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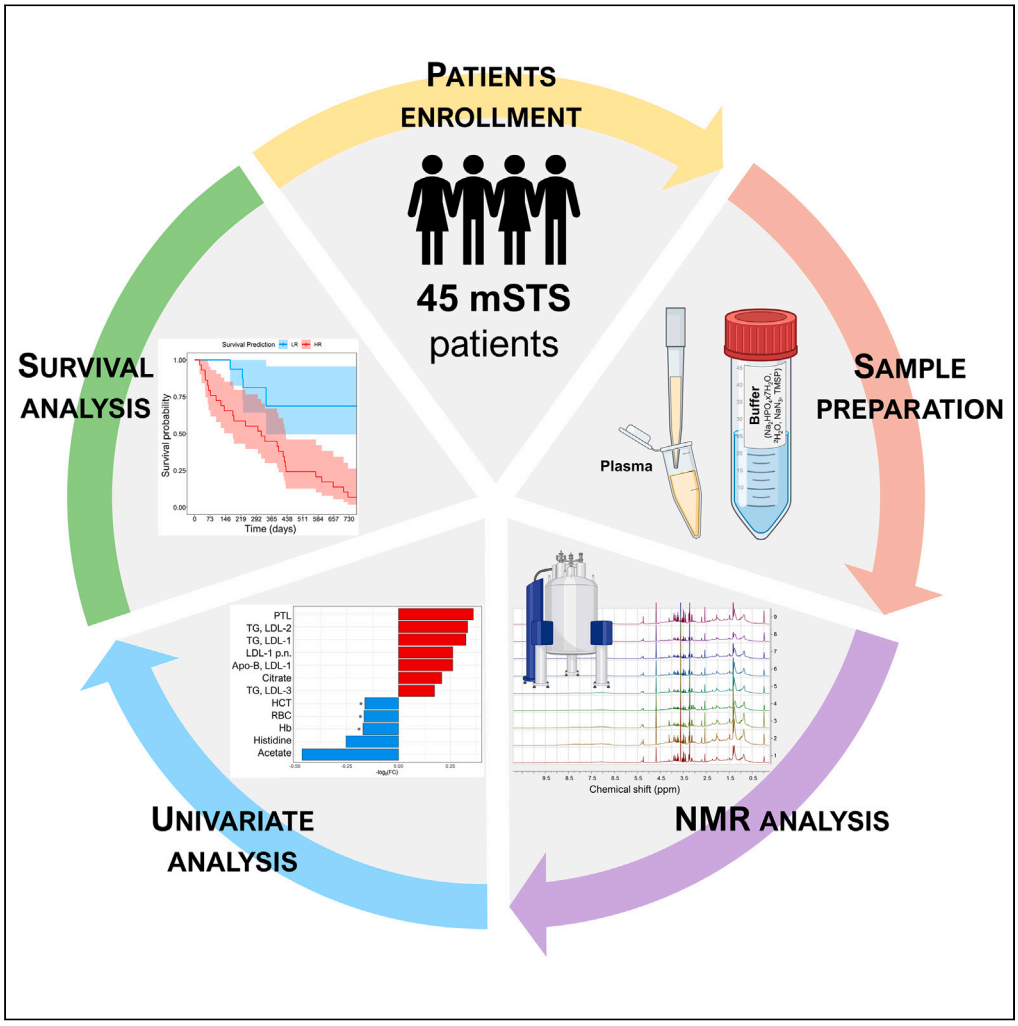

Alessia Vignoli, Gianmaria Miolo, Leonardo Tenori, ..., Simona Scalone, Claudio Luchinat, Giuseppe Corona

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Highlights

Soft tissue sarcomas are rare malignant tumors that are difficult to prognosticate

A score combining acetate, TG LDL-2, and RBC count predicts 2-year survival

This score is statistically significant and independent of other prognostic factors

A nomogram based on these 3 biomarkers has been developed to inform the clinical use

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Novel metabolomics-biohumoral biomarkers model for predicting survival of metastatic soft-tissue sarcomas

Alessia Vignoli,[1,6] Gianmaria Miolo,[2,6] Leonardo Tenori,[1,3] Angela Buonadonna,[2] Davide Lombardi,[2] Agostino Steffan,[4] Simona Scalone,[2] Claudio Luchinat,[3,5] and Giuseppe Corona[4,7,*]

SUMMARY

Soft tissue sarcomas (STSs) are rare malignant tumors that are difficult to prognosticate using currently available instruments. Omics sciences could provide more accurate and individualized survival predictions for patients with metastatic STS. In this pilot, hypothesis-generating study, we integrated clinicopathological variables with proton nuclear magnetic resonance (^{1}H NMR) plasma metabolomic and lipoproteomic profiles, capturing both tumor and host characteristics, to identify novel prognostic biomarkers of 2-year survival. Forty-five metastatic STS (mSTS) patients with prevalent leiomyosarcoma and liposarcoma histotypes receiving trabectedin treatment were enrolled. A score combining acetate, triglycerides low-density lipoprotein (LDL)-2, and red blood cell count was developed, and it predicts 2-year survival with optimal results in the present cohort (84.4% sensitivity, 84.6% specificity). This score is statistically significant and independent of other prognostic factors such as age, sex, tumor grading, tumor histotype, frailty status, and therapy administered. A nomogram based on these 3 biomarkers has been developed to inform the clinical use of the present findings.

INTRODUCTION

Soft tissue sarcomas (STSs) are a heterogeneous group of malignant tumors of mesenchymal origin. Despite being rare as compared to other tumors, their incidence continues to steadily increase, and they are associated with relevant morbidity and mortality, in particular in young adults where certain sarcoma subtypes are among the leading causes of death.[1,2]

The prediction of survival for patients diagnosed with STS is challenging as the currently available tools such as tumor-node-metastasis staging systems are limited in their accuracy.[3] To address the limitations of the current approach, there is a growing need for personalized prognostic markers able to provide a more accurate and individualized prediction of STS patient outcomes, considering individual patient characteristics.

By utilizing personalized markers in treatment, oncologists can approach STS patients with a more informed and effective strategy. This can result in more efficient and effective treatment plans, customized to meet the unique needs of each patient, and ultimately improve their quality of life. Omics sciences could represent one potential answer to this need. Circulating blood metabolites and lipoproteins provide a picture of patients and of their biological characteristics within the dynamic context of cancer processes, considering both the effects the tumor exerts upon the host and the effects the host exerts upon the tumor.[4] Thus, metabolite/lipoprotein alterations could be used as potential biomarkers of cancer prognosis and patient survival.[5–8]

In the current pilot, hypothesis-generating study we utilized blood plasma metabolomics and lipoproteomics, via proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, coupled with standard blood analysis, to predict 2-year survival in a cohort of 45 metastatic STS (mSTS) patients. We proposed a 3-biomarker-based model and compared its performance with that of other prognostic parameters (age, sex, tumor grading, tumor histotype, frailty status, and trabectedin therapy). This new model shows to be more accurate (accuracy 84.4%) with respect to the tumor grade (accuracy 77.8%). Moreover, we proposed a nomogram based on the three biomarkers that could allow clinicians to have an easy but personalized prediction of patient outcomes.

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Table 1. Demographic and clinical characteristics of the study population

	Study Population (45 patients)	2-year survived (13 patients)	2-year deceased (32 patients)
Age (yrs), median (min-max)	66 (37–90)	66 (37–76)	69 (37–90)
Sex (M), n (%)	24 (53)	9 (69)	15 (47)
BSA, median (min-max)	1.9 (1.4–2.8)	2 (1.8–2.5)	2 (1.4–2.8)
BMI, median (min-max)	26.5 (15.6–45.1)	27 (22.9–36.8)	26 (15.6–45.1)
Trabectedin therapy (2° line), n (%)	29 (64)	6 (46)	23 (72)
Frail, n (%)	19 (42)	7 (54)	12 (38)
Histotype, n (%)			
L-sarcomas	19 (42)	9 (69)	10 (31)
Other sarcomas	26 (58)	4 (31)	22 (69)
Grading, n (%)			
G1	1 (2)	1 (8)	0 (0)
G2	12 (27)	7 (54)	5 (16)
G3	32 (71)	5 (38)	27 (84)

RESULTS

Characteristics of enrolled patients

The analyses were conducted on 45 mSTS patients; their survival status was evaluated at two years from sample collection. At that moment 32 patients were deceased (71%) and 13 patients were alive (29%). Demographic and clinical characteristics of the study population are reported in Table 1 for the entire population and stratified by survival status.

Univariate analysis of metabolites, lipoproteins-related parameters, and clinical data

A total of 31 metabolites were quantified in all plasma spectra and used for our analyses. These include amino acids and their derivatives (alanine, creatine, creatinine, glutamine, glycine, histidine, isoleucine, leucine, methionine, N,N-Dimethylglycine, ornithine, phenylalanine, sarcosine, tyrosine, valine), carboxylic acids (acetate, citrate, formate, lactate, succinate), keto bodies and derivatives (3-hydroxybutyrate, acetoacetate, acetone, pyruvate), and other compounds such as glucose, mannose, dimethylsulfone, trimethylamine-N-oxide, 3-Methyl-2-oxovalerate, GlycA, and GlycB. Univariate analysis of these data (Table S1) showed that only acetate (area under the receiver operating characteristic curve [AUROC]: 0.74, p value: 0.01), citrate (AUROC: 0.70, p value: 0.04), and histidine (AUROC: 0.69, p value: 0.04) differ significantly between 2-year deceased and survived patients (although none of them remains statistically significant after false discovery rate [FDR] correction).

The univariate analysis of the panel of 112 lipoprotein-related parameters quantified (Table S2) showed that the levels of triglycerides subfraction low-density lipoprotein (LDL)-2 (AUROC: 0.81, p value: 0.001), triglycerides subfraction LDL-1 (AUROC: 0.74, p value: 0.01), triglycerides subfraction LDL-3 (AUROC: 0.72, p value: 0.02), Apolipoprotein-B (Apo-B) subfraction LDL-1 (AUROC: 0.70, p value: 0.04), and LDL-1 particle number (AUROC: 0.69, p value: 0.04) were statistically different (only before FDR correction) between 2-year deceased and survived patients.

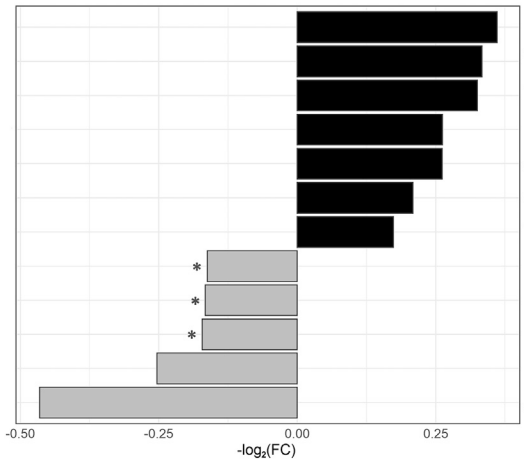
A panel of 32 demographic data (age, weight, height, BSA, BMI) and biohumoral blood parameters (complete blood count, urea, uric acid, aspartate transaminase [AST], alanine transaminase [ALT], albumin, alkaline phosphatase [FAL], lactate dehydrogenase [LDH], bilirubin T, bilirubin D, protein T, Na^+, K^+, Cl^-, calcium, C-reactive protein, vitamin D) were measured in all patients. Univariate analysis (Table S3) demonstrated that the levels of red blood cell (RBC) count (AUROC: 0.82, p value: 0.0009), hematocrit (HCT, AUROC: 0.81, p value: 0.001), hemoglobin (Hb, AUROC: 0.80, p value: 0.002), and platelet count (PTL, AUROC: 0.70, p value: 0.03) significantly differ between 2-year deceased and survived patients, and, with the exception of PTL, they remain significant even after FDR correction.

The trends of the 12 parameters that globally discriminate between the two groups of patients are reported in Figure 1. We chose to consider and discuss both results adjusted and not adjusted for multiple comparisons to decrease the risk of missing promising biomarkers. However, we are aware that this could increase the risk of a type I error.

Metabolites, lipoprotein-related parameters, and clinical data (demographic and biohumoral parameters) were analyzed in relation to the histotypes via univariate analysis. Histidine (AUROC: 0.70, p value: 0.02), LDL-Cholesterol (AUROC: 0.68, p value: 0.04), triglycerides LDL-4 subfraction (AUROC: 0.68, p value: 0.04), BMI (AUROC: 0.71, p value: 0.01), LDH (AUROC: 0.70, p value: 0.03), BSA (AUROC: 0.68, p value: 0.04), and weight (AUROC: 0.68, p value: 0.04) differ significantly between leiomyosarcoma/liposarcoma (L-sarcomas) and other sarcomas, although none of them remains statistically significant after FDR correction.

Construction of a combined score predictive for 2-year mortality

The most discriminating metabolite (acetate), lipoprotein-related parameter (triglycerides, LDL-2), and clinical variable (RBC count) between 2-year deceased and survived patients were integrated into a unique score by logistic regression (none of these features significantly differ between L-sarcomas and other sarcomas). The following equation describes the calculated model:



	Accuracy	Sensitivity	Specificity	AUROC
PTL	67%	59%	85%	0.7
Triglycerides, LDL-2	**82%**	**78%**	**92%**	**0.81**
Triglycerides, LDL-1	64%	53%	92%	0.75
LDL-1 Particle Number	53%	34%	100%	0.69
Apo-B, LDL-1	53%	34%	100%	0.7
Citric acid	71%	69%	77%	0.7
Triglycerides, LDL-3	69%	66%	77%	0.72
HCT	76%	72%	85%	0.81
RBC	**78%**	**75%**	**85%**	**0.82**
Hb	76%	78%	69%	0.8
Histidine	58%	44%	92%	0.69
Acetic acid	**71%**	**69%**	**77%**	**0.74**

Figure 1. Values of -log$_2$ fold change (FC) of the 12 parameters that differ between 2-year deceased and survived patients

Positive/negative values have higher/lower concentration in plasma samples from deceased patients with respect to survived patients. Parameters that remain statistically significant after FDR correction are marked with asterisks. The accuracy, sensitivity, specificity, and AUROC of each variable are reported. Each optimal cut point was determined by maximizing the Youden-Index.

$$log\left(\frac{p}{1-p}\right) = 1.8 - 0.55[Acetic\ acid] + 1.59[Triglycerides\ LDL2] - 1.32[RBC]$$

This combined score was able to predict 2-year survival with optimal cross-validated results: AUROC 0.875, sensitivity 84.4%, specificity 84.6%, and accuracy 84.4%. This score results in an increase of about 10% sensitivity compared with each single variable. Furthermore, analyzing this combined score with Kaplan-Meier curves (Figure 2), a clear discrimination is evident between the two groups of patients, demonstrated by a p value of 0.0001 and a hazard ratio (HR) of 5.15. When assessed individually, acetate and triglycerides LDL-2 did not reach the statistical significance after FDR correction in the comparison between survivors and deceased patients. They, however, contributed to the model performance significantly. This means that only the combination of the three variables is potentially useful as prognostic test.[9,10]

Interestingly, the 2-year survival status was significantly different between high- and low-risk groups of patients identified by the 3-biomarker combined score even when patients were stratified according to the trabectedin therapy or the tumor histotype (Figure 3).

Comparison of the combined score with other prognostic factors

The predictive performance of the 3-biomarker combined score was compared with that of age, sex, tumor grading, tumor histotype, frailty status, and trabectedin therapy via Cox proportional hazards model. The prognostic effect of the 3-biomarker combined score was independent of the other covariates, and it is the only one statistically significant as shown by the Cox multivariate analysis (Figure 4). Among the tested covariates, only the tumor grade showed near significance, with grade 3 (G3) patients associated with the worst prognosis (as expected). In the study population, the proposed model showed higher HR (4.15 vs. 2.67) as well as higher accuracy (84.4% vs. 77.8%) as compared to tumor grade in predicting 2-year survival.

Nomogram for 2-year risk of death estimation

The nomograms tool is frequently adopted for cancer prognosis since it simplifies complex statistical predictive models to a single numerical estimate model able to predict the probability of an event, such as 2-year cancer death. We built a nomogram model using acetate concentration, triglycerides LDL-2 concentration, and RBC count (Figure 5). These three variables are independent and differently concur in the nomogram score making their combination more clinically important to predict 2 years patient outcome. To use the nomogram, locate the patient's RBC count and draw a line straight up to the points axis to establish the score associated with an RBC count. Repeat for the other two covariates (acetate and triglycerides LDL-2 concentrations). Add the scores for each covariate together and locate the total score on the total points axis; finally, from the total score draw a line straight down to the risk of death axis to obtain the survival probability. Acetate concentration, being the less discriminating covariate, can contribute to the total points with a maximum of 27 points. Triglycerides LDL-2 concentration and RBC count can contribute with a maximum of 100 points and 80 points, respectively. A total score <61 points is associated with a risk of death <10%, and a total score >104 points is associated with a risk of death >90%.

DISCUSSION

Inter-patient variability in response, time to progression, and overall survival (OS) can be only partially explained by clinicopathological characteristics. Therefore, an increasing attention should be focused also on systemic biochemical and omic variables to accurately predict

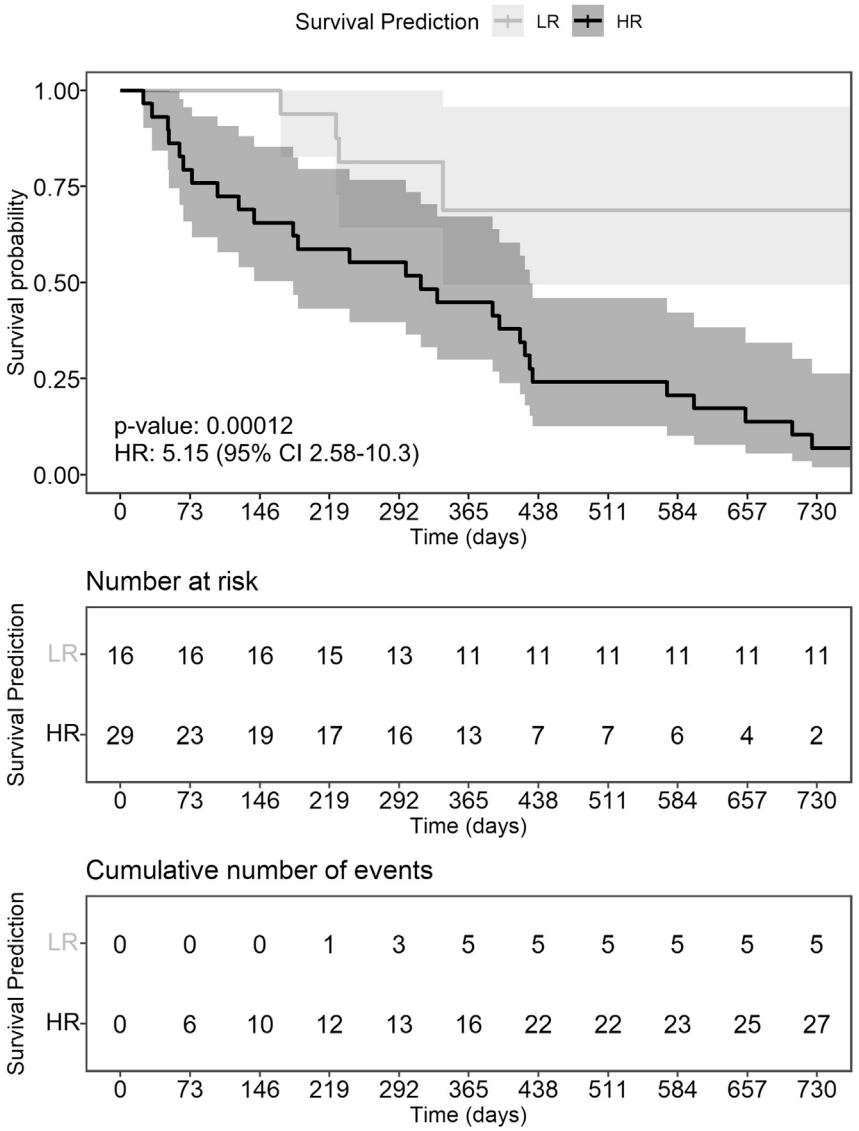


Figure 2. Overall mSTS patients according to risk score of combined acetate, triglycerides, LDL-2, and red blood cell count biomarkers

High-risk (black) group is significantly clustered with respect to the low (gray)-risk group with hazard ratios of 5.15. The number at risk: number of patients stratified according to the combined score at each time point. Cumulative number of events: total number ofdeceased patients at each time point for each risk group based on the combined score. p values are calculated with the log-rank test. LR: predicted by the combined score at low risk of death; HR: predicted by the combined score at high risk.

the different clinical trajectories of cancer patients. It is emerging that tumor and host features can deeply interact with each other, determining clinical phenotypes strongly associated with the pharmacological response and overall clinical outcomes.[11,12] These phenotypes can be revealed by applying omic approaches able to integrate both tumor and host characteristics into biochemical fingerprints of biological matrices.[13] Their identification is particularly important for STS, which represents a heterogeneous group of rare tumors. These neoplasms are complex to monitor by time course tissue biopsies, thus making them particularly difficult to classify and prognostically manage.

Analysis of biosamples via nuclear magnetic resonance (NMR) spectroscopy is particularly suitable for clinical investigations since it is high throughput, intrinsically quantitative over a wide dynamic range, and reproducible.[14,15] In this study, 31 plasma metabolites and 112 lipoprotein-related parameters, quantified by NMR, were combined with 32 demographic and blood variables to identify specific circulating metabolic features associated with 2-year survival in 45 mSTS patients undergoing trabectedin treatment. The Cox regression analysis revealed that low RBC count, reduced plasma levels of acetate, and high triglycerides LDL-2 subfraction were independent factors for poor prognosis in mSTS patients. The low RBC count, as expected, was found strongly related to both HCT and Hb levels and poor OS. The relationship

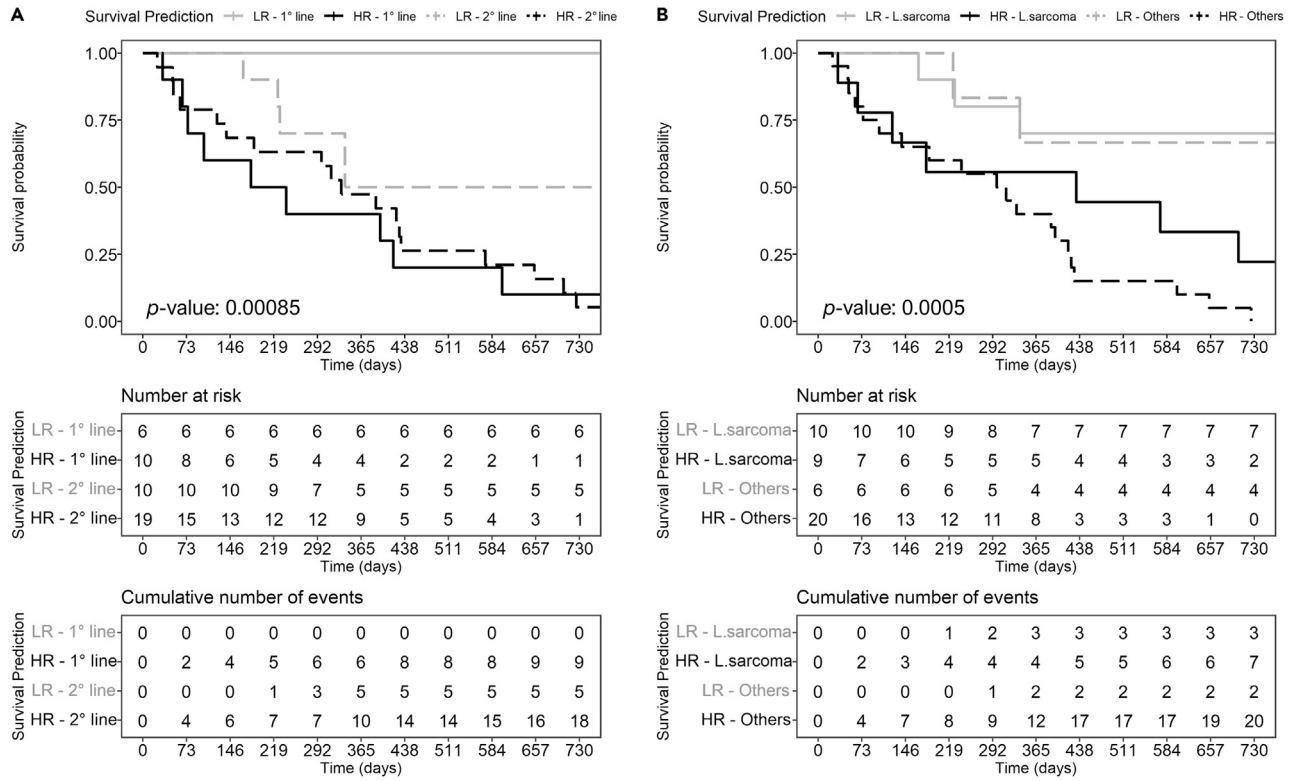


Figure 3. Overall survival as function of combined score for high and low risk mSTS patients

The patients are stratified by (A) trabectedin therapy: low risk for first-line treatment (gray line), high risk for first-line treatment (black line), low risk for second-line treatment (gray dashed line), high risk for second-line treatment (black dashed line) and by (B) Histotype: low risk for patients with L. sarcoma (gray line), high risk for patients with L.sarcoma (black line), low risk for patients with other sarcomas (gray dashed line), high risk for patients with other sarcomas (black dashed line). LR: predicted by the combined score at low risk of death; HR: predicted by the combined score at high risk.

between RBC count, Hb levels, and OS has not been uniquely established; however, the results of the present investigation seem to confirm a deep interconnection between pre-treatment anemia and the prognosis of mSTS patients, as previously observed.[16–21] Reduced levels of Hb promote tumor hypoxia, which represents the initial point for triggering microenvironmental structural modifications. These modifications are characterized by the formation of abnormal neoangiogenic networks as well as cell-cycle functional modifications.[22] The latter lead to metabolic rewiring of tumor cells mainly involving *de novo* lipid biosynthesis.[23,24] The low level of acetate observed in patients with poor OS may emerge directly from such specific metabolic modifications that make the cancer phenotype more aggressive.[23,25] Circulating acetate derives principally from dietary sources and gut prokaryotic metabolism.[26] In the mSTS patients investigated, BMI and biochemistry parameters such as AST, ALT, urea, and albumin resulted not significantly different between the groups with good and poor prognosis. This indicates that neither anorexia or cachexia nor other pathophysiological malnutrition conditions were present. Moreover, no further metabolic differences in gut-derived metabolites (e.g., trimethylamine-N-oxide, formate) have been found, which would have implied a differential microbiome metabolism or dysbiosis among the two groups of patients.

The acetate is a pivotal player in tumor metabolism.[27–29] Acetyl-coenzyme A (acetyl-CoA) is an active precursor of cellular lipid biosynthesis that under physiological conditions derives almost exclusively from glucose metabolism. Conversely, under metabolic stress conditions, as in neoplastic diseases, acetyl-CoA derives from acetate, reflecting the cancer cell metabolic reprogramming.[30] Indeed, STSs, like ovarian, breast, and lung cancers, are often characterized by high expression levels of enzymes involved in acetate metabolism such as the acetyl-CoA synthetase (ACSS) family.[31–34] Moreover, acetyl-CoA, besides its role as a bridge molecule between glycolysis, tricarboxylic acid (TCA) cycle, and lipid synthesis, represents the key acetyl donor for cell-cycle proteins: notably, histones, hypoxia-inducible factor2 alpha, transcription factor EB, and interferon regulatory factor 4,[26,34–36] all affecting tumor growth and development. In this context, the low circulatory acetate observed in poor-prognosis patients can be explained by a higher tumor cellular uptake of this metabolite for promoting the lipid biosynthesis associated with cancer cells survival and progression.[37] In attempt to support this hypothesis, we analyzed genetic dataset from the STSs repository of The Cancer Genome Atlas (TCGA) to explore the potential alterations associated with the ACSS2 gene. Interestingly, in a cohort of 22 STS patients, including both soft tissue tumors and sarcoma, no genetic mutations in the ACSS2 gene have been reported in the tumor tissues. However, all cases exhibited copy number variations (CNVs) of the ACSS2 gene that enhanced the function of the ACSS2 enzyme (gain-of-function) which indirectly support the increased consumption of plasma acetate for cancer needs as documented in

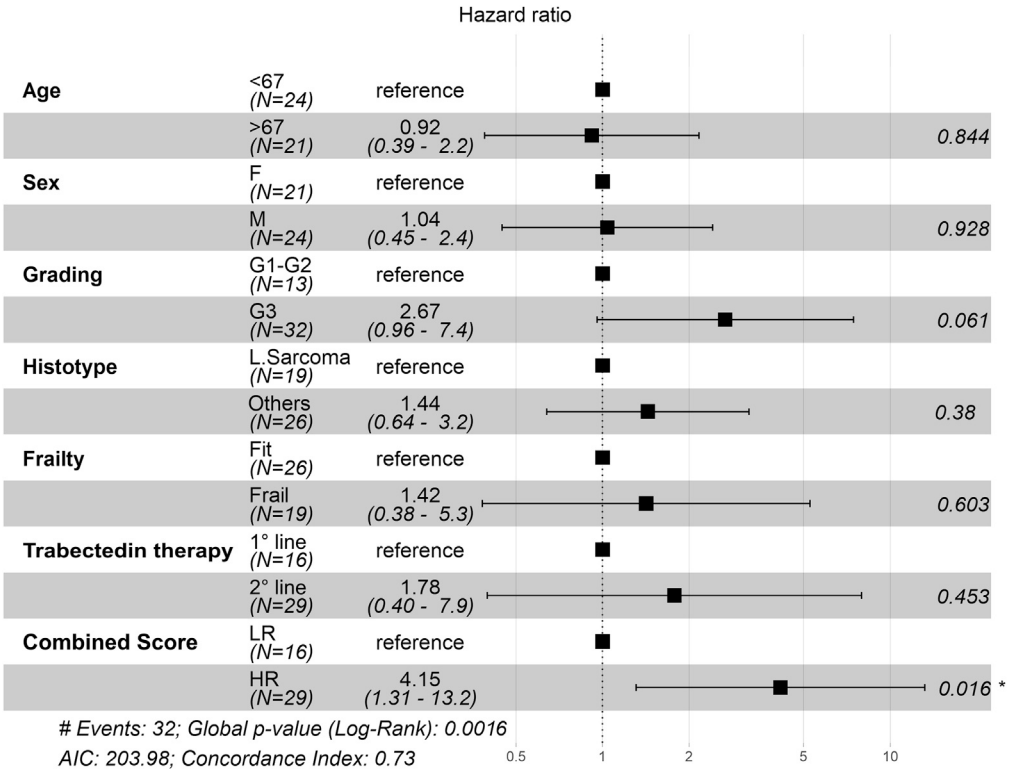


Figure 4. Forest plot for Cox proportional hazards model

The figure provides a forest plot reporting the hazard ratio (HR) and the 95% confidence intervals of the HR for each covariate included in the Cox proportional hazards model. Magnitude of significance is denoted with asterisks (*).

our study for patients with more aggressive and poor prognostic STSs. Our findings so provide valuable evidence concerning the regulatory mechanisms of ACSS2 expression in tumor development and progression of STSs.

Besides acetate and RBC count, plasma LDL triglycerides levels, in particular LDL-1, LDL-2, and LDL-3 subtractions, were found increased in mSTS patients with poor prognosis. The LDL lipoproteins together with very low-density lipoproteins (VLDLs) are responsible for the delivery of triglycerides and cholesterol to peripheral tissues.[38,39] Tumor cells have higher lipids demand, which involves a greater uptake of cholesterol and triglycerides mediated by a higher expression of LDL receptors.[40,41] High levels of LDL and VLDL triglycerides derived from tumor-host interplay were recently observed in human epidermal growth factor receptor 2 (HER2) positive breast cancer, suggesting that mobilization of the triglyceride LDL subfractions may represent a surrogate index of aggressiveness.[42] Analogously, the increase of triglycerides-LDL cargo in plasma of mSTS patients may reflect a unique host tumor-induced metabolic reprogramming aimed at fueling the tumor for its lipid needs.[43,44]

In summary, the three selected parameters (high plasma triglycerides-LDL, together with the low blood level of RBC count and plasma acetate) appear to be the result of host-tumor dynamic metabolic interconnections associated with highly aggressive tumor features. The integration of these three biomarkers into a combined model allowed us to discriminate, with high sensitivity and specificity, patients with high and low risk of 2-years death (Figure 2). This combined score outperformed conventional variables such as age, sex, tumor histotype, and grading, currently applied to build nomograms useful to predict OS and the risk of distant metastases after surgery.[45] To date, many different nomograms, even dynamic, including surgical margins and chemotherapy/radiotherapy treatments, have been developed;[3,46–48] however, their prognostic scores are still based exclusively on clinical-pathological parameters. In this study, for the first time to the best of our knowledge, a nomogram that incorporates circulating biomarkers (RBC count, acetate, triglycerides LDL-2) has been developed to predict 2-year OS of mSTS patients, independently of clinicopathological characteristics and previous treatments.

Limitations of the study

This investigation suggests integrating metabolomics and lipoproteomics with clinical analysis as a prognostic tool for studying tumor-host interactions, and monitoring mSTS patients to contribute to the selection of the best clinical options for each individual patient.

This pilot hypothesis-generating study has some limitations: it includes only mSTS patients, where the tumor effects on the host metabolism might be more pronounced than in early-stage patients, and the small sample size would necessitate further larger and independent validations. The limited sample size prevented us from performing sex-based analyses and specific analysis for each histological subtype and

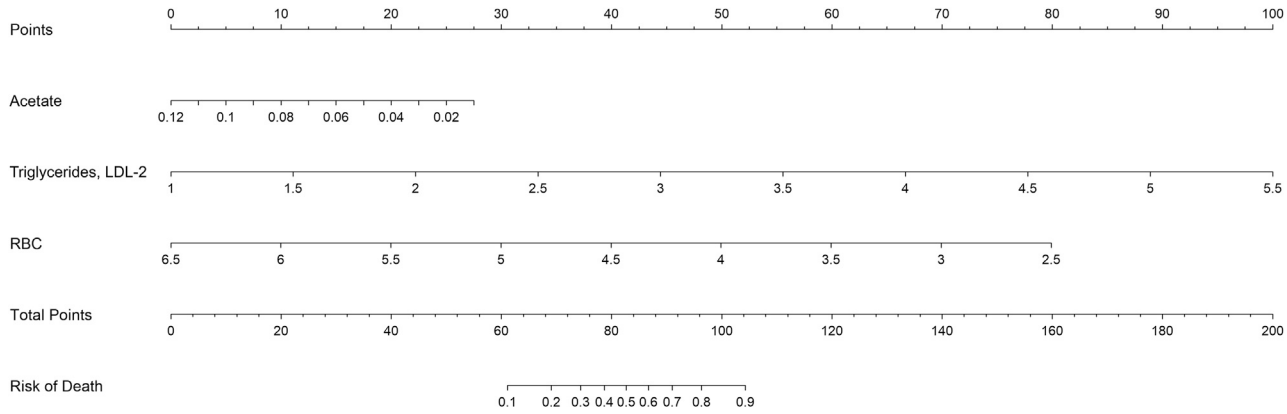


Figure 5. Nomogram predicting 2-year cancer-specific survival of patients with soft tissue sarcomas

The nomogram summed the points identified on the scale for each of the three independent prognostic variables (acetate, triglycerides LDL-2, RBC count).

from clearly interpreting the impact of classical prognostic factors. However, it suggests that a limited panel of clinical and metabolomic parameters could be used as a statistically significant predictor of 2-year survival.

The strength of the proposed prognostic model/nomogram lies in its stratification power. When applied to a group of mSTS patients with heterogeneous clinicopathological characteristics and clinical outcomes, it proves to be a useful tool in a real-world clinical setting.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
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- ADDITIONAL RESOURCES

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107678.

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AUTHOR CONTRIBUTIONS

Conceptualization: GC, GM, AB, DL, AV, LT, CL. Patients Management: GM, AB, DL, SS, AS. Data curation: GC, GM, AV. Laboratory analysis: GC, AS, AV. Statistical Analysis: AV, LT. Data Interpretation: GC, GM, AV, LT. Supervision: LT, CL, CG, GM. Writing—original draft: GC, GM, AV. Writing-review, editing and approval: AV, LT, CL, GM, AB, DL, SS, AS, CG. AV contributed to the present study before her full-time involvement in the project PNRR PE8 Age-It (funded under the decree n. 341/2022 of the Italian Ministry of University).

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Plasma	Centro di Riferimento Oncologico di Aviano (CRO). The study was approved by the CRO Institutional Ethical Committee.	no. 2015.004CE; NCT04394728
Chemicals, peptides, and recombinant proteins		
Sodium phosphate dibasic heptahydrate	Sigma-Aldrich	CAS: 7782-85-6
Deuterium oxide	Sigma-Aldrich	CAS: 7789-20-0
3-(Trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt	Sigma-Aldrich	CAS: 24493-21-8
Sodium Azide	Sigma-Aldrich	CAS: 26628-22-8
Software and algorithms		
Microsoft R Open	Microsoft Corporation	4.0.2
Bruker IVDr Quantification in Plasma/Serum B.I.Quant-PS platform	Bruker	2.0.0
Bruker IVDr Lipoprotein Subclass Analysis platform	Bruker	1.0.0

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, G. Corona (giuseppe.corona@cro.it).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This paper does not report any original code.
- The data reported in this paper will be shared by the lead contact upon reasonable request.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The study was carried out in accordance with the principles of the Declaration of Helsinki and was approved by the CRO Institutional Ethical Committee (no. 2015.004CE, 09/04/2015, NCT04394728). All participants signed written informed consent.

This monocentric study enrolled 45 mSTS patients receiving trabectedin treatment as first (n=16) or second (n=29) line of treatment. Leiomyosarcoma and liposarcoma (L-sarcomas) were the most prevalent histological subtypes accounting for about 42% (n=19) of the cases. Other mSTS (n=26) included: malignant peripheral nerve sheath tumor (n=4), mixofibrosarcoma (n=4), undifferentiated pleomorphic sarcoma (n=4), not otherwise specified sarcoma (n=4), chondrosarcoma (n=2), synovial sarcoma (n=2), endometrial stromal sarcoma (n=2), malignant fibrohistiocytoma (n=2), desmoplastic small-round-cell tumor (n=1) and carcinosarcoma (n=1). The largest portion of the STS presented poorly differentiated G3 grade tumors, with only a 29% of patients that showed moderate G2 grade differentiation. Overall, 53% (n=24) of patients showed a good general health condition, with a EOCG performance status (PS) of 0, while the remaining 47% (n=21) had PS between 1 and 2. Normal hematological, renal, and liver functions were requested to receive trabectedin therapy, together with a 3-week interval free from previous chemotherapy. Patients had a median age of 66 (range 37-90) years, with a superimposable percentage of males (53%) and females (47%) (Table 1). Nineteen mSTS patients did not receive anthracyclines and ifosfamide combination since they were considered frail according to the following criteria: age >75 years (n=10), PS 2 (n=4), previous anthracyclines chemotherapeutic regimens (n=2) or documented cardiological disfunctions (n=3). Sixteen patients of this frail group underwent first line treatment with trabectedin. The status of the disease along treatment was re-evaluated by computed tomography scan every three months.

No restrictions on sex were placed on the inclusion of patients. There were no restrictions on race or ethnicity, but all patients who were included were Italian.

METHOD DETAILS

Sample collection

The following clinical information were collected from the patient's medical record: age, sex, height, weight, body mass index, body surface area.

Plasma samples were collected from a peripheral vein in 7.5 mL sterile vacutainers containing ethylenediaminetetraacetate (EDTA). Samples from 45 mSTS patients were collected, after fasting overnight, following the standard operating procedures for metabolomics,[49] and immediately frozen at −80°C.

NMR analysis

Plasma samples were thawed at room temperature and shaken before use. A total of 350 µL of sodium phosphate buffer (75 mM Na2HPO4x7H2O; 20% (v/v) 2H2O, 6.1 mM NaN3; 4.6 mM 3-(Trimethylsilyl)propionate-2,2,3,3-d4; pH 7.4) was added to 350 µl of each sample. After homogenization by vortexing for 30 s, 600 µL of each mixture were transferred into a 5 mm NMR tube.

NMR spectra were acquired at a Bruker 600 MHz spectrometer (Bruker BioSpin) operating at 600.13 MHz proton Larmor frequency equipped with an automatic and refrigerated (6°C) sample changer (SampleJet, Bruker BioSpin). To ensure high spectral quality and reproducibility, the spectrometer was calibrated daily.

All samples were acquired at 310 K using a standard nuclear Overhauser effect spectroscopy pulse sequence NOESY 1Dpresat (noesygppr1d on Bruker spectrometers) to detect signals of both low and high-molecular weight molecules.[50] Each NOESY experiment was acquired using 32 scans plus 4 dummy scans, an acquisition time of 2.7 s, a relaxation delay of 4 s and a mixing time of 0.01 s.

A line-broadening factor of 0.3 Hz was applied to each free induction decay before the application of the Fourier transform. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated at the anomeric glucose signal δ 5.24 ppm using TopSpin 3.6 (Bruker BioSpin GmbH, Rheinstetten, Germany).

QUANTIFICATION AND STATISTICAL ANALYSIS

A panel of 27 metabolites was unambiguously identified and quantified using Bruker IVDr Quantification in Plasma/Serum B.I.Quant-PS platform™ (version 2.0.0). The regions of glycoproteins, that comprises both the NMR signals for GlycA at δ 2.04 and GlycB at δ 2.08, and the region of the signals of 3-methyl-2-oxovalerate and mannose were quantified via peak integration.

Identification and quantification of 112 lipoprotein-related parameters were performed utilizing the Bruker IVDr Lipoprotein Subclass Analysis platform™ (version 1.0.0).[51] This tool is not diagnostically validated, but it is a standardized platform for NMR pre-clinical research. Using this platform, information about the content of triglycerides, cholesterol, free cholesterol, phospholipids, Apo-A1, Apo-A2 and Apo-B100 of the main fractions and subfractions of VLDL, IDL, LDL and HDL classes can be obtained.

All data analyses were performed using Microsoft R Open (version 4.0.2). The metabolites with values lower than the limit of quantification (LOQ) and missing clinical data were imputed by means of a Random Forest approach as implemented in the R package "missForest"[52] using 500 trees and default parameters. The imputation procedure was repeated 100 times and then the average matrix was calculated. Covariates with more than 25% of observation under the LOQ were excluded. Of note, none of the three covariates (acetate, triglycerides LDL-2, RBC count) used for the prediction of patient survival presented missing data.

Data regarding metabolites, lipoprotein-related and clinical parameters were integrated with the aim of predicting two-years survival of patients. The area under the receiver operating characteristic curve (AUROC) was calculated for each variable using the function "colAUC" included in the R package "caTools". The Wilcoxon rank-sum test was used to infer differences between metabolites/lipoprotein-related parameters/clinical data in the two-years survived or deceased patients. *P*-values were adjusted for multiple testing using the false discovery rate (FDR) procedure, described by Benjamini, Heller, Yekutieli,[53] with the Benjamini-Hochberg correction at α=0.05. Since, peculiarly, in our dataset there are several similar raw *p*-values, using the abovementioned procedure, several adjusted *p*-values resulted equal.

A logistic regression model based of the most significant metabolite (acetate), lipoprotein-related parameter (triglycerides, LDL-2) and clinical variable (red blood cell count) was computed using the function "glm" (R package "stats"). This model was internally validated by means of leave-one-out cross-validation scheme, and to delineate high risk of death, the cut-off for the combined score was optimized at 0.63 using the function "coords" of the R package "pROC" that maximized the Youden's J statistic. The ability of this score to predict 2-years survival was assessed using Kaplan–Meier curves, with additional calculation of the hazard ratio (HR) and *p*-value assessed by Log-Rank test. The performances and the independence of the combined score were evaluated by calculating Cox proportional hazards regression models[54] (R library "Survival") and each model significance was assessed through a likelihood-ratio test.

The nomogram for 2-years risk of death estimation was calculated using the function "nomogram" included in the R library "rms".

ADDITIONAL RESOURCES

The study was approved by the CRO Institutional Ethical Committee (no. 2015.004CE, 09/04/2015, NCT04394728 https://classic.clinicaltrials.gov/ct2/show/NCT04394728).