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Small extracellular vesicles as biomarkers in sarcoma follow-up: Protocol for a pilot study

Valentin Vautrot¹ Dr; Céline CHARON-BARRA²; Alice Hervieu³; Aurélie Bertaut⁴ Dr; Isen Naiken¹; Emilie Rederstoff²; Nicolas Isambert^{5, 6, 7} Pr; Jessica Gobbo^{3, 1}

¹ INSERM UMR 1231, HSP-pathies, Label Dijon FR

² Unit of Pathology, Department of Biology and Pathology of Tumours, Georges-François Leclerc Centre Dijon FR

³ Department of Medical Oncology, Early Phase Unit, Georges-François Leclerc Centre Dijon FR

⁴ Unit of Methodology, Biostatistics and Data Management, Georges-François Leclerc Centre Dijon FR

⁵ Department of Oncology, Pôle Régional de Cancérologie, University Hospital of Poitiers Poitier FR

⁶ INSERM, Centre d'Investigation Clinique 1402 Poitier FR

⁷ PRODICET UR24144 Poitier FR

Corresponding Author:

Jessica Gobbo

INSERM UMR 1231, HSP-pathies, Label

JGobbo@cgfl.fr

Dijon

FR

Abstract

Background: Sarcomas are rare cancer with heterogeneous group of tumours. Metastasis is often fatal, and current prognostic methods are limited. Recent findings show that sarcoma cells release small extracellular vesicles. Studying them can help assess cancer development, progression, and treatment effectiveness.

Objective: This study aims to demonstrate the potential of small extracellular vesicles for monitoring the disease and predicting recurrence risk.

Methods: A multicentric, prospective pilot study that will include 30 adults' patients with localized or metastatic sarcoma. Small extracellular vesicles will be isolated from blood samples and protein and miRNA concentration will be determined. Research will last, on average, 6 months for patients with localized sarcoma and 4 months for patients with metastatic sarcoma. The study is sponsored by the Georges-François Leclerc Center and is currently ongoing.

Results: Our innovative approach could improve sarcoma patient management through a non-invasive technique to predict treatment response and/or disease progression. Clinical Trial: ClinicalTrials.gov identifier NCT03800121. Registered January 11, 2019, <https://clinicaltrials.gov/ct2/show/study/NCT03800121>

Keywords: Pilot-study, liquid biopsy, exosomes, sarcoma, cancer monitoring.

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Original Manuscript

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2 **Protocol for a pilot study**

3 Valentin Vautrot[1,2], Alice Hervieu[3], Aurélie Bertaut[4], Céline Charon-Barra[5], Isen
4 Naiken[3,6], Emilie Rederstoff[5], Nicolas Isambert[7,8,9, *], Jessica Gobbo[1, 2, 3, 6,10*,**]

5 [1] INSERM UMR 1231, HSP-pathies, Label "Ligue National contre le Cancer" and Label
6 d'Excellence LipSTIC, Dijon, France.

7 [2] University of Burgundy, Faculty of Medicine and Pharmacy, Dijon, France.

8 [3] Department of Medical Oncology, Early Phase Unit, Georges-François Leclerc Centre, Dijon,
9 France.

10 [4] Unit of Methodology, Biostatistics and Data Management, Georges-François Leclerc Centre,
11 Dijon, France.

12 [5] Unit of Pathology, Department of Biology and Pathology of Tumours, Georges-François Leclerc
13 Centre, 1 rue du Professeur Marion, 21000, Dijon, France.

14 [6] Faculty of Medicine and Pharmacy, University of Burgundy-Franche-Comté, Dijon, France

15 [7] Department of Oncology, Pôle Régional de Cancérologie, University Hospital of Poitiers,
16 Poitiers, France.

17 [8] INSERM, Centre d'Investigation Clinique 1402, Poitiers, France.

18 [9] PRODICET UR24144, Poitiers, France

19 [10] INSERM, CIC1432, Module plurithématique, U2P, Dijon, France.

20 *Co-direction

21 **Corresponding author: Jessica Gobbo, Centre Georges-François Leclerc, 1, Rue du Professeur
22 Marion, 21079 Dijon; +0033 8 73 77 66, JGobbo@cgfl.fr

23 ABSTRACT

24 Background:

25 Sarcomas are rare cancer with heterogeneous group of tumours. They affect both genders across all
26 age groups and present significant heterogeneity, with more than 70 histological subtypes. Despite
27 tailored treatments, the high metastatic potential of sarcomas remains a major factor in poor patient
28 survival, as metastasis is often the leading cause of death. Currently, metastatic risk assessment relies
29 mainly on histological grading, yet this method has limitations due to the disease's heterogeneity.
30 Advances in genomic and transcriptomic research have identified potential molecular signatures, but
31 these approaches lack reproducibility and prognostic reliability. Therefore, new biomarkers are
32 essential for improving risk prediction and therapy adaptation.

33 Recent studies highlight that sarcoma cells secrete extracellular vesicles (EVs), particularly small EVs
34 (sEVs). These nanovesicles, abundant in bodily fluids such as blood, urine, and saliva, play a crucial
35 role in tumor development, growth, and metastasis. sEVs contain proteins and nucleic acids that
36 mirror tumor characteristics. Given their presence in blood, sEVs offer a promising avenue for non-
37 invasive molecular cancer analysis via liquid biopsy. Preliminary studies in Ewing sarcoma have
38 shown substantial alterations in sEVs-derived transcripts, underscoring their potential in tracking
39 disease progression and treatment efficacy.

40 **Objectives :** This study aims to investigate whether sEVs can serve as reliable biomarkers for
41 monitoring sarcoma progression and predicting recurrence risk.

42 **Methods:** This prospective, multicentric pilot study will enroll adult patients diagnosed with
43 localized or metastatic : liposarcomas, leiomyosarcomas (LMS), or undifferentiated pleomorphic
44 sarcomas (UPS) at three French cancer centers. . The study's primary goal is to quantify sEVs and
45 analyze their protein and RNA content in blood patients with localized or metastatic sarcomas before
46 and after treatment. Small extracellular vesicles will be isolated from plasma samples and protein and
47 miRNA concentration will be determined. Research will last, on average, 6 months for patients with
48 localized sarcoma and 4 months for patients with metastatic sarcoma. The study is sponsored by the

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49 Georges-François Leclerc Center and is currently ongoing.

50 **Results:** We expect to identify differences in exosome levels based on disease stage and
51 observe correlations between exosome dynamics and treatment response. If confirmed, these
52 findings could establish sEV as non-invasive biomarkers for monitoring therapy effectiveness
53 and disease progression in sarcoma patients.

54 **Conclusion:** This study could establish a novel, non-invasive biomarker for sarcoma prognosis and
55 treatment monitoring. If successful, a nationwide study will be launched to confirm findings in a larger
56 patient cohort, potentially revolutionizing sarcoma management and improving patient outcomes.

57 **Trial Registration:** ClinicalTrials.gov identifier NCT03800121. Registered January 11, 2019,
58 <https://clinicaltrials.gov/ct2/show/study/NCT03800121>

59 **Keywords:** Pilot-study, liquid biopsy, exosomes, sarcoma, cancer monitoring.

60 Introduction Sarcomas are rare cancers representing 1-2% of all adult cancers with more
61 than 5,000 cases per year in France recently[1]. They affect men and women of all ages and
62 constitute a heterogeneous group of tumours, with more than 70 different histological types
63 and subtypes[2]. Despite adapted therapies, the metastatic risk of these diseases has currently
64 a major negative impact on patient survival. Metastatic dissemination of tumour cells is
65 responsible in most cases for patient death. The estimation of sarcoma's metastatic risk is
66 complex given the histological heterogeneity of this pathology. It is therefore essential that, at
67 diagnosis, a reliable evaluation of metastatic potential may be determined, to better adapt the
68 therapeutic strategy. Currently, the estimation of this risk is based essentially on histological
69 criteria, namely the tumour grade. In recent years, large-scale genome and transcriptome
70 analysis allowed the identification of tumour molecular signatures predictive of prognosis.
71 However, results reproducibility can be discussed and biological and prognostic significance
72 remains limited. It is therefore necessary to find new biomarkers.

73 It has recently been found that sarcoma tumour cells secrete extracellular vesicles (EV's)[3]
74 including small EV's (sEV). In this study protocol we will use the term exosomes to refer to
75 small EV's. Exosomes are nanovesicles secreted by all the cells of the human body and
76 found in abundance in different biological fluids such as blood, urine, saliva and breast milk.
77 Recently, the role and involvement of exosomes in sarcomas'[4,6] development has been
78 demonstrated[5]. Indeed, exosomes seem to play an important role in tumorigenesis, growth,
79 tumour progression and metastases appearance. Exosomes contain many proteins and nucleic
80 acids (DNA, RNA, microRNA), reflecting tumour characteristics. It has been shown that the
81 amount of exosomes can be correlated with the grade of tumour malignancy[7,8]. Present in
82 the blood, they offer the possibility of a non-invasive analysis of cancer cell molecular
83 information by liquid biopsy. Recent preclinical studies have demonstrated strong changes in
84 more than 1,200 transcripts present in exosomes derived from Ewing3 sarcoma cells.

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85 Therefore, study of plasma-derived exosomes in sarcoma patients has a high potential to
86 evaluate cancer pathogenesis, progression and treatment efficacy.

87 The aim of this study is to demonstrate the potential of exosomes as biomarkers for
88 monitoring disease progression and predicting treatment response in both metastatic and
89 non-metastatic sarcoma patients.

90

91 METHODS**92 Study population**

93 Adult patients with a diagnosed sarcoma, such as liposarcomas, leiomyosarcomas (LMS),
94 undifferentiated pleomorphic sarcoma (UPS) and managed at Georges-François Leclerc
95 Center in Dijon or at Jean Minjoz Center in Besançon, or CHU Poitiers.

96 Ethics approval and consent to participate

97 This study, supported by the Centre Georges-François Leclerc and approved by the local
98 ethics committee, was authorized by the French Health Agency (ANSM- Agence Nationale de
99 la Sécurité du Médicaments et des produits de santé”, n° 2018-A01393-52) in May 2018, and
100 by the French Ethical Research Committee (CPP- “Comité de Protection des Personnes, Ile de
101 France 1”) in October 2018. The clinical trial has been registered at ClinicalTrials.gov with
102 the identifier NCT03800121. Last version dated from 19/05/2021 of the study protocol is 1.4.
103 Participants must provide informed consent in agreement with the Declaration of Helsinki.
104 Subjects will be informed of the objectives of the project and the risks and benefits of the
105 explorations to be carried out. None of the tests will pose risks that could endanger
106 participants life. Confidentiality of participant data will be guaranteed at all times in
107 agreement with the CNIL MR01 reference methodology registered for CGFL (1878714v0,
108 30/07/2015).

109

110 Study objectives and endpoints

111 The main objective of this pilot study is to quantify exosomes from plasma and to analyse
112 protein and RNA content, in patients with sarcoma, with localized or metastatic disease.
113 Analysis will be performed before and after surgery in localized disease and before and after
114 1st line chemotherapy in metastatic sarcoma. Some patients may have received neoadjuvant
115 chemotherapy / radiotherapy.

116 The secondary objectives are to:

- 117 1 Determine whether the initial exosome concentration, protein and RNA profile vary in
118 the plasma with localized or metastatic stage of the disease.
- 119 2 Determine if the exosome concentration as well as protein and RNA profile varies
120 upon treatment.
- 121 3 Determine if the initial exosome concentration (T0) is associated with a response to
122 treatment.
- 123 4 Determine whether changes in exosome concentration before and after treatment is
124 associated with a response to treatment.

125 The primary endpoint of the study is the quantification of exosome concentration in the blood,
126 as well as the measurement of protein and RNA levels after exosome lysis. Measures will be
127 performed before and after surgery in case of localized disease and before and after
128 chemotherapy in case of metastatic disease.

129 The secondary endpoints will be exosome blood concentration, as well as protein and RNA
130 concentrations measured after exosome lysis. Treatment response and tumour progression
131 (locoregional or appearance of metastases) will be assessed by MRI or CT scan using
132 RECIST V1.1 criteria.

133 Study design

134 Pilot, prospective, multicentric, non-randomized study, without direct individual benefit.

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135 Study is supported by the INCa platform CLIP² at the Georges-François Leclerc Center in
136 Dijon, CHU Poitiers and Jean Minjoz Center in Besançon. Study inclusion period will be 48
137 months and study total duration will be 60 months. Study design is depicted in Figure 1.
138 Research will last in average 6 months for patients with localized sarcoma and 4 months for
139 patients with metastatic sarcoma.

140 Investigators receive an informative notice on EXOSARC study and will be provided eligible
141 patients samples. Upon patient written consent, serology to HIV, HCV, and HBV will be
142 tested (Figure 1). Only patients with negative serology for these infectious agents will be
143 included, to protect technicians from biological risks, in agreement with laboratory
144 procedures. Exact inclusion and exclusion criteria are available in table 1.

145 **Table 1. EXOSARC study inclusion and exclusion criteria****Inclusion criteria**

Men and women diagnosed with localized or metastatic soft tissue sarcoma (liposarcoma, LMS or UPS).

Adult patients (≥ 18 years old) with histologically confirmed sarcoma, including but not limited to liposarcomas, leiomyosarcomas (LMS), and undifferentiated pleomorphic sarcoma (UPS).

Previous treatment of the disease with chemotherapy, radiotherapy or surgery is allowed if it has been completed for more than 12 months at the time of inclusion.

Age ≥ 18 years old.

For metastatic or locally advanced (inoperable) sarcoma, patients for whom first-line metastatic chemotherapy is indicated.

Affiliated to French social security social or beneficiary of such a regimen.

Written informed consent.

Exclusion Criteria

Patient with other synchronous tumors

Patient with sarcoma in irradiated tissue.

Patient with a history of cancer other than sarcoma in the 5 years preceding sarcoma diagnosis.

Patient unable to undergo medical follow-up for geographical, social or psychological reasons,

Person benefiting from an adult protection system (including guardianship and trusteeship).

Positive serology to HIV and/or HBV and/or HCV.

Pregnant or lactating woman.

Patients unable to understand, read and/or sign an informed consent.

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151 Patient Identification and Recruitment:

152 Patients will be identified and recruited from participating oncology centers through:

153 • Diagnosis codes (ICD-10) and pathology reports from hospital databases.

154 • Referral by treating oncologists and multidisciplinary sarcoma teams based on

155 eligibility criteria.

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- Screening during routine clinical visits for newly diagnosed sarcoma patients or those scheduled for treatment.

Sampling

32 ml of blood (8x 4ml EDTA tubes) will be collected at each sampling for exosome analysis in both cohorts.

- For cohort 1; patients with localized sarcoma and undergoing neoadjuvant chemotherapy; a blood samples will be collected before surgery at cure 1 (sample 1) and at the last cure preceding surgery (sample 2). Then at 1 month (sample 3) and 6-8 months post-surgery (sample 4).
- For cohort 2; patients with metastatic or locally advanced inoperable sarcoma; a blood sample will be collected before initial chemotherapy administration (Sample 1). And then, at the third (sample 2) and at the 6th chemotherapy treatment (sample 3).

Note that sample collection will take place before chemotherapy treatment even if cure schedule is changed or postponed. Samples will be kept for a maximum of 2 hours at 4 °C.

Blood collection and storage

Plasma will be collected by centrifugation of blood samples (EDTA tube) (2200 rpm for 15 minutes at 4°C). All the three components obtained will be aliquoted and stored on site, at -80°C.

Extracellular vesicle/ Exosome analysis

Thawed plasma samples will process to centrifugation at 2,000 g , 4°C ,20 minutes. Exosome will be extract with Total sEV Precipitation Reagent (4484450, Thermo Fisher Scientific) for 10 minutes following homogenization. Part of the extracellular vesicles will be used to determine their concentration and distribution profile using Nanotracking Analysis(NS300, Malvern). The data will analyze using Nanosight NTA 3.2 Analytical Software (Malvern Instruments, Malvern, the UK). The

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other part will be used for the analysis of extracellular vesicle contents (proteins, RNA) and stored at -80° C. Content analysis (proteins/RNA) will be performed using measured using Western blot analysis, an enzyme-linked immunosorbent assay (ELISA) and RNAseq (illumina) approaches. It is expected that all collected blood will be used for extracellular vesicles and content analysis.

Data collection

The following data will be collected during the study, at the time of inclusion and during medical follow-up: demographic and clinical data (age, sex, weight, height, performance status, ...); medical history, date of diagnosis; 1st line treatment, disease evaluation reports (biological, imaging,), exosome analysis data (total exosome concentration, HSP70 exosome concentration and multiple miRNA dosing) will be sent as a standard report from the laboratory in charge of these analysis (Inserm UMR1231– 7 boulevard Jeanne d’Arc, 21000, Dijon). All data will be recorded in a Case Record Form (CRF).

Sample size

A total of 30 patients, for whom all samples required for the study have been collected, should be included, distributed as follows:

- Group 1: 15 patients with localized disease, for whom the 4 samples required for the study were collected.
- Group 2: 15 patients with metastatic disease, for whom the 3 samples required for the study were collected.

These numbers will provide preliminary data before considering a larger study. The primary objective of this study is to measure the markers of interest in patients. Given that the study focuses on rare cancers and that statistical analyses are only secondary objectives, no statistical power calculation has been performed for this study. Patients for whom the total number of required samples was not achieved will be replaced.

Statistical analysis

Plasma exosomes as well as exosomal protein and RNA concentrations will be described by

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208 their mean (with standard deviation) and their median (with extent). Comparison of baseline
209 concentrations of subjects with localized disease versus metastatic disease will be performed
210 using non-parametric Wilcoxon tests. Comparison of pre- and post-treatment (surgical or
211 drug) concentrations, will be performed using non-parametric tests for matched data. Analyses
212 will be carried out by subgroup (metastatic and localized) and on the whole population.
213 Relative exosome variation before and after treatment will be compared in responders and
214 non-responders using non-parametric Wilcoxon tests. A Bonferroni correction will be applied
215 to the p-value to adjust the significance threshold during multiple comparisons.

216 The area under the ROC curve (AUC) modelling the response to treatment in function of
217 initial exosome concentration will be plotted. AUC with 95% confidence interval will be
218 provided. Model will be adjusted to the type of disease (localized or metastatic). An exosome
219 concentration threshold, predictive of response to treatment will be determined. Performance
220 (i.e. sensitivity, specificity and predictive values) associated with this threshold will be
221 calculated. A similar approach will be followed concerning exosome concentration before and
222 after treatment. Different protein and RNA-derived markers will be analysed. Performance
223 associated to the absence or presence of these markers to predict response to treatment will be
224 determined for each of the markers. Analyses will be performed under the software SAS 9.4.

225 **Patient and Public Involvement**

226 The patient committee of the National League Against Cancer (“Comité de patients en
227 recherche clinique en cancérologie de la Ligue contre le cancer”) was consulted on the
228 information notice that will be given to patients asked to participate in this trial. This
229 committee brings together patients, former patients or patient relatives who have a shared
230 interest in Oncology medical research. The purpose of this consultation was to read and
231 improve the patient information notice on the design and aim of the study, so that patients can
232 clearly understand and decide whether to participate, in agreement with the principle of
233 informed consent.

234 **RESULTS**

235 This study is expected to provide valuable insights into the role of exosomes in sarcomas, both in
236 localized and metastatic stages. We anticipate that exosome concentration, as well as protein and RNA
237 profiles, will differ between localized and metastatic disease, reflecting distinct tumour biology.
238 Furthermore, we expect to observe variations in exosome concentration and content before and after
239 treatment, potentially correlating with treatment response. A higher baseline exosome concentration
240 may be associated with a poorer or more aggressive disease profile, while changes in exosome levels
241 following treatment could serve as predictive biomarkers for therapeutic efficacy. The integration of
242 exosome analysis with imaging-based assessments (MRI or CT scan using RECIST V1.1 criteria) will
243 help establish correlations between exosome dynamics and tumour progression. If confirmed, these
244 findings could pave the way for the use of exosomes as non-invasive biomarkers for monitoring
245 sarcoma treatment response and disease progression, ultimately improving patient management.

246 **DISCUSSION**

247 As of 2025, despite the strong diagnostic and prognostic potential of exosomes in sarcomas [3,7–10],
248 no prospective study has yet demonstrated the feasibility and clinical relevance of this concept. Our
249 team has recently patented a technique to detect exosomes in various biological fluids, such as blood
250 and urine [6] (Patent WO/2015189395A1, Inserm Transfer). We have also launched a study on
251 exosomes from solid tumours, analyzing their protein content and microRNA (Exodiag,

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252 NCT02662621), which has confirmed the technical feasibility of exosome research in humans.
253 Notably, this study is currently the only ongoing clinical trial on sarcomas listed on ClinicalTrials.gov.
254 Consistent with preclinical findings, we believe that studying plasma exosomes in rare cancers like
255 sarcomas could significantly improve therapeutic management.

256 Looking ahead to 2030, our innovative approach has the potential to offer a non-invasive method for
257 predicting treatment response and disease progression. If our pilot study yields positive results, we
258 plan to conduct a nationwide study to validate these findings in a larger cohort.

259 List of Abbreviations

260 LMS: leiomyosarcomas; UPS: undifferentiated pleomorphic sarcoma; MRI: Magnetic
261 resonance imaging; CT scan: Computed Tomography scanner; EV's: extracellular vesicles,
262 sEV : small extracellular vesicles, RECIST: Response Evaluation Criteria in Solid Tumours;
263 HIV: Human Immunodeficiency Virus; HCV: Hepatitis C virus; HBV: Hepatitis B virus;
264 CRF: Case Record Form; ROC : Receiver Operating Characteristic; AUC: Area Under Curve.

265

266 **Data Availability**

267 The investigator undertakes, on behalf of himself and all individuals involved in the conduct
268 of the trial, to ensure the confidentiality of all information provided by the Centre Georges
269 François Leclerc until the publication of the trial results. This confidentiality obligation shall
270 not apply to information that the investigator is required to disclose to patients as part of their
271 participation in the trial, nor to information that has already been published.

272 The investigator commits not to publish, disclose, or use, in any manner whatsoever, directly
273 or indirectly, any scientific or technical information related to the trial.

274 Any partial or total reproduction of this document is prohibited without the authorization of
275 the Centre Georges François Leclerc. However, in accordance with Article R 5121-13 of the
276 French Public Health Code, the center and the investigator may provide information related to
277 the trial to: the Minister of Health, public health inspector physicians, public health inspector
278 pharmacists, the Director General and inspectors of the ANSM (French National Agency for
279 Medicines and Health Products Safety).

280 The trial shall not be subject to any written or oral comments without the sponsor's approval
281 (CGFL); all information communicated or obtained during the trial shall be the exclusive
282 property of the sponsor, who shall have full discretion over its use.

283 At the end of the study, research-related documents (Investigator File, completed and
284 validated CRFs, and other research documents) will be archived by the sponsor and the
285 investigator sites in accordance with Article 2 of the decree of August 11, 2008, which sets the
286 retention period at 15 years.

287 **Competing interests**

288 The authors declare that they have no competing interests.

289 Funding

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291 Cancer”, “Institut National du Cancer”, “Centre Georges-François Leclerc“, NanoDiag,
292 “Cancéropôle Est” and “Conseil Régional Bourgogne-Franche-Comté”.

293 Authors’ contributions (CRediT)

294 Supervision : JG, NI , Methodology : AB, Writing – original draft : JG, NI, CB, VV, ER, AH,
295 IN, Writing – review & editing : JG, IN.

296 JG and NI initiated and designed this protocol, with AB providing expertise on the
297 methodology of the study. All authors edited the protocol and agreed with the final
298 manuscript.

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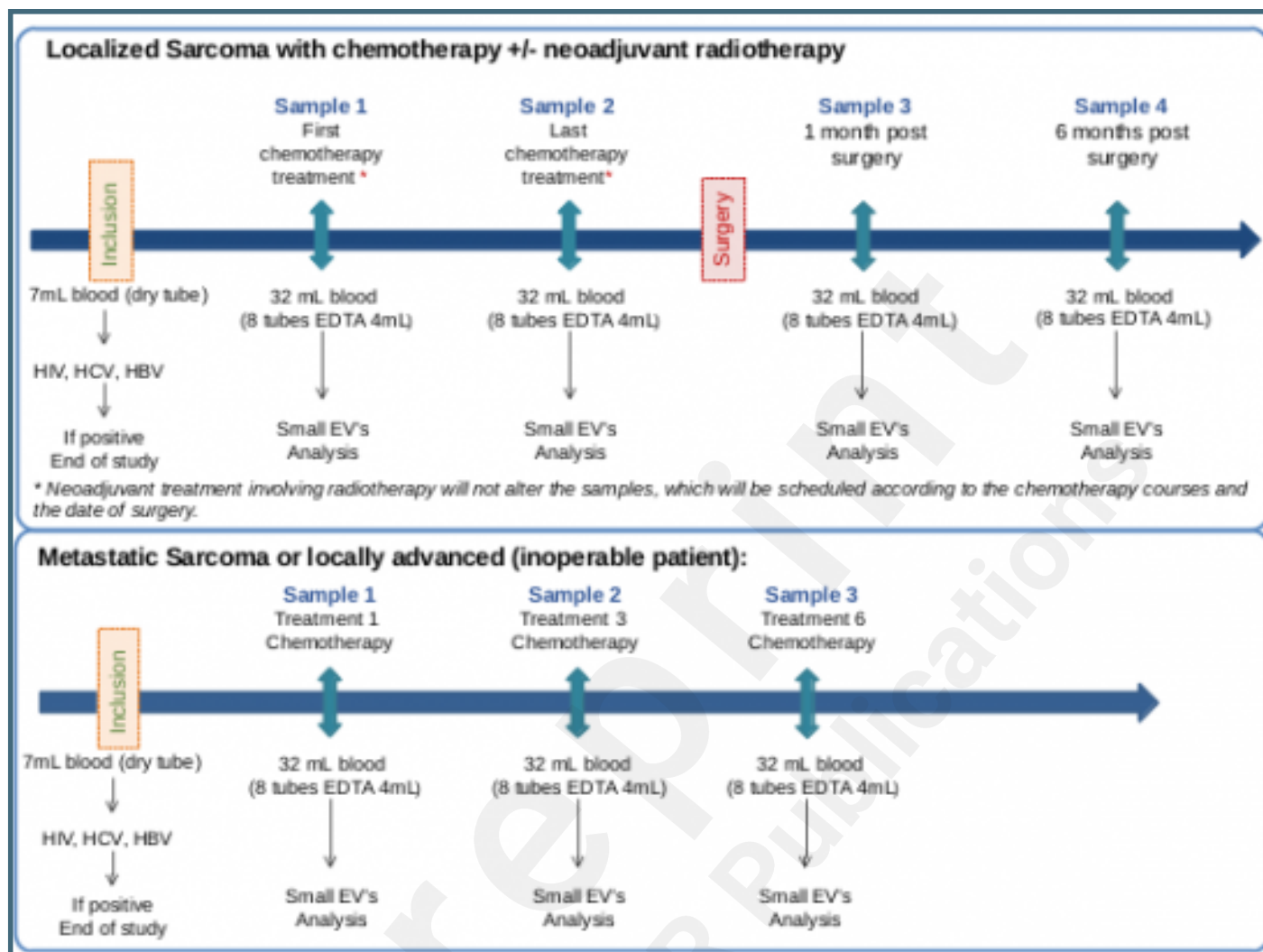
332 **Figure 1:** Study Design



Supplementary Files

Figures

Study Design.



CONSORT (or other) checklists

Spirit-Consort.

URL: <http://asset.jmir.pub/assets/fea2342088d6359d9bd9b61c9e145f02.pdf>

Consort Checklist.

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