTHER THAN DISEASE PROGRESSION

1. RATIONALE AND BACKGROUND

Sirolimus is considered one of the most effective antitumor agents for epithelioid hemangioendothelioma (EHE) and is administered worldwide (Gronchi et al., 2021; S. Stacchiotti et al., 2021). Sirolimus is used off-label for this indication and current supporting evidence is limited to retrospective data.

In 2021, Stacchiotti et al. presented a retrospective series of 37 patients with progressive EHE showing a RECIST overall response rate (ORR) of 11%, a m-PFS of 13 months, and a 12-month PFS rate of 54%, with a median follow-up of 41.5 months (Silvia Stacchiotti et al., 2021). Thirteen patients with advanced EHE treated with sirolimus, who had previously shown disease stability but discontinued therapy for reasons other than progression, experienced poor outcomes after stopping sirolimus (Giani et al., 2024, 2023). Nine of the 13 patients progressed, 2 died, and only 2 remained progression-free, with a median post-progression PFS of 3.02 months (95% CI, 2.2-3.8). However, among six patients who were rechallenged with sirolimus, one died 37 months later, while five remained alive; the best response was RECIST stable disease in five patients and progression in one. In these patients, at 81.3-months median follow up (IQR: 62.2-143.1), median post-rechallenging PFS was unreached. Although limited by small numbers, this study suggests that a subset of EHE patients can achieve durable benefit from sirolimus rechallenge, further supporting its antitumor effect in EHE.

Disease progression after stabilization/response on sirolimus treatment would be a strong indication that previous disease stabilization cannot be reasonably attributed to the natural history/indolence of the tumour but to the activity of sirolimus. This will allow prospective observation of disease behaviour following sirolimus discontinuation.

2. OBJECTIVES

2.1. Primary objective

The primary aim of Sub-study 2 is to allow prospective observation of disease behaviour following sirolimus discontinuation for any reason other than evidence of disease progression. The aim is to prospectively demonstrate, albeit indirectly, that after disease stabilization during sirolimus treatment, disease progression resumes upon discontinuation, thus indicating that the previous disease stabilization was not due to the natural history/indolence of the tumour but to the drug's activity.

2.2. Secondary objectives

To allow prospective observation of disease behaviour following sirolimus re-challenge for those patients restarting the drug at disease progression. This latter occurrence would be another strong indication that the previous disease stabilization was not due to the natural history/indolence of the tumour but to the activity of sirolimus.

3. STUDY DESIGN

This is a prospective study embedded within the “PROSPECTIVE INTERNATIONAL OBSERVATIONAL STUDY ON EPITHELIOID HAEMANGIOENDOTHELIOMA: 1/DESCRIBING THE CLINICAL PRESENTATION, NATURAL HISTORY, AND TREATMENT OUTCOMES; 2/EVALUATING CYTOKINES AND HORMONES AS BIOMARKERS;

3/GENERATING PATIENT-DERIVED PRECLINICAL MODELS AS A TOOL TO ASSESS THE ACTIVITY OF ANTICANCER AGENTS AND VALIDATE NOVEL THERAPEUTIC TARGETS". This study will analyse the data of all evaluable patients affected by advanced EHE who will discontinue sirolimus for any reason other than evidence of disease progression (Treatment-free Cohort), and patients affected by advanced EHE who will re-challenge sirolimus for the evidence of disease progression following sirolimus discontinuation (Re-Treatment Cohort), after entering the main Prospective International Observational Study (i.e. patients in Groups B and D as described on Page 5 of the main protocol) and following the approval of Amendment 6 Patients who will have discontinue or re-challenge sirolimus within the main study before the activation of Sub-study 2 and those included in the main protocol after reaching the target for Sub-study 2 will not be part of this analysis. Sub-study 2 will consider only patients who discontinue sirolimus for whatever reason (e.g., patient request, physician choice, toxicity, others) provided that the patient has had:

- a sirolimus treatment duration of at least 6 months; and
- radiological/clinical evidence of disease stability/response at the time of stopping sirolimus (i.e., a subset of patients in Group B and D, as described on Page 5 of the main protocol).

4. ELIGIBILITY CRITERIA

These will consist of general criteria required for participation in the international prospective study (4,1) and additional criteria specific for Sub-study 2 (4.2):-

4.1. General criteria required for participation in the international prospective study:

4.1.1. *Inclusion criteria*

1. Histological and molecularly confirmed diagnosis of EHE according to 2020 WHO classification, performed on biopsy or surgical specimen
2. Signed informed consent
3. Adequate patient compliance to treatment or follow up

4.1.2. *Exclusion criteria*

1. Impossibility to ensure adequate compliance

4.2. Additional criteria specific for Sub-study 2:

4.2.1. *Patients who discontinue sirolimus – Treatment-free Cohort*

4.2.1.1. Inclusion criteria

1. Age =/>18 years
2. Treatment with sirolimus for at least 6 months at the time of discontinuation
3. No evidence of disease progression by RECIST v1.1 (i.e., confirmed RECIST stable disease or response) at the time of sirolimus discontinuation or within 4 weeks prior to sirolimus discontinuation
4. No anticancer chemotherapy, immunotherapy, or any investigational drug therapy following the discontinuation of sirolimus

4.2.1.2. Exclusion criteria

1. Anticancer chemotherapy or immunotherapy or any investigational drug therapy concomitant to sirolimus or after sirolimus discontinuation

2. Dementia or significantly altered mental status (e.g., psychiatric disorder) that would prevent the understanding or rendering of informed consent and compliance with the requirements of this study.

3. Any severe and/or uncontrolled medical conditions that might jeopardize the interpretation of the study results.

4. Immunocompromised patients, e.g., patients who are known to be serologically positive for human immunodeficiency virus (HIV).

5. Active viral hepatitis (HBV or HCV infection). Active hepatitis B virus (HBV) is defined by a known positive HBV surface antigen (HBsAg) result. Patients with a past or resolved HBV infection (defined as the presence of hepatitis B core antibody and absence of HBsAg) are eligible. Patients positive for hepatitis C virus (HCV) antibody are eligible only if polymerase chain reaction is negative for HCV RNA.

6. Other malignancy unless curatively treated with no evidence of disease for ≥3 years except adequately treated non-melanoma skin cancer, curatively treated in situ cancer of the cervix, ductal carcinoma in situ (DCIS), stage 1, grade 1 endometrial carcinoma, or other malignancy considered as definitely cured by the investigator.

7. Any other severe and/or uncontrolled concurrent medical disease that in the opinion of the investigator could cause unacceptable safety risks or compromise compliance with the protocol (e.g. impairment of gastrointestinal (GI) function, or GI disease that may significantly alter the absorption of the study drugs).

8. Radiation therapy concomitant to sirolimus or after sirolimus discontinuation.

4.2.2. Patients who re-challenge sirolimus – Re-treatment Cohort

4.2.2.1. Inclusion criteria

1. Age =/>18 years
2. Evidence of disease progression by Response Evaluation Criteria In Solid Tumors (RECIST) v1.1 or by Serosal Changes and Outcomes Reporting (RESCORe) at the time of sirolimus rechallenge
3. After sirolimus rechallenge regular radiologic assessment at least every 3-4 months until evidence of RECIST 1.1 progression or sirolimus definitive discontinuation
4. No anticancer chemotherapy, immunotherapy, or any investigational drug therapy following the discontinuation of sirolimus

4.2.2.2. Exclusion criteria

1. Anticancer chemotherapy or immunotherapy or any investigational drug therapy concomitant to sirolimus or after sirolimus rechallenge
2. Dementia or significantly altered mental status (e.g., psychiatric disorder) that would prevent the understanding or rendering of informed consent and compliance with the requirements of this study.
3. Any severe and/or uncontrolled medical conditions that might jeopardize the interpretation of the study results.
4. Immunocompromised patients, e.g., patients who are known to be serologically positive for human immunodeficiency virus (HIV).

5. Active viral hepatitis (HBV or HCV infection). Active hepatitis B virus (HBV) is defined by a known positive HBV surface antigen (HBsAg) result. Patients with a past or resolved HBV infection (defined as the presence of hepatitis B core antibody and absence of HBsAg) are eligible. Patients positive for hepatitis C virus (HCV) antibody are eligible only if polymerase chain reaction is negative for HCV RNA.

6. Other malignancy unless curatively treated with no evidence of disease for ≥3 years except adequately treated non-melanoma skin cancer, curatively treated in situ cancer of the cervix, ductal carcinoma in situ (DCIS), stage 1, grade 1 endometrial carcinoma, or other malignancy considered as definitely cured by the investigator.

7. Any other severe and/or uncontrolled concurrent medical disease that in the opinion of the investigator could cause unacceptable safety risks or compromise compliance with the protocol (e.g. impairment of gastrointestinal (GI) function, or GI disease that may significantly alter the absorption of the study drugs).

8. Radiation therapy concomitant to sirolimus or after sirolimus rechallange.

5. STUDY ENDPOINTS

5.1. Patients who discontinued sirolimus for reasons other than progression – Treatment-free Cohort

5.1.1. Main end-point

Progression-free Survival (PFS-1) according to RECIST1.1 from sirolimus discontinuation.

5.1.2. Secondary end-points

- ORR according to RECIST 1.1

- Post-discontinuation OS (pdOS)
- Quality of life changes during treatment according to EORTC QLQ-C30 questionnaire and ESAS.

5.1.3. Exploratory end-points

- PFS-1 according to RESCORe (Appendix 4) and pain progression (Appendix 3) following sirolimus discontinuation
- Correlation between GDF-15 levels at predefined timepoints (baseline at the time of sirolimus discontinuation, at first disease radiological evaluation following sirolimus discontinuation, at first evidence of RECIST disease progression following sirolimus discontinuation) and PFS-1 according to RECIST 1.1 and pdOS
- Correlation between serosal effusion and PFS-1 according to RECIST 1.1 and pdOS.

5.2. Patients who restart sirolimus for secondary progression – Re-treatment Cohort

5.2.1. Main end-point

Progression-free Survival (PFS-2) according to RECIST 1.1, after sirolimus re-challenge.

5.2.2. Secondary endpoints

- ORR according to RECIST 1.1
- Duration of response according to RECIST 1.1 (DoR)
- Post-sirolimus rechallenge OS (psrOS)

- Quality of life changes during treatment according to EORTC QLQ-C30 questionnaire and ESAS

5.2.3. Exploratory end-points

- ORR by RESCORe (Appendix 4) and pain changes (Appendix 3)
- PFS-2 by RESCORe (Appendix 4) and pain changes (Appendix 3)
- Correlation between GDF-15 levels at predefined timepoints (at baseline prior to sirolimus rechallenge, at first disease radiological evaluation following sirolimus restart, at first evidence of RECIST best response, at first evidence of disease progression and at the time of sirolimus definitive interruption) and PFS-2 according to RECIST 1.1 and psrOS.

6. PATIENTS

Subjects will be analyzed according to their cohort (Patients who discontinue sirolimus – Treatment-Free Cohort; Patients who re-challenge sirolimus – Re-Treatment Cohort).

6.1. Treatment-Free Cohort

The Evaluable Analysis Set (EAS) will include all subjects who, after entering Sub-study 2:

1. Have undergone a baseline radiological assessment following sirolimus discontinuation, including CT scans, MRI, and bone scans as clinically indicated for all sites of disease.
2. Undergo regular radiological assessments of all disease sites every 3–4 months thereafter, until evidence of disease progression or death and/or have at least 3 radiological assessment following the baseline.

6.2. Re-Treatment Cohort

The EAS will include all subjects who, after entering Sub-study 2, re-start sirolimus and:

1. Have undergone a baseline radiological assessment before sirolimus re-challenge, including CT scans, MRI, and bone scans as clinically indicated for all sites of disease.

2. Undergo regular radiological assessments of all disease sites every 3–4 months thereafter, until evidence of disease progression or death and/or have at least 3 radiological assessment following the baseline

3. Have restarted oral sirolimus at the daily dose of 5 mg (as for the instructions provided in the technical data sheet and consistent with what is presented in the main protocol).

4. Sirolimus blood levels have been regularly monitored: at baseline, before starting treatment, 2 weeks after treatment initiation and every 4 – 8 weeks thereafter and have been maintained between 15 and 40 ng/dL, by adjusting the daily intake of the drug accordingly to achieve this range.

7. CLINICAL ASSESSMENT, DATA COLLECTION AND TREATMENT

As for what is foreseen in the main protocol, radiologic images (CT and/or MRI scans) will be uploaded onto the centralized XNAT platform and centrally reviewed by a dedicated sarcoma radiologist. The XNAT Platform is a cross-platform, open-source tool designed to support imaging research with its core function to manage the import, archiving, processing, annotation and secure distribution of imaging and related study data. The radiological review will assess response to systemic treatments by RECIST, and by RESCORe (Appendix 4).

In Re-Treatment Cohort, sirolimus will be started after baseline assessment of all sites of disease by CT scan and / or MRI and/or bone scan as clinically indicated performed within 4 weeks from the first dose of the drug. Sirolimus will be initiated at the daily dose of 5 mg,

according to the instructions provided in the technical data sheet and consistent with what is presented in the main protocol. Sirolimus will be continued until disease progression, unacceptable toxicity, or a patient's refusal to continue treatment.

Pain and QoL evaluation will be performed at the baseline, and at least at the same timepoints of the radiological assessments, according to Pain Assessment Criteria (Appendix 3).

8 STATISTICAL CONSIDERATION

8.1. Sample size

An observational single-group prospective design will be applied, including those patients who will discontinue sirolimus for any reason other than disease progression. To detect a one-year PFS-1 of 50% vs 20% in the historical control group (Giani et al., 2024, 2023) at one-sample log-rank test with 5% Type I error (two-sided) under the assumption of exponential distribution of PFS times, a sample size of 15 patients will allow us to achieve a power of 82.6%.

8.2. Statistical analyses

The statistical analyses will be carried out using SAS® Studio (version 5.2., SAS Institute, Cary, NC) and R software (last available version, R Foundation for Statistical Computing, Vienna, Austria).

Standard descriptive statistics will be calculated to describe the study patients: Means, medians, standard deviations and ranges will be computed, as appropriate, to describe the distribution of numerical variables; frequencies and percentages will be calculated for the categorical variables. Unless otherwise specified, missing data will not be replaced.

For each endpoint, the analyses will be done using the corresponding evaluable population.

8.3. Patients who discontinued sirolimus for reasons other than progression

8.3.1. Main end-point

Progression-free Survival (PFS-1) according to RECIST 1.1 from sirolimus discontinuation. PFS-1 time will be calculated from the date of sirolimus discontinuation to the date of progression according to RECIST 1.1 or death from any cause, whichever will occur first; time will be censored at the date of the last follow-up for the patients alive and without progression before the study cut-off. One sample log-rank test will be used to test the difference in 12-month PFS-1 with the 20% hypothesized in the historical control

8.3.2. Secondary end-points

- ORR according to RECIST 1.1: point estimate will be calculated as the number of patients with CR and PR divided by the total number of patients who discontinued sirolimus; the exact 95% confidence interval will also be estimated.

- Post-discontinuation OS (pdOS): time will be calculated from the date of sirolimus discontinuation to the date of death for any cause; time will be censored at the date of the last follow-up for the patients alive before the study cut-off.

- Quality of life changes during sirolimus discontinuation interval according to EORTC QLQ-C30 questionnaire and ESAS. For both EORTC QLQ-C30 and ESAS questionnaires descriptive analyses will be performed: mean and standard deviation at each time will be estimated and, to represent trends over time, plots of mean scores will be depicted.

8.3.3. Exploratory end-points

- PFS-1 according to RESCORe (Appendix 4) and pain progression (Appendix 3) following sirolimus discontinuation: time will be calculated from the date of sirolimus discontinuation to the date of progression according to RECIST 1.1 or death from any cause, whichever will occur first; time will be censored at the date of the last follow-up for the patients alive and without progression before the study cut-off.

- Correlation between GDF-15 levels at predefined timepoints (baseline at the time of sirolimus discontinuation, at first disease radiological evaluation, disease progression) and PFS-1 according to RECIST 1.1 and pdOS: Descriptive analyses of GDF-15 time profile using appropriate plots (such as line plots and boxplots to show the distribution of values each time point for the different stratification groups) and descriptive statistics (such as mean, standard deviation , median, interquartile range (IQR), minimum, and maximum values at each time point) will be performed stratifying for PFS-1 status (progression or death present vs no events) and OS status (alive, death). These analyses have exploratory purposes, and no statistical testing will be performed.

- Correlation between serosal effusion and PFS-1 according to RECIST 1.1 and pdOS. Kaplan-Meier PFS-1 and pdOS curves will be estimated in the 2 subgroups serosal effusion present or absent. These analyses have exploratory purposes, and no statistical testing will be performed.

8.4. Patients who restart sirolimus for secondary progression

8.4.1. Main end-point

Progression-free Survival (PFS-2) according to RECIST 1.1, after sirolimus re-challenge: time will be calculated from the date of sirolimus restart to the date of progression or death from

any cause, whichever will occur first; time will be censored at the date of the last follow-up for the patients alive and progression-free before the study cut-off.

8.4.2. Secondary endpoints

- ORR according to RECIST 1.1: point estimate will be calculated as the number of patients with CR+PR divided by the total number of patients who restart sirolimus; the exact 95% confidence interval will also be estimated.

- Duration of response according to RECIST 1.1 (DoR): DoR evaluation will be based on responders only and will be assessed regardless of treatment modifications of any kind. DoR time will be calculated from the date of the first documented response (complete or partial response) to the date of disease progression or death from any cause (in case of patients who die before evidence of disease progression); time will be censored at the date of the last follow-up for the patients alive before the study cut-off.

- Post-sirolimus rechallenge OS (psrOS): time will be calculated from the date of the first dose of sirolimus rechallenge to the date of death for any cause; time will be censored at the date of the last follow-up for the patients alive before the study cut-off.

- Quality of life changes during treatment according to EORTC QLQ-C30 questionnaire and ESAS: For both EORTC QLQ-C30 and ESAS questionnaires descriptive analyses will be performed: mean and standard deviation at each time will be estimated and, to represent trends over time, plots of mean scores will be depicted.

8.4.2. Exploratory end-points

- ORR by RESCORe criteria (Appendix 4) and pain assessment criteria (Appendix 3): a point estimate will be calculated as the number of patients with CR+PR divided by the total number of patients who restart sirolimus; the exact 95% confidence interval will also be estimated.

- PFS-2 by RESCORe (Appendix 4) and pain changes (Appendix 3): time will be calculated from the date of sirolimus restart to the date of progression or death from any cause, whichever will occur first; time will be censored at the date of the last follow-up for the patients alive and progression-free before the study cut-off.

- The PFS-1, pdOS, DoR, PFS-2, and psrOS curves will be estimated with the Kaplan-Meier method. Summaries of Kaplan-Meier and crude cumulative incidence curves will be estimated at specific timepoints of clinical interest, together with the 95% confidence intervals, along with median times and corresponding interquartile ranges.

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APPENDIX 3

PAIN ASSESSMENT CRITERIA (PAC)

BACKGROUND

In cancer patients with neoplastic pleural or peritoneal involvement, pain can be classified by assessing etiology and clinical characteristics and by pain syndrome recognition following established criteria, such as the IASP Task Force (ITF) (Caraceni et al., 1999; Portenoy and Ahmed, 2018), ICD-11 (Perrot et al., 2019) and expert recommendations (Caraceni, 2001). For an accurate definition of these characteristics, specialist evaluation (Palliative Care or Pain Specialist) is recommended, in particular for those patients with severe pain. Through clinical criteria, we can describe typical pain patterns in patients with epithelioid hemangioendothelioma (EHE) and, particularly but not exclusively, pain related to the presence of serosal / pleural involvement.

In patients affected by EHE with pleural involvement, the typical syndrome manifests as nociceptive somatic pain in the ipsilateral hemithorax, described as constrictive, persistent, and associated with exacerbations. These exacerbations can occur either due to pleural stretching (deep breaths) or in the absence of clear triggers; moreover, this excruciating pain may reduce the mobility of the hemithorax and of the homolateral shoulder. Sometimes, it is also possible to observe a neuropathic component, described as a well localized superficial pain, often radiating to the anterior chest wall due to involvement of the intercostal nerves (from T1 to T11). This pain may be evoked by pressuring the Valleix points due to irritation of compressed nerve fibers. Depending on local spread of the disease (i.e, to paravertebral structures) patients can also report poorly localized back pain, exacerbated when lying down, and disturbing sleep. Pain characteristics may vary depending on the sites of disease progression; for example, patients may develop visceral pain, described as deep retrosternal and poorly localized pain, if heart,

pericardium, large vessels and/or esophagus are involved.

In EHE patients with peritoneal involvement, nociceptive somatic or visceral pain can be observed in different abdominal quadrants, described as constrictive, persistent, and associated with exacerbations. Exacerbations may have characteristics of colic pain, even leading to sub-occlusive or occlusive intestinal conditions. In the case of peritoneal disease, the neuropathic component is typically absent but may manifest in the case of retroperitoneal nodal involvement.

Pain in EHE can also be due to the spread of the disease to different distant tissues, such as liver, bone or nervous tissues.

Usually, pain related to serosal infiltration rapidly worsens and it is the worst pain intensity that is the main factor affecting the quality of life of these patients, while average pain intensity can be moderate and constant. This condition is often described as breakthrough pain (Caraceni et al., 2004). It is not uncommon to find poor response to opioids, while frequently there is a good response to NSAIDs (e.g., ketorolac).

When pain is severe independently from its cause a specialist evaluation is still recommended to accurately define the pain etiology and characteristics. The Palliative Care Specialist or Pain Therapist should also set the appropriate therapy for the pain features.

Pain assessment criteria

To assess disease status by means of clinical criteria and to accurately determine pain syndromes and assess pain progression, patients' pain evaluations should be conducted by a Palliative Care or Pain Specialist. At each follow-up visit, the patient will complete a modified Brief Pain Inventory (BPI) (Serlin et al., 1995), with pain assessment referring to the last week

instead of the last 24 hours. The 7-day interval was chosen as it provides more information than the 24-hour interval. Additionally, we selected Worst Pain Intensity (WPI) as the reference parameter rather than Average Pain Intensity (API) since it is the former that has a more significant impact on these patients' quality of life and overall pain. This is consistent with previous studies reporting the WPI as possibly more impactful than API on pain evaluation (Atkinson et al., 2010; Harris et al., 2007).

The relationship between pain and tumor will be defined based on the site of pain and the components of the pain syndrome. Tumor-related pain will be then assessed according to the following criteria. In the event of multiple painful lesions, the site associated with the highest level of WPI will be used as the response parameter, but all other pains will be recorded.

The WPI must be > 4 in order to be used as a parameter of response, as this pain intensity provides a threshold for clinically significant WPI.

Baseline pain evaluation for pain response should be done from 3 to 7 seven days after starting an adequate analgesic treatment and before starting anti-neoplastic treatment; response evaluation will be done at least 6 weeks after the administration of specific therapy. Within this time frame, it should be possible to estimate the drug activity. Pain assessment and response should be implemented also in patients who are without pain at baseline, and who develop pain while on treatment using the same criteria.

Pain intensity assessment

The status of the disease will be primarily evaluated by pain intensity assessment. According to literature, the 30% change cut-off was chosen to identify a clinically relevant pain change (Corli

et al., 2013; Dworkin et al., 2008; Farrar et al., 2001). Previous studies have also found other cut-offs, such as a 2-point reduction on a scale from 0 to 10, as clinically significant when evaluating pain response. However, we have opted for a percentage threshold as we believe it to be the most reliable across all pain ranges (Corli et al., 2013).

Analgesic drug assessment

Pain response criteria cannot disregard analgesic drug assessment. While an appropriate pain therapy will be started before the baseline evaluation, it is very likely that dosage adjustment and/or drug change will be needed throughout the following weeks. Therefore, keeping in consideration the analgesic therapy modification will be essential to assess tumor response throughout pain.

If a switch in opioid is performed, the equianalgesic dose between the current and previous drug will be used to evaluate the disease status. As it is not possible to assess an equianalgesic dose of NSAIDs, for this family, the total dose will be taken as reference, although these patients often require the use of NSAIDs with high anti-inflammatory potency (e.g., ketorolac) (Buckley and Brogden, 1990; Patrignani and Patrono, 2015). Since different NSAIDs have various therapeutic dosages, if a switch in NSAIDs is performed, it will not be possible to assess the disease status using the NSAIDs daily dose. The assessment will be resumed at the next visit.

When assessing the analgesic daily dose, it is necessary to take into consideration the total rescue pain drug dose added to the background therapy dose. To determine the daily dose of rescue pain medication, the total amount administered over the previous 7 days is calculated and subsequently divided by 7, providing the average daily dose required for rescue analgesia.

For the criteria regarding opioid/NSAID dose increase or reduction, the doses of the two drug families will be evaluated separately. A significant increase or reduction (see criteria) in one of

the two drug families will be sufficient to define PD or PR respectively. Although unlikely, it is possible that the trend of doses of the two drug families is not consistent (e.g., opioid reduction and NSAID increase). In this case, the change with the greater magnitude will be relevant for evaluation if the opposite change is no more than 10%. If the two dose changes are inconsistent and exceed 10% of the respective dose, in the absence of significant changes in WPI (see criteria), SD status will be maintained.

Pain Assessment Criteria

The criteria are the following:

- COMPLETE RESPONSE (CR), WPI=0 with no more than a 10% increase in opioid/NSAID daily dose;
- PARTIAL RESPONSE (PR), at least a 30% decrease in WPI with no more than a 10% increase in opioid/NSAID daily dose, and/or at least a 30% reduction in opioid/NSAID daily dose with no more than a 1-point increase in WPI;
- STABLE DISEASE (SD), WPI and/or opioid/NSAID daily dose variations that do not meet PR or PD criteria;
- PROGRESSIVE DISEASE (PD), at least a 30% increase in WPI with no more than a 10% reduction in opioid/NSAID daily dose, and/or at least a 30% increase in opioid/NSAID daily dose with no more than a 1-point reduction in WPI and/or the development of new disease related pain with a WPI more than 4.

In the event that the patient experiences pain progression at a different metastatic site from the one initially selected as the target to monitor pain changes, the reference pain can be changed, taking into consideration the anatomic location affected by the pain associated with the highest WPI. This evaluation must be performed by a Palliative Care or Pain Specialist.

To ensure accurate pain evaluation, patients will be instructed to document WPI, baseline

analgesic regimen, and usage of rescue pain medication in a structured paper diary every evening. Palliative Care/Pain specialist follow up will be performed at least once a month for the first six months to monitor pain progression and optimize pain management. These FU may be conducted via telemedicine, if clinically appropriate. After six months, the FU visit should be performed within one week from the oncological FU visit. Pain assessment-based evaluations of disease status will be performed during the visit nearest to each scheduled radiological assessment (refer to Appendix 2)

Exclusion or Drop-out criteria

Since these criteria are based on the clinical evaluation of the patient, there may be circumstances in which tumor-related pain assessment is to be considered not evaluable. In detail, the unavailability of the assessment can be defined in presence of one (or more) of the following:

- inability of the patient to define the intensity of his/her own pain (e.g., in cases of cognitive impairment);
- increase of pain due to factors unrelated to tumor progression (e.g., tumor region infection);
- significant lack of compliance with analgesic therapy;
- every situation unrelated to the tumor that may affect or jeopardize the correct pain evaluation according to the clinician;
- evaluation conducted by someone other than a Palliative Care Specialist or a Pain Therapist.

Examples:

- CR: Baseline WPI of 8/10 with oral daily morphine 100 mg → WPI at the visit 0/10 with oral daily morphine 100/110 mg

- PR: Baseline WPI of 8/10 with oral daily morphine 100 mg → WPI at the visit 5/10 with oral daily morphine 100/110 mg

Or

- PR: Baseline WPI of 5/10 with oral daily morphine 100 mg → WPI at the visit 6/10 with oral daily morphine 60 mg

Or

- PR: Baseline WPI of 5/10 with oral daily morphine 100 mg and oral daily ketorolac 60 mg → WPI at the visit 6/10 with oral daily morphine 60 mg and oral daily ketorolac 65 mg

- SD: Baseline WPI of 8/10 with oral daily morphine 100 mg → WPI at the visit 6/10 with oral daily morphine 100/110 mg (trend towards PR, but still not meeting the criteria)

Or

- SD: Baseline WPI of 5/10 with oral daily morphine 100 mg → WPI at the visit 6/10 with oral daily morphine 80 mg (trend towards PR, but still not meeting the criteria)

Or

- SD: Baseline WPI of 5/10 with oral daily morphine 100 mg → WPI at the visit 10/10 with oral daily morphine 0 mg (pain exacerbation due to inappropriate reduction of analgesic therapy)

Or

- SD: Baseline WPI of 8/10 with oral daily morphine 100 mg → WPI at the visit 2/10 with oral daily morphine 150 mg (pain reduction due to response to increased analgesic therapy)

Or

- SD: Baseline WPI of 7/10 with oral daily morphine 100 mg and oral daily ketorolac 60 mg → WPI at the visit 6/10 with oral daily morphine 50 mg and oral daily ketorolac 90 mg (inconsistent variations in opioid and NSAID doses exceeding the 10% of the starting dose, with pain without significant variations)

Or

- SD: Baseline WPI of 8/10 with oral daily morphine 100 mg → WPI at the visit 9/10 with oral daily morphine 100/110 mg (trend towards PD, but still not meeting the criteria)

Or

- SD: Baseline WPI of 5/10 with oral daily morphine 100 mg → WPI at the visit 6/10 with oral daily morphine 120 mg (trend towards PD, but still not meeting the criteria)

- PD: Baseline WPI of 8/10 with oral daily morphine 100 mg → WPI at the visit 8/10 with oral daily morphine 130 mg

Or

- PD: Baseline WPI of 8/10 with oral daily morphine 100 mg and oral daily ketorolac 90 mg → WPI at the visit 8/10 with oral daily morphine 130 mg and oral daily ketorolac 85 mg

Or

- PD: Baseline WPI of 5/10 with oral daily morphine 100 mg → WPI at the visit 8/10 with oral daily morphine 100 mg

Or

- PD: Baseline upper right arm WPI of 5/10 with oral daily morphine 100 mg → upper right arm WPI at the visit 6/10 with oral daily morphine 110 mg plus the development of a constrictive, persistent, pain in the right hemithorax, associated with exacerbations with a WPI 6/10

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APPENDIX 4

Response Evaluation by Serosal Changes and Outcomes Reporting (RESCORe) criteria

1. RATIONALE AND BACKGROUND

Aggressive forms of EHE are often associated with systemic symptoms (tumor-related pain, fever, fatigue, shortness of breath and weight loss) and serosal involvement, either in the form of effusion, thickening of serosal layers (either focal or diffuse) or both.

In such cases, disease progression appears to be temporally correlated with the onset or worsening of serosal involvement, which can occur without evidence of new lesions or threshold growth of existing ones and cannot therefore be detected by Response Evaluation Criteria In Solid Tumors (RECIST) 1.1 (Frezza et al., 2021; Lau et al., 2011; Rosenbaum et al., 2020).

Refinement of radiologic response criteria is an emerging field of investigation in the sarcoma community.

RECIST 1.1 have recently been proven suboptimal predictors of clinical outcomes in patients affected by high-risk soft-tissue sarcomas of the extremities and superficial trunk (Vanzulli et al, 2025).

Disease-specific radiologic response criteria have also been proposed for malignant mesothelioma, based on the assumption that this tumor displays a peculiar pattern of growth which is not adequately captured by RECIST (Byrne et al, 2004; Armato et al, 2018).

This study aligns with such trajectory of research by proposing an alternative exploratory response assessment score, the Response Evaluation by Serosal Changes and Outcomes Reporting (RESCORe) criteria (see also Table 1).

2. DEFINITION

- **RESCORe Complete Response (CR)** was assigned if CR for RECIST 1.1 and no serosal involvement (defined as either focal/diffuse thickening of serosal layers, effusion or both) was observed or had completely resolved if present at prior studies;
- **RESCORe Partial Response (PR)** was assigned in two distinct scenarios:
    a. PR according to RECIST 1.1 and no new effusion or existing effusion did not increase ≥ 40%* and no new nor unequivocal increase of existing serosal layers involvement;
    b. Reduction in the sum of the longest diameters of target lesions 10% < x < 30% and serosal effusion reduced ≥ 66%* and no new nor unequivocal increase of existing serosal layers involvement;
- **RESCORe Progressive Disease (PD)** was assigned if PD per RECIST 1.1 or new effusion or existing effusion increased ≥ 40%* (or up to complete occupation of pleural/peritoneal cavity) or new/unequivocal increase of serosal layers involvement;
- **RESCORe Stable Disease (SD)** was assigned in cases not qualifying for either CR, PR or PD.

*** volumetric assessment

The thresholds above were partially derived from previous work by Orsatti et al, who compared the performances of uni-, bi- and tri-dimensional response criteria in the setting of rhabdomyosarcoma.

3. EVALUABLE PATIENTS

RESCORe criteria are not applicable in case of:

1. Active interventions to treat and/or prevent serosal involvement, such as thoracentesis/paracentesis or chest/abdominal drainages;

2. Clinical evidence supporting non-neoplastic origin of effusion, for example confirmed bacterial/viral pneumonia, thoracic traumas, cardiac failure, or suspected drug-induced effusion.

A standardized, EHE-tailored checklist is proposed to ensure all relevant findings are investigated by the reporting radiologist:

1. Pleural effusion observed: Y/N;

   1.1 If Y to 1: Pleural effusion already observed at latest prior: Y/N;

   1.2 If Y to 2: Please specify % variation of pleural effusion (volumetric assessment; including comparison with all available priors); if stable, specify duration of stability;

   1.3 If N to 2: Please alert principal provider of care;

2. Pleural thickening (either focal or diffuse) observed: Y/N;

   2.1 If Y to 5: Pleural thickening already observed at latest prior: Y/N;

   2.2 If Y to 6: Please describe any changes in pleural involvement (including comparison with all available priors);

   2.3 If N to 6: Please alert principal provider of care

3. Peritoneal involvement observed: Y/N;

   3.1 If Y to 9: Peritoneal effusion already observed at latest prior: Y/N;

   3.2 If Y to 10: describe any changes in peritoneal effusion (including comparison with all available priors);

   3.3 If N to 10: Please alert principal provider of care

Legend: Y = yes; N = no

RESCORe category	Target lesions	Non-target lesions	New lesions	Serosal involvement
CR	-Disappearance of all lesions -LN axis < 10 mm	Disappearance of all lesions	No	No or completely resolved
PR	Decrease ≥ 30% of SLDs from baseline (≥ 4 weeks)	No progression	No	No new involvement, effusion increase < 40%, no unequivocal increase in serosal layers involvement
PR	Decrease 10% < x < 30% of SLDs from baseline (≥ 4 weeks)	No progression	No	No new involvement, effusion reduced ≥ 66%, no unequivocal increase in serosal layers involvement
PD	≥ 20% increase of LSD from nadir with an absolute increase ≥ 5 mm	Unequivocal progression in lesion size	Yes	New involvement or effusion increase ≥ 40% or unequivocal increase in serosal layers involvement
SD	Does not qualify for CR, PR nor PD, nadir as reference	Persistence of one or more	No	No new involvement, effusion changes within thresholds, no unequivocal increase in serosal layers involvement

Table 1. RESCORe criteria.
CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; LN: lymph node; SLD: sum of longest diameters.

FIGURES

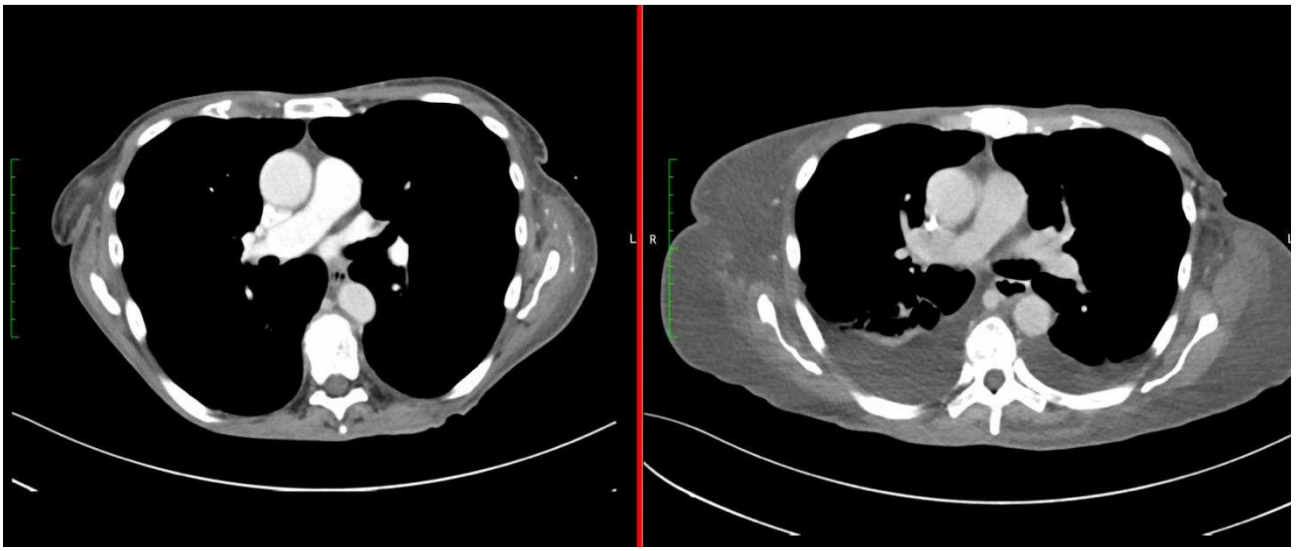


Figure 1. Development of bilateral pleural effusion in a patient with aggressive EHE

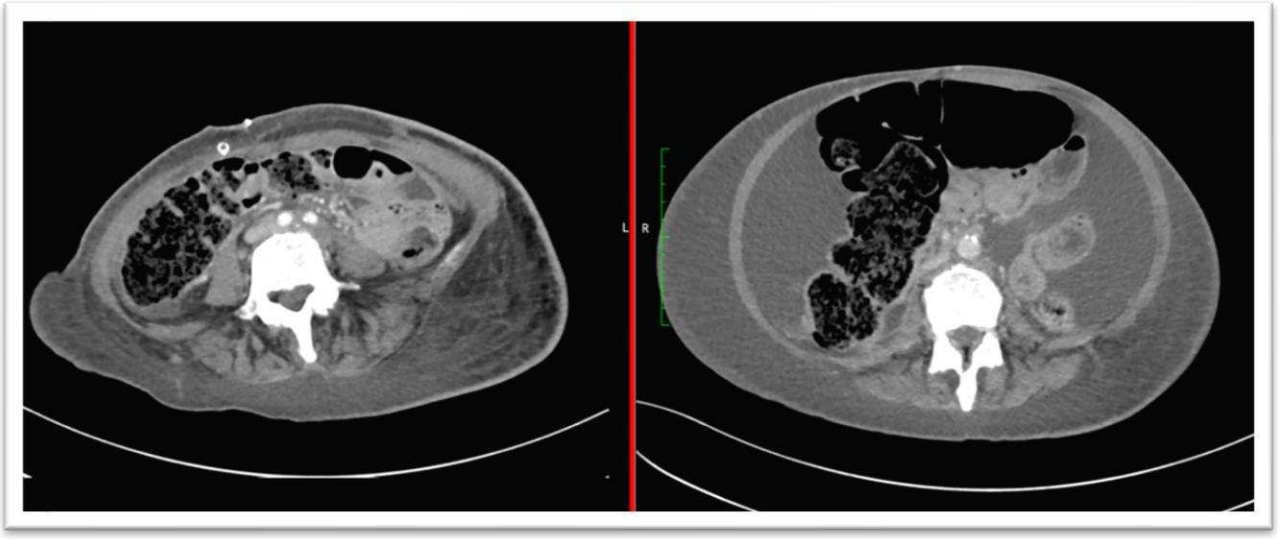


Figure 2. Development of peritoneal effusion in a patient with aggressive EHE

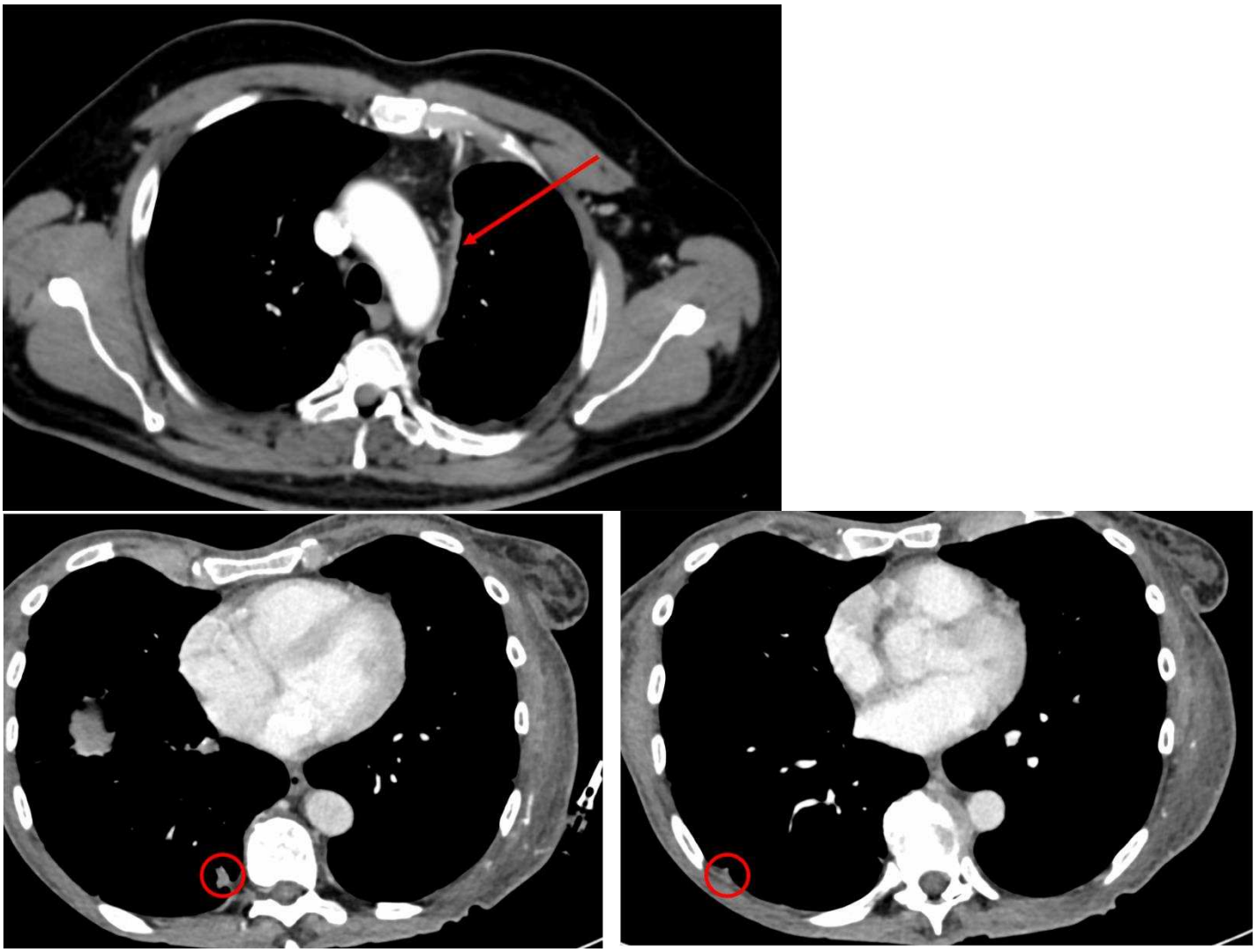
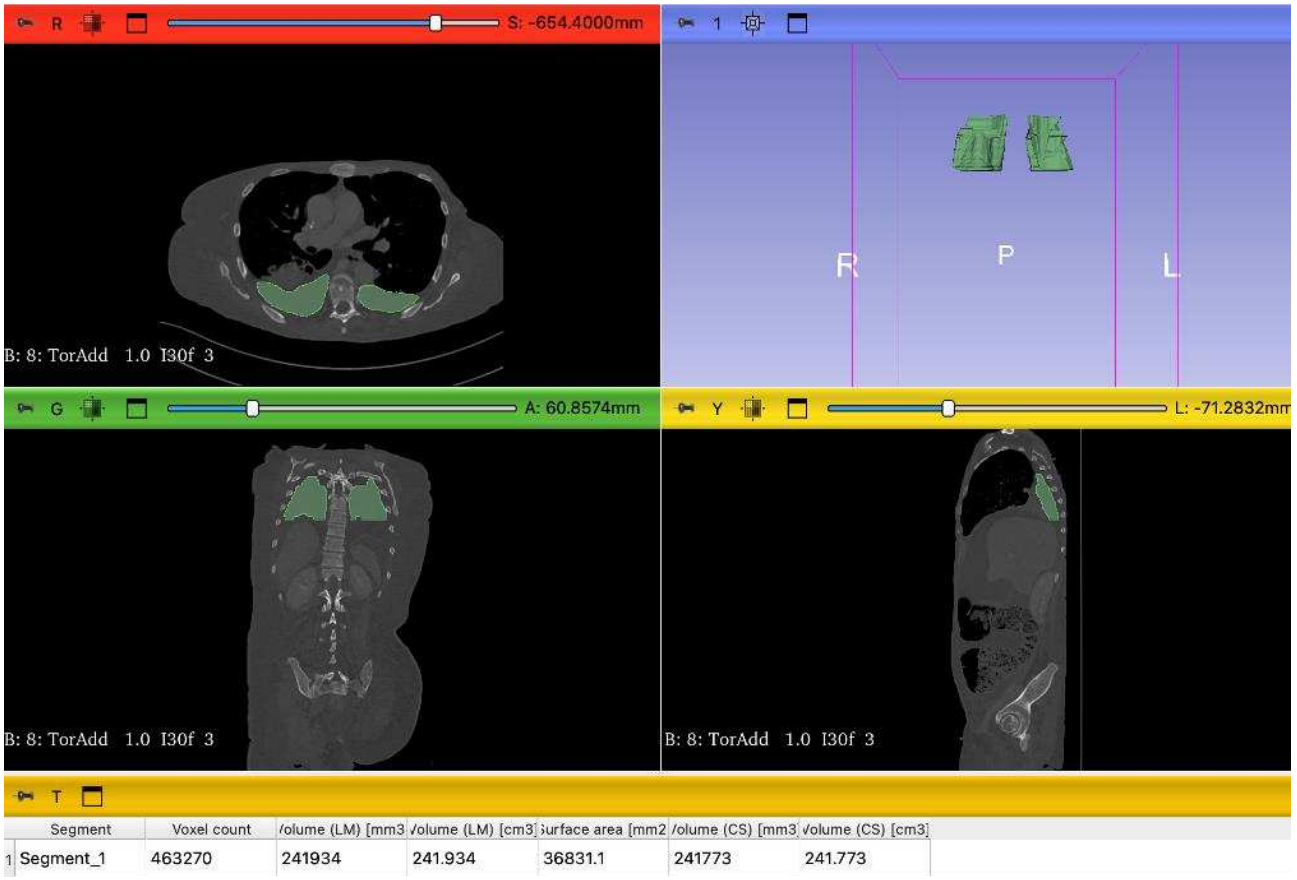
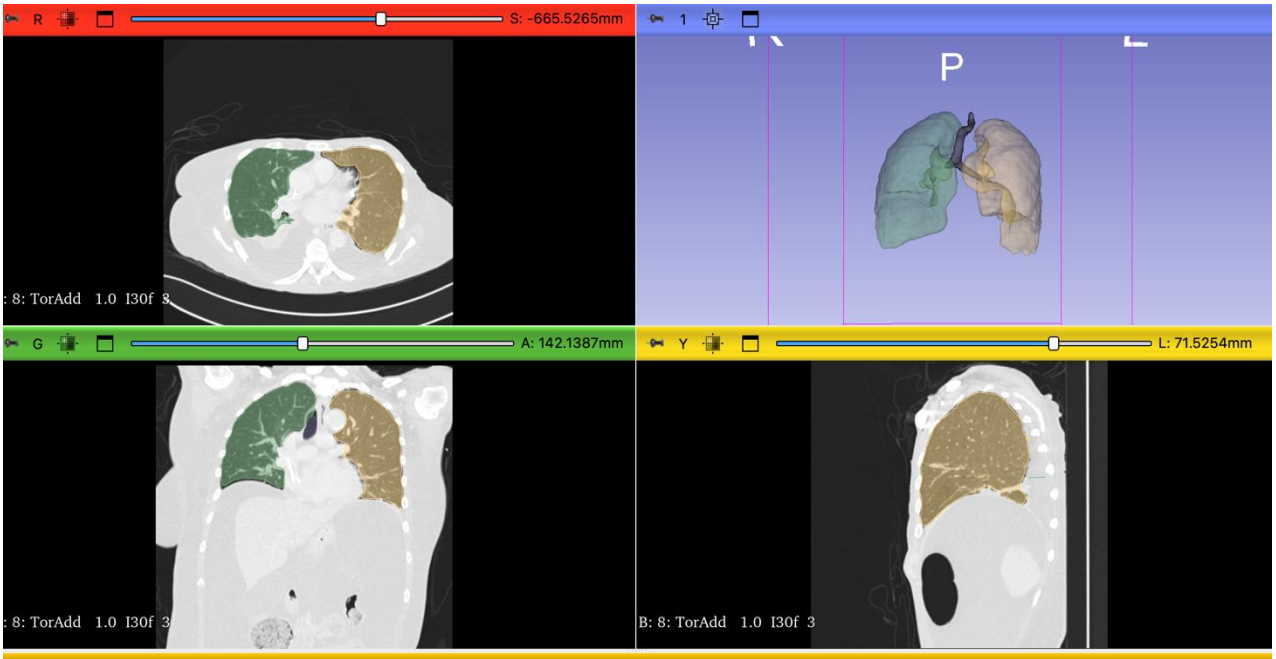


Figure 3. Early signs of pleural involvement may be represented by subtle thickening of pleural layers



Segment	Voxel count	Volume (LM) [mm3]	Volume (LM) [cm3]	Surface area [mm2]	Volume (CS) [mm3]	Volume (CS) [cm3]
1 Segment_1	463270	241934	241.934	36831.1	241773	241.773

Figure 4. Quantification of pleural effusion can be performed with the aid of dedicated tools for image segmentation, such as 3DSlicer.



Segment	Voxel count (LM)	Volume (LM) [mm3]	Volume (LM) [cm3]	Voxel count (SV)	Volume (SV) [mm3]	Volume (SV) [cm3]	Minimum [Hounsfield unit]	Maximum [Hounsfield unit]	Mean [Hounsfield unit]
right lung	1505548	871179	871.179	1474495	853211	853.211	-1024	933	-741.919
left lung	1601339	926608	926.608	1588000	918890	918.89	-1024	1160	-738.333
other	23286	13474.4	13.4744	22685	13126.6	13.1266	-1024	142	-913.572

Figure 5. Pleuro-parenchymal lung involvement may also be inferred from computing residual ventilated lung with the aid of semi-automated segmentation tools

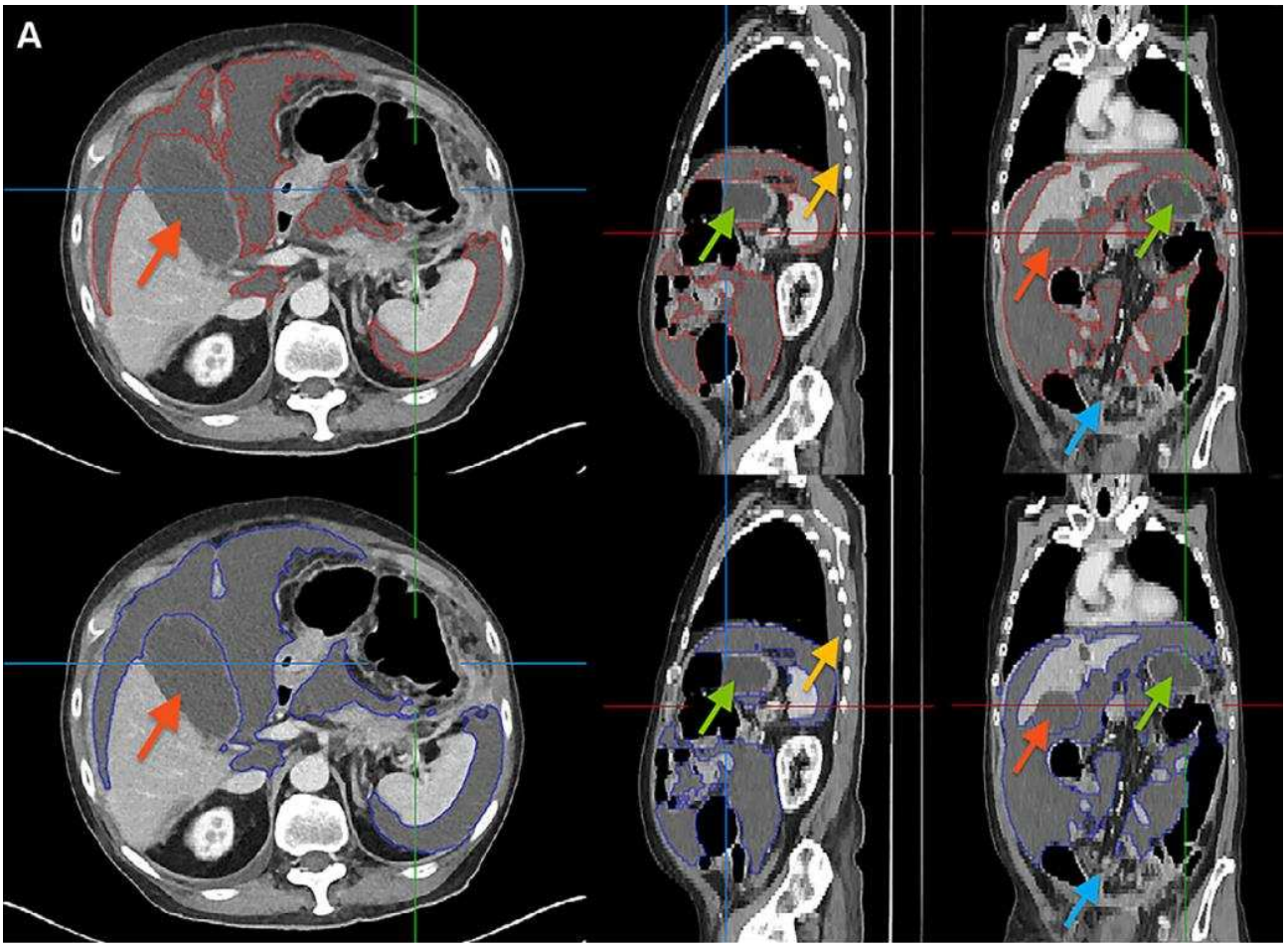


Figure 6. Quantification of peritoneal effusion can be performed with the aid of dedicated tools for fully automated image segmentation (Hou et al, 2024).

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