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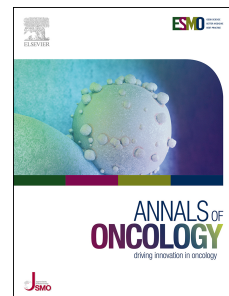
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1 Original article.

2 **ctDNA quantification improves estimation of outcomes in patients with high**
3 **grade osteosarcoma: a translational study from the OS2006 trial.**

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53

54 **Running title**

55 Risk Prediction with Circulating tumor DNA in Osteosarcoma

56

57 This study was presented in part at AACR 2023.

58

59 **Manuscript information.**

60 Figures: 3, Supplementary figures: 7

61 Tables: 2, Supplementary tables: 4

62 Supplemental Material

63

64 **Abbreviations**

65 ctDNA: circulating tumor DNA, lpWGS: low passage whole genome sequencing, CNA:

66 Copy number alteration, CPA: copy number abnormality score, diagCPA: CPA at

67 diagnosis, surgCPA: CPA at surgery, eotCPA: CPA at end of treatment

68

69 **Abstract** (259/300)

70 Purpose: Osteosarcoma stratification relies on clinical parameters and histological
71 response. We developed a new personalized stratification using less invasive
72 circulating tumor DNA (ctDNA) quantification.

73 Patients and Methods: Plasma from patients homogeneously treated in the prospective
74 protocol OS2006, at diagnosis, before surgery and end of treatment, were sequenced
75 using low passage whole genome sequencing (lpWGS) for copy number alteration
76 detection. We developed a prediction tool including ctDNA quantification and known
77 clinical parameters to estimate patients' individual risk of event.

78 Results: ctDNA quantification at diagnosis (diagCPA) was evaluated for 183 patients
79 of the protocol OS2006. diagCPA as a continuous variable was a major prognostic
80 factor, independent from other clinical parameters, including metastatic status
81 (diagCPA HR=3.5, $p=0.002$ and 3.51, $p=0.012$, for PFS and OS). At the time of surgery
82 and until the end of treatment, diagCPA was also a major prognostic factor
83 independent from histological response (diagCPA HR=9.2, $p<0.001$ and 11.6,
84 $p<0.001$, for PFS and OS). Therefore, the addition of diagCPA to metastatic status at
85 diagnosis or poor histological response after surgery improved the prognostic
86 stratification of patient with Osteosarcoma. We developed the prediction tool PRONOS
87 to generate individual risk estimations, showing great performance with . ctDNA
88 quantification at the time of surgery and the end of treatment still required improvement
89 to overcome the low sensitivity of lpWGS and to enable the follow up of disease
90 progression.

91 Conclusions: The addition of ctDNA quantification to known risk factors improves the
92 estimation of prognosis calculated by our prediction tool PRONOS. To confirm its value
93 an external validation in the Sarcoma 13 trial is underway.

94 **Keywords:** ctDNA, Osteosarcoma, prognostic score, stratification

95

96 **Context - Highlights**

- 97
- Circulating tumor DNA detection (ctDNA) is a non-invasive procedure
- 98
- The use of low passage whole genome sequencing is cost-effective
- 99
- This procedure represents a considerable step in reducing therapeutic burden
- 100
- and trauma for patient with cancer
- 101
- PRONOS is a tool that estimates relapse risk using ctDNA at diagnosis and
- 102
- known clinical risk factor in osteosarcoma

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103 Introduction

104 Osteosarcoma is the most common primary bone tumor in children and young adults
105 ¹. Overall survival remains around 70%, with no successful new therapy introduction in
106 the last decades and with an important treatment burden for survivors ²⁻⁵.

107 Metastasis identification and chemosensitivity, evaluated by percentage of tumor
108 necrosis after presurgical chemotherapy, are the main prognostic factors.
109 Unfortunately, they are insufficient to predict patient outcome robustly enough to
110 identify in an earlier stage high-risk patients to introduce new anticancer agents and
111 low risk patients to decrease treatment burden ^{2,3}. Hence, we urgently need new robust
112 and early biomarkers to better predict treatment failure, that could be combined with
113 powerful clinical risk factors.

114 Liquid biopsy is an increasingly studied field for stratification in sarcoma ⁶⁻⁹. By using
115 the simple, fast and cost effective approach of low passage whole genome sequencing
116 (lpWGS) it allows for the detection and quantification of circulating tumor DNA (ctDNA)
117 to stratify patients ¹⁰⁻¹³. The vast majority of osteosarcoma carries copy number
118 alterations (CNA) that can be detected in the plasma. and its detection has previously
119 been correlated with outcome ^{6, 14, 15}. Several analytic workflows can be used to
120 process low definition sequencing data, such as IchorCNA, and WisecondorX, with
121 different strategies to account for the noise generated by the sequencing ¹⁶⁻¹⁹. In this
122 work, we show that ctDNA quantification from patients treated within the prospective
123 OS2006 trial is a strong prognostic factor, independent of other known clinical
124 parameters. We developed a prognostic model using ctDNA at diagnosis and known
125 risk factors that significantly improves patients' stratification from diagnosis and
126 throughout the treatment.

127

128 **Patients and Methods**

129 *Patient and clinical data*

130 Plasma samples were collected in the prospective OS2006 trial, a randomized phase-
131 III trial for newly diagnosed osteosarcoma ^{2, 3}. Briefly, patients received either a
132 methotrexate based regimen with VP16 and ifosfamide, or a Doxorubicin, cisplatin and
133 ifosfamide regimen according to their age as previously described ^{2, 3, 20}. Zoledronate
134 was randomized to be added or not to standard chemotherapy, with a 1:1 ratio. This
135 study was carried out in accordance with the ethical principles of the declaration of
136 Helsinki and with Good Clinical Practice guidelines. A specific informed consent for
137 blood and tumor samples was obtained from patients or legal representatives. Study
138 was approved by an ethics committee and the institutional review boards. Clinical data
139 were collected as reported previously ^{2, 3}. Histological response to neoadjuvant
140 chemotherapy of the primary tumor was defined as “good response” (GR, <10% viable
141 cells) or “poor response” (PR, \geq 10% viable cells). It is important to note that the
142 selection of samples was not based on any specific criteria. It was based on the
143 availability of samples at the time of analysis.

144 *Sample processing and sequencing*

145 Samples were selected based on the availability of more than 1.5mL of plasma.
146 Samples were prospectively collected in EDTA tubes and processed on site before first
147 cycle of chemotherapy, before surgery and at end of treatment and processed as
148 described in the **Supplementary method**. Whole genome sequencing was performed
149 to a mean target coverage of 0.5X.

150 *Bioinformatic analysis*

151 Data processing is detailed in the **Supplementary method**. Briefly, upon read
152 alignment we compared two algorithms, IchorCNA and WizecondorX with data from
153 comparative genomic hybridization (CGH) from the tumor samples. More information
154 regarding the algorithm comparison is provided in the **Supplementary material**.

155 To differentiate high versus low CPA score, we used an unsupervised clustering based
156 on gaussian mixture modelisation (**Supplementary material, supplementary figure**
157 **S3A**).

158 *Clinical endpoints*

159 The primary endpoint was the progression free survival (PFS) as defined as the delay
160 between the baseline time and the occurrence of progression or death of the patient.
161 CPA measurements at diagnostic, surgery, or at the end of treatment served as the
162 baseline time for different analyzes to evaluate the prognostic potential of CPA
163 measurement at different time points. The secondary endpoint was the overall survival
164 (OS) defined as the time between the baseline and the death of the patient. The follow-
165 up of the patients who did not encounter the event of interest was censored at the date
166 of the end of follow-up.

167 *Prognosis analysis*

168 Univariable survival analyses were performed using the Kaplan-Meier estimator. The
169 expected patient survival probabilities in different strata were compared using the Log-
170 rank test. Multivariable analyses were performed using the Cox proportional hazard
171 model adjusted for the CPA and for the known clinical prognostic factors in
172 osteosarcoma. We considered the CPA as a continuous variable and a categorical
173 variable (low and high) to compare different functional forms of the CPA ~ response
174 relationship (**Supplementary method, Supplementary figure S3B**). We compared
175 the related models using the Bayesian information criterion (BIC). A result with an
176 alpha risk of <5% was considered as statistically significant.

177 *Predictive score*

178 As the standard statistical approach aims to overfit the data to explain its variability,
179 the resulting model may underperform for a prediction task. We assessed in parallel
180 the additional value of the CPA for the patient prognosis prediction compared to a
181 reference clinical model (including only the risk factors considered in the current clinical
182 practice) to its CPA-enhanced version. We assessed the discrimination of those
183 models' performance using the area under the time-dependent receiver operating
184 characteristic curve (AUtdROC), and the calibration using the integrated calibration
185 index (ICI) and graphical representations²¹⁻²³. Those indices were computed for the
186 prognosis predictors (OS and PFS) at 5 years from the different clinical milestones
187 (diagnosis, surgery, and end of treatment) and their 95% confidence interval was
188 computed using nested bootstrap with 500 external samples and 100 internal samples
189²⁴⁻²⁶. Transparent reporting of a multivariable prediction model for individual prognosis
190 or diagnosis (TRIPOD) recommendation were used for optimism correction²⁵. More

191 details about their computation, optimism correction and the test procedure are
192 provided in the **supplementary methods**. We also provide a web interface for
193 graphical representation of the individual patient's prediction within this model for
194 precision medicine.

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195 **RESULTS**196 *Patients*

197 Plasma samples collected from 183 children, adolescents, and adults treated in
198 OS2006 study were included ^{2, 3}. The ctDNA cohort represents the original OS2006
199 cohort regarding clinical characteristics and prognostic factors, except for age with a
200 slightly young ctDNA cohort (14years and 8 months vs 15 years and 4 months) and
201 chemotherapy that could be related to a larger participation of the pediatric centers to
202 the plasma collection. In total, 465 samples were sequenced: 178 at diagnosis, 144
203 pre-surgery and 143 at the end of treatment. Matched tumor copy number profiles from
204 CGH array were available for 91 patients. The clinical characteristics of our cohort were
205 similar between our study and the original OS2006 cohort (**Table 1**).

206 *Optimization of circulating tumor fraction estimation in plasma from osteosarcoma*
207 *patient*

208 To validate a workflow able to recapitulate the CNA profile of the tumor in the plasma
209 at diagnosis and evaluate ctDNA content, we compared copy number profiles from
210 IchorCNA and WizecondorX with CGH from the tumor samples (**Supplementary**
211 **figure S1 and S2**) ^{16, 17}. We favored WizecondorX 1- to avoid manual post analytic
212 curation of the data, often used with IchorCNA to improve specificity and 2- because
213 WizecondorX Copy Number Abnormality (CPA) score is a continuous variable with low
214 values being the result of the sequencing noise (no null value). WizecondorX's calling
215 on CNAs was then compared with the one obtained by CGH on the primary tumor. We
216 found that the similarity between CNA between bulk tumor and plasma was highly
217 correlated with CPA score (**Supplementary figure S2C**). Therefore, CPA score
218 represents a combination of ctDNA content and confidence of ctDNA detection. We
219 computed the CPA score using WizecondorX from low coverage WGS data to measure
220 the global abnormality of each genome, expressed as a CPA score, before treatment,
221 before surgery and at end of treatment and observed that the cohort mean CPA score
222 decreased throughout treatment (**Supplementary figure S2D**), probably reflecting the
223 decrease of the tumor burden in response to chemotherapy. CPA score was used as
224 a continuous variable for the following analysis. However, to help the constitution of
225 risk groups, we also defined the "high" and "low" CPA score groups based on a non-

226 supervised, outcome agnostic clustering method which yielded a cutoff score of 0.6
227 **(Supplementary Methods, Supplementary figures S3A).**

228 *ctDNA fraction is a major prognostic factor at diagnosis*

229 Early prognostic biomarkers are essential to adapt patients' treatment. At diagnosis,
230 metastatic status is the main known prognostic factor in osteosarcoma as confirmed
231 by univariable and multivariable analysis (**Figure 1A-B and Supplementary table 1**).
232 However, we found that diagCPA as a continuous variable is also independently
233 associated with outcome. There was a trend toward a difference in mean diagCPA
234 between patients with and without metastases, despite no interaction in the
235 multivariable analysis (**Figure 1C and supplementary table 2**). Using diagCPA as a
236 binarized variable ("high" vs. "low"), we constituted four groups of patients according
237 to the presence of metastasis and ctDNA level and found a significant impact on PFS
238 and OS (**Figure 1D-E, Table 2**). Interestingly, the metastatic status and the diagCPA
239 score were associated to similar independent increases of the PFS and the OS risk,
240 as highlighted by the overlap of the survival curves of patients with metastases at
241 diagnostic or with a high CPA score. Those results suggest that 3 risk groups can be
242 defined in osteosarcoma: a low-risk defined by the "absence of metastasis and low
243 CPA score", an intermediate-risk defined by the "presence of either metastasis or high
244 CPA score", and a high risk defined the "presence of both metastasis and high CPA
245 score". Multivariable analysis confirmed that diagCPA as a binarized variable was
246 associated with outcome (**Figure 1F-G**). Of note, correlation between diagCPA and
247 tumor size was very weak and unable to explain the level of ctDNA detected on its own
248 **(Supplementary figure 4).**

249

250 *ctDNA fraction estimation improves the clinical prognostic characterization of*
251 *osteosarcoma patient at the time of surgery*

252 Histological response evaluated after neoadjuvant chemotherapy (i.e., at the time of
253 surgery) is the main known prognostic factor of osteosarcoma which we confirmed by
254 performing similar univariable and multivariable analyses using the date of surgery as
255 baseline time point. Interestingly, surgCPA did not show a significant association with
256 either PFS or OS and therefore should not be used for risk assessment. On the other
257 hand, diagCPA as a continuous variable was also shown to be independently

258 associated with PFS at this time point, despite the strong influence of histological
259 response on outcome. (**Figure 2 A-B and supplementary table S1**). There was only
260 a significant interaction observed between histological response and diagCPA for
261 overall survival. However this result needs to be interpreted with caution due to the
262 high statistical instability as reflected by the extremely wide confidence interval
263 (**Supplementary table S2**). Metastatic status was only significantly associated with
264 PFS. (**Figure 2 A-B**). **Figure 2C-D** represent the CPA at diagnosis and surgery
265 according to the metastatic status and histological response. As previously described,
266 using the diagCPA as a binarized (“high” vs. “low”) variable we constituted four groups
267 of patients according to the histological response and diagCPA. We found a significant
268 impact on survival defining risk groups (**Figure 2E-F, Table 2**). The prognostic impact
269 of the binarized diagCPA was confirmed in multivariable analysis (**Figure 2G-H**).

270 *ctDNA at the end of treatment*

271 To evaluate the prognostic value of different ctDNA sampling timepoints, we assessed
272 the predictive value of CPA calculated at diagnosis, time of surgery and end of
273 treatment by univariate and multivariate analysis. EotCPA as a continuous variable
274 was not associated with prognosis and should not be used for risk stratification. The
275 results were similar with analysis at surgery with only diagCPA and poor histological
276 response to chemotherapy (PR) were associated with PFS and OS (**Supplementary**
277 **Figure S5 and supplementary table S1 and 2**).

278 The group analysis at the end of treatment, with four groups according low/high
279 diagCPA and histological response, also found a significant association with PFS and
280 OS (**Supplementary Figure S5 D-G, Table 2**). At the end of treatment, survival of
281 patient with high diagCPA was significantly worse only in the PR group
282 (**Supplementary Figure S5 F-G**).

283

284 *Patient outcome prediction*

285 Patient stratification is based on the risk estimation from clinical factors and robust
286 biomarkers. To define a patient classifier that considers all those factors, we developed
287 a prognostic prediction multivariable model to discriminate the patients who
288 progressed (or died) within 5 years from diagnosis, surgery, or end of treatment. The

289 assessed models included the clinical factors and different functional forms of the
290 diagCPA (categorical, linear, and non-linear) (**Supplementary figure S3B**). The
291 ctDNA analysis at surgery and at the end of treatment were not used they were not
292 associated with prognosis. These models were compared to the reference model,
293 comprised only of the known clinical factors, to evaluate the additional value of
294 diagCPA for the patient prognostic prediction within current clinical practice.

295 Regarding only the clinical score, the addition of the histological response improved
296 the clinical score discrimination of more than 10% of AUtdROC, from 0.61 to 0.79 for
297 the 5-year PFS, from 0.63 to 0.77 for the 5-year OS, confirming that it is a major
298 predictor of the patient prognosis (**Supplementary table 3-4, Supplementary figure**
299 **S6 A-B**).

300 The addition of the diagCPA also improved the clinical score discrimination at
301 diagnosis of more than 8% of AUtdROC for the 5-year PFS, irrespective of the
302 functional form of diagCPA. It also improved the clinical score including the histological
303 response for the two other time points by $\approx 5\%$, confirming its value as a predictor in
304 addition to standard clinical factors. Similar trends were observed for the 5-year OS,
305 but the lower number of events than PFS limited statistical power for accurate model
306 comparison.

307 Model calibration is critical to the interpretation of the generated score into survival
308 probability estimation by clinicians. The different models were thus assessed to identify
309 the most robust predictor. While the model with non-linear relationship presented the
310 best discrimination, the integrated calibration index (ICI) and calibration plots
311 (**Supplementary table 5-6, Supplementary figure S7 A-C**) indicated that there was
312 more discrepancy between its predictions and the 5-year PFS observed than the other
313 functional form (underestimation of the high risk probabilities and overestimation of the
314 low risk probabilities). As the linear form is more statistically parsimonious than the
315 binarized diagCPA, which requires estimating an additional parameter (the low/high
316 threshold), the linear diagCPA form model was selected for our predictive score.

317 Since multivariable score assessment is difficult to implement in practice due to the
318 number of variables and their potentially continuous nature, we developed PRONOS:
319 a free research tool to evaluate patient risk. It allows for prediction at diagnosis using
320 standard diagnostic clinical factors and diagCPA and, when available, histological

321 response to chemotherapy to provide updated predictions throughout treatment.
322 PRONOS calculates a patient's individual risk according to its clinical profile and
323 diagCPA (**Figure 3**). We expect that PRONOS could be easily integrated in standard
324 clinical care of osteosarcoma, after an external and prospective validation, to optimize
325 the care of future patients.

326

327

328 **Discussion**

329 In this work, we showed in the largest prospectively collected cohort of patients with
330 osteosarcoma treated within the same clinical trial, that our workflow and tool named
331 PRONOS, combining shallow sequencing of cell free DNA at diagnosis and clinical
332 factor is a fast, non-invasive, and reliable biomarker to predict the patient outcome.
333 CPA score at diagnosis is a strong prognostic factor, independent from the major
334 known clinical factors that are metastatic status or histological response. Our approach
335 is not suitable for longitudinal analysis of ctDNA, which would require a different
336 strategy.

337 ctDNA is a promising biomarker for stratification in sarcoma that needs in depth
338 validation ⁶⁻⁹. Our analysis shows that ctDNA detection at diagnosis stands out as a
339 major independent prognostic factor that can be combined to provide a better risk
340 stratification for precision medicine, whereas the longitudinal analysis (surgCPA and
341 eotCPA) was not exploitable. Shallow WGS had previously been used in 72 localized
342 osteosarcoma at diagnosis, showing a trend of ctDNA detection on survival ⁶. A
343 methylation assay designed for osteosarcoma was also recently studied for ctDNA
344 detection in 72 pre-surgery samples. While the authors showed a worse prognosis of
345 patient with detectable ctDNA in univariable analysis, it was not significant in
346 multivariable analysis ²⁷. Other approaches based on detection of SNV or structural
347 variants using a patient specific panel or whole exome sequencing (respectively 10
348 and 36 patients) were used during treatment or at relapse, without any information on
349 its prognostic impact ^{28, 29}. An approach using cfDNA fragmentation used in Ewing
350 sarcoma and other sarcomas (8 patients with osteosarcoma) increased CNA detection,
351 and showed correlation with outcome in Ewing sarcoma ³⁰.

352 In this work, we show that diagCPA is a^{17, 19}n accurate reflection of bulk tumor CNA
353 and that the signal intensity, recapitulated by the CPA score, is a good marker of ctDNA
354 fraction and the confidence of ctDNA detection. WizecondorX, in contrast to IchorCNA,
355 uses the whole cohort to compute CPA and is therefore, to a certain extent, adapted
356 to the biological feature of the cohort as previously published. WizecondorX is an
357 efficient automated tool to estimate ctDNA content, creating a reproducible and reliable
358 factor for prognostic. For practical reasons, patients' stratification requires the
359 simplification of ctDNA score into "High" or "Low" groups. We showed this
360 categorization underperforms for the prediction of patient response compared to its
361 continuous form. The latter was thus preferred for the development of a multivariate
362 model of risk estimation.

363 In this study, we demonstrated that ctDNA level at diagnosis impacts outcome and
364 have an additive prognostic value to known major prognostic factors. In addition, our
365 study motivates the identification of a very high-risk group, representing approximately
366 15% of patients at surgery or the end of treatment, that are most likely to benefit from
367 new alternative therapies. For example, a nested randomized trial was set-up in the
368 current French osteosarcoma first-line trial SARCOME13 (NCT03643133), to
369 randomize the adjunction of Mifamurtide to the standard chemotherapy after surgery
370 for high risk patients only, including poor responders³¹. It is important to highlight that
371 ctDNA evaluated with our approach is only relevant at diagnosis.

372 As precision medicine requires patient stratification, we propose to define the patients'
373 risk using the multivariable predictive score PRONOS, which includes the CPA score
374 at diagnosis and clinical factors familiar to physicians in their decision-making. This
375 tool is the main achievement of this work, which represents an innovative approach to
376 patient stratification. For example, at the time of surgery, the patient's relapse risk will
377 be estimated based on histological response, metastatic status, diagCPA and the fact
378 that the patient did not already experience disease progression. To illustrate the
379 potential of clinical implementation of PRONOS, we created a demo open-source
380 online companion application (https://pronosgr.shinyapps.io/pronos_valuesbox/) that
381 could be used for patient stratification in clinical studies, as well as real-life clinical
382 practice. Investigators using PRONOS can decide the threshold of what is considered
383 of "High risk" in term of predicted PFS or OS.

384 Our study has some limitations. Despite the excellent performance of our prognostic
385 prediction multivariable model we implemented in PRONOS, an external validation is
386 required before its routine use in clinic. It is planned to be conducted in the recently
387 closed Sarcome13 study (NCT03643133). Moreover, PRONOS is available online as
388 a research tool, for demonstration (**Figure 3**). It is to notice that despite the fact that
389 the samples are originated from a trial, only a portion was analyzed for ctDNA. As
390 mentioned in the method, the samples were not selected based on any other criteria
391 than availability at the moment of the study. However, this sample was representative
392 of the trial in term of major risk factors and survival rates, suggesting that this pragmatic
393 selection did not cause a selection bias. On the technology side, the main limit is its
394 inability to analyze CPA score throughout treatment. More sensitive approaches are
395 needed for applications like minimal residual disease detection to monitor maintenance
396 therapy or early identification of poor histological response.

397 Our work represents a major achievement in the development of an early, non-
398 invasive, rapid, and cost-effective new prognostic biomarker for osteosarcoma. One of
399 the key advantages is that PRONOS is an easy to implement in clinical practice,
400 “hands-off” pipeline. It needs to be validated in an independent cohort, but can be used
401 to customize future studies by setting the risk threshold according to the clinical
402 objective.

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415 **Conflict of Interest.**

416 No conflict of interest to declare.

417

418 **References**

- 419 1. Trama A, Botta L, Foschi R, et al: Survival of European adolescents and young
420 adults diagnosed with cancer in 2000-07: population-based data from EURO CARE-5.
421 *Lancet Oncol* 17:896–906, 2016
- 422 2. Gaspar N, Occean B-V, Pacquement H, et al: Results of methotrexate-etoposide-
423 ifosfamide based regimen (M-EI) in osteosarcoma patients included in the French
424 OS2006/sarcome-09 study. *European Journal of Cancer* 88:57–66, 2018
- 425 3. Piperno-Neumann S, Le Deley M-C, Rédini F, et al: Zoledronate in combination with
426 chemotherapy and surgery to treat osteosarcoma (OS2006): a randomised,
427 multicentre, open-label, phase 3 trial. *Lancet Oncol* 17:1070–1080, 2016
- 428 4. Smeland S, Bielack SS, Whelan J, et al: Survival and prognosis with osteosarcoma:
429 outcomes in more than 2000 patients in the EURAMOS-1 (European and American
430 Osteosarcoma Study) cohort. *Eur J Cancer* 109:36–50, 2019
- 431 5. Janeway KA, Grier HE: Sequelae of osteosarcoma medical therapy: a review of rare
432 acute toxicities and late effects. *The Lancet Oncology* 11:670–678, 2010
- 433 6. Shulman DS, Klega K, Imamovic-Tuco A, et al: Detection of circulating tumour DNA
434 is associated with inferior outcomes in Ewing sarcoma and osteosarcoma: a report
435 from the Children’s Oncology Group. *British Journal of Cancer* 119:615–621, 2018
- 436 7. Lak NSM, Voormanns TL, Zappeij-Kannegieter L, et al: Improving Risk Stratification
437 for Pediatric Patients with Rhabdomyosarcoma by Molecular Detection of
438 Disseminated Disease [Internet]. *Clin Cancer Res* , 2021[cited 2021 Sep 20] Available
439 from: [https://clincancerres.aacrjournals.org/content/early/2021/09/06/1078-](https://clincancerres.aacrjournals.org/content/early/2021/09/06/1078-0432.CCR-21-1083)
440 0432.CCR-21-1083

- 441 **8.** Ruhen O, Lak NSM, Stutterheim J, et al: Molecular Characterization of Circulating
442 Tumor DNA in Pediatric Rhabdomyosarcoma: A Feasibility Study. *JCO Precis Oncol*
443 6:e2100534, 2022
- 444 **9.** Abbou S, Klega K, Tsuji J, et al: Circulating Tumor DNA Is Prognostic in
445 Intermediate-Risk Rhabdomyosarcoma: A Report From the Children's Oncology
446 Group. *JCO* JCO.22.00409, 2023
- 447 **10.** Abbou SD, Shulman DS, DuBois SG, et al: Assessment of circulating tumor DNA
448 in pediatric solid tumors: The promise of liquid biopsies. *Pediatric Blood & Cancer*
449 0:e27595
- 450 **11.** Cescon DW, Bratman SV, Chan SM, et al: Circulating tumor DNA and liquid biopsy
451 in oncology. *Nature Cancer* 1:276–290, 2020
- 452 **12.** Alix-Panabières C, Pantel K: Liquid Biopsy: From Discovery to Clinical Application.
453 *Cancer Discov* 11:858–873, 2021
- 454 **13.** Krumbholz M, Eiblwieser J, Ranft A, et al: Quantification of Translocation-Specific
455 ctDNA Provides an Integrating Parameter for Early Assessment of Treatment
456 Response and Risk Stratification in Ewing Sarcoma. *Clin Cancer Res* 27:5922–5930,
457 2021
- 458 **14.** Klega K, Imamovic-Tuco A, Ha G, et al: Detection of Somatic Structural Variants
459 Enables Quantification and Characterization of Circulating Tumor DNA in Children
460 With Solid Tumors. *JCO Precis Oncol* 2018, 2018
- 461 **15.** Shulman DS, Crompton BD: Using Liquid Biopsy in the Treatment of Patient with
462 OS. *Adv Exp Med Biol* 1257:95–105, 2020

- 463 **16.** Adalsteinsson VA, Ha G, Freeman SS, et al: Scalable whole-exome sequencing of
464 cell-free DNA reveals high concordance with metastatic tumors [Internet]. Nat
465 Commun 8, 2017[cited 2017 Nov 15] Available from:
466 <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5673918/>
- 467 **17.** Raman L, Dheedene A, De Smet M, et al: WisecondorX: improved copy number
468 detection for routine shallow whole-genome sequencing. Nucleic Acids Res 47:1605–
469 1614, 2019
- 470 **18.** Sharbatoghli M, Fattahi F, Aboulkheyr Es H, et al: Copy Number Variation of
471 Circulating Tumor DNA (ctDNA) Detected Using NIPT in Neoadjuvant Chemotherapy-
472 Treated Ovarian Cancer Patients. Front Genet 13:938985, 2022
- 473 **19.** Raman L, Van Der Linden M, Van Der Eecken K, et al: Shallow whole-genome
474 sequencing of plasma cell-free DNA accurately differentiates small from non-small cell
475 lung carcinoma. Genome Med 12:35, 2020
- 476 **20.** Piperno-Neumann S, Ray-Coquard I, Occean B-V, et al: Results of API-AI based
477 regimen in osteosarcoma adult patients included in the French OS2006/Sarcome-09
478 study. Int J Cancer 146:413–423, 2020
- 479 **21.** Blanche P, Dartigues J-F, Jacqmin-Gadda H: Estimating and comparing time-
480 dependent areas under receiver operating characteristic curves for censored event
481 times with competing risks. Stat Med 32:5381–5397, 2013
- 482 **22.** Austin PC, Harrell Jr FE, van Klaveren D: Graphical calibration curves and the
483 integrated calibration index (ICI) for survival models. Statistics in Medicine 39:2714–
484 2742, 2020

- 485 **23.** McLernon DJ, Giardiello D, Calster BV, et al: Assessing Performance and Clinical
486 Usefulness in Prediction Models With Survival Outcomes: Practical Guidance for Cox
487 Proportional Hazards Models. *AnnInternMed* 176:105–114, 2023
- 488 **24.** Efron B, Tibshirani R: Improvements on Cross-Validation: The .632+ Bootstrap
489 Method. *Journal of the American Statistical Association* 92:548–560, 1997
- 490 **25.** Moons KGM, Altman DG, Reitsma JB, et al: Transparent Reporting of a
491 multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD):
492 explanation and elaboration. *Ann Intern Med* 162:W1-73, 2015
- 493 **26.** Noma H, Shinozaki T, Iba K, et al: Confidence intervals of prediction accuracy
494 measures for multivariable prediction models based on the bootstrap-based optimism
495 correction methods. *Statistics in Medicine* 40:5691–5701, 2021
- 496 **27.** Lyskjær I, Kara N, De Noon S, et al: Osteosarcoma: Novel prognostic biomarkers
497 using circulating and cell-free tumour DNA. *Eur J Cancer* 168:1–11, 2022
- 498 **28.** Barris DM, Weiner SB, Dubin RA, et al: Detection of circulating tumor DNA in
499 patients with osteosarcoma. *Oncotarget* 9:12695–12704, 2018
- 500 **29.** Berlanga P, Pierron G, Lacroix L, et al: The European MAPPYACTS trial: Precision
501 Medicine Program in Pediatric and Adolescent Patients with Recurrent Malignancies.
502 *Cancer Discovery* candisc.1136.2021, 2022
- 503 **30.** Peneder P, Stütz AM, Surdez D, et al: Multimodal analysis of cell-free DNA whole-
504 genome sequencing for pediatric cancers with low mutational burden. *Nat Commun*
505 12:3230, 2021

506 **31.** Brard C, Piperno-Neumann S, Delaye J, et al: Sarcome-13/OS2016 trial protocol:
507 a multicentre, randomised, open-label, phase II trial of mifamurtide combined with
508 postoperative chemotherapy for patients with newly diagnosed high-risk
509 osteosarcoma. *BMJ Open* 9:e025877, 2019

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512 **Figure legends**513 **Figure 1.** Risk analysis using ctDNA detection at the time of diagnosis.

514 **A-B)** Multivariable analysis for progression free survival (PFS, **A**) and overall survival
515 (OS, **B**) with CPA as a continuous variable. N=174 patients evaluable for clinical
516 parameters and diagCPA. **C)** Violin plot showing CPA score at diagnosis according to
517 metastatic status. Individual values are symbolized by green dots. Red dots represent
518 the median, black lines the mean and red lines the standard deviation. The high/low
519 threshold of 0.6 calculated by unsupervised clustering is represented by a discontinued
520 blue line. **D-E)** Kaplan-Meier curves for PFS (**D**) and OS (**E**) according to metastasis
521 and ctDNA status. diagCPA is defined “high” or “low” based on the threshold previously
522 defined. **F-G)** Multivariable analysis considering diagCPA as a binarized variable
523 “low/high” for PFS (**F**) and OS (**G**). N=174 patients evaluable for clinical parameters
524 and diagCPA.

525 Met: metastasis; PR: poor histological response; GR: good histological response;
526 diagCPA: CPA score at diagnosis.

527 **Figure 2.** Risk analysis using ctDNA detection at the time of surgery.

528 **A-B)** Multivariable analysis for progression free survival (PFS, **A**) and overall survival
529 (OS, **B**) with ctDNA content as a continuous variable at diagnosis (diagCPA) and
530 before surgery (surgCPA). N=129 patients evaluable for all clinical parameters
531 including histological response, diagCPA and surgCPA. **C-D)** Violin plot showing CPA
532 score at diagnosis (**C**) and before surgery (**D**), according to metastatic status and
533 histological response. Individual values are symbolized by green dots. Red dots
534 represent the median, black lines the mean and red lines the standard deviation. The

535 threshold of 0.6 is calculated by unsupervised clustering is represented by a
536 discontinued blue line. **E-F**) Kaplan-Meier curves for PFS (**E**) and OS (**F**) according to
537 histological response and diagCPA. diagCPA is defined “high” or “low” based on the
538 threshold previously defined. **G-H**) Multivariable analysis considering diagCPA as a
539 binary variable “low/high” for PFS (**G**) and OS (**H**). N=162 patients evaluable for clinical
540 parameters and diagCPA. surgCPA was not considered in this analysis since it was
541 non significantly associated in the multivariable analysis with the continuous variable.
542 Met. At Diag. : presence of metastasis at diagnosis; Response: histological response;
543 diagCPA: CPA score at diagnosis; surgCPA: CPA score at the time of surgery.

544 **Figure 3.** Individual risk prediction by PRONOS. Screen captures of PRONOS tool for
545 patient’s individual risk prediction. A-C) Simulated patient survival estimations
546 displaying individual progression free survival (PFS) risk prediction (left panes), and
547 individual overall survival (OS) risk prediction (right panes). Test patient 1 risk
548 estimated at diagnosis with metastatic disease and diagCPA = 0.95 (A). Test patient
549 2 risk estimated at surgery, with metastatic disease, good histological response and
550 diagCPA = 0.35 (B). Test patient 3 risk estimated at end of treatment without
551 metastasis, good histological response and diagCPA = 0.35 (C). Only diagCPA, age,
552 gender and metastasis status are considered for diagnosis test time. This model
553 includes histology only at the time of surgery and at the end of treatment. A demo of
554 PRONOS is available at https://pronosgr.shinyapps.io/pronos_valuesbox/. This demo
555 of the predictive application allows estimating the survival and event probability of
556 patients, according to the CPA score, the metastases at diagnosis and the histological
557 response to treatment. Pronos is a research tool built on the OS2006 study. CPA
558 should be generated with the use of OS2006 data in order to provide representative
559 results of the model. It is explicitly intended for research purposes and is not to be

560 employed for medical or clinical applications. The development team disclaims any
561 responsibility for such use.

562 **Tables 1.** Patient characteristics.

563 **Table 2.** Influence of CPA score, metastatic status, and histological response on PFS
564 and OS.

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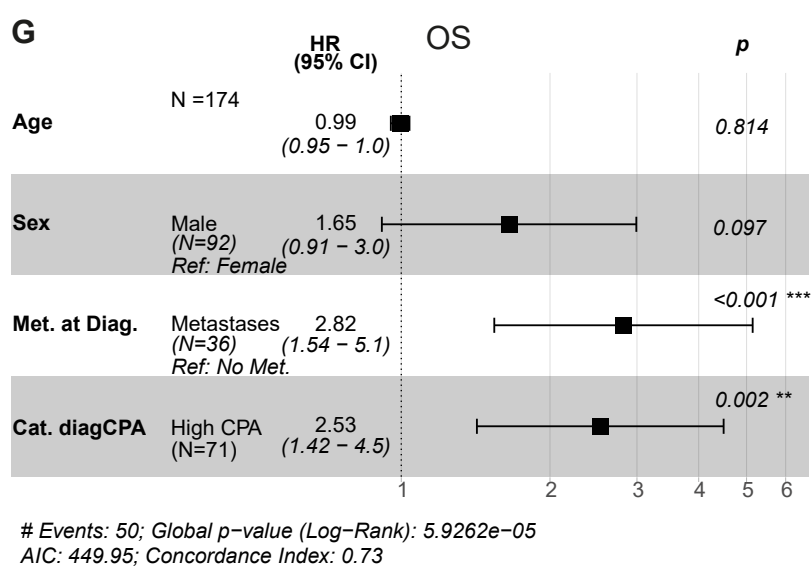
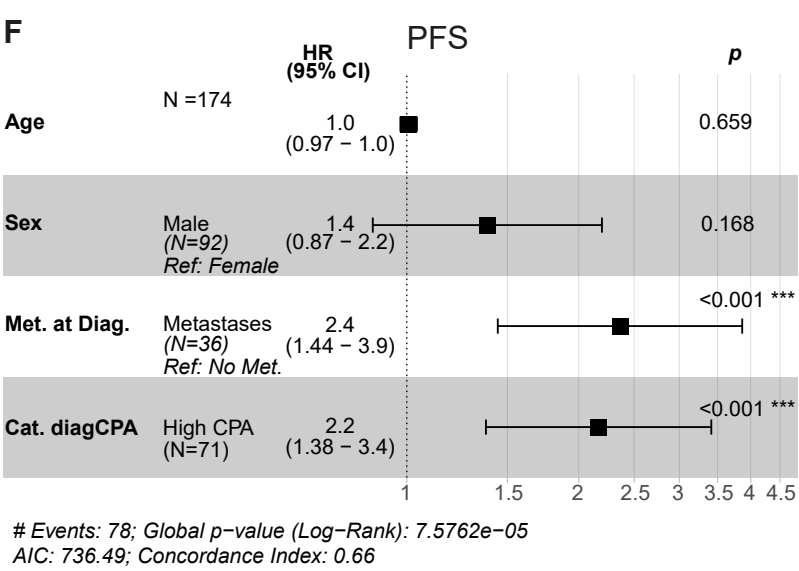
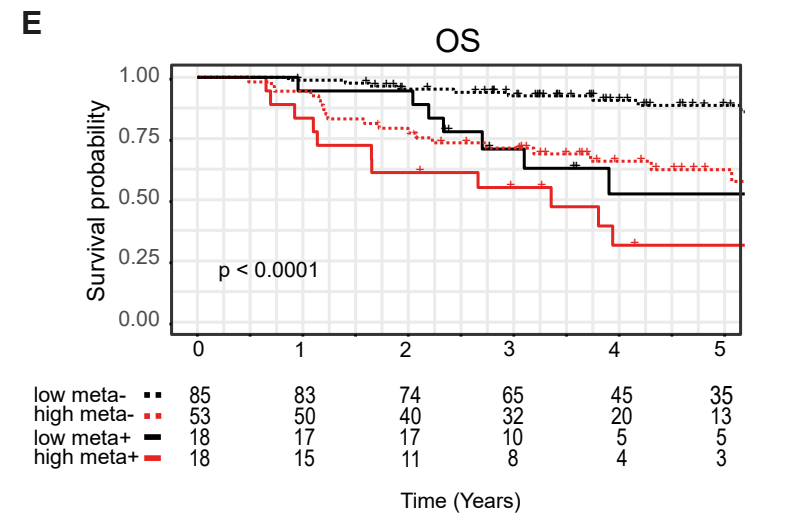
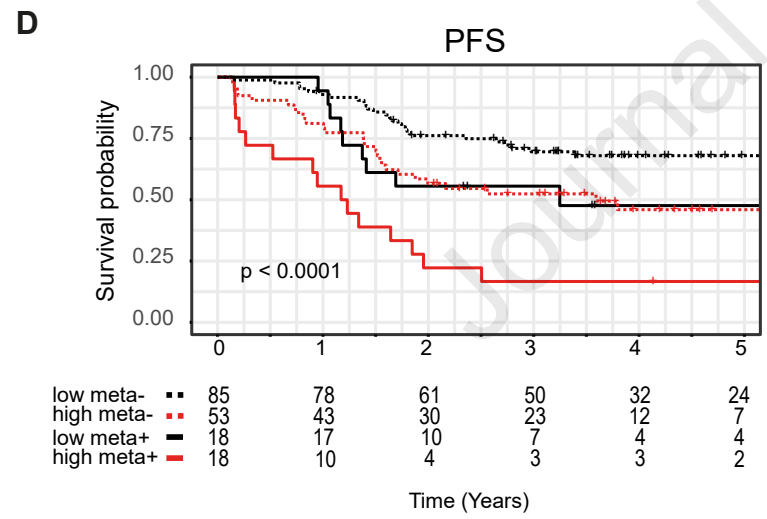
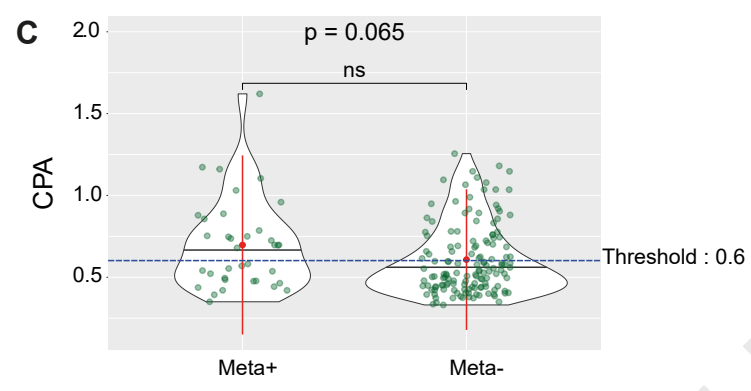
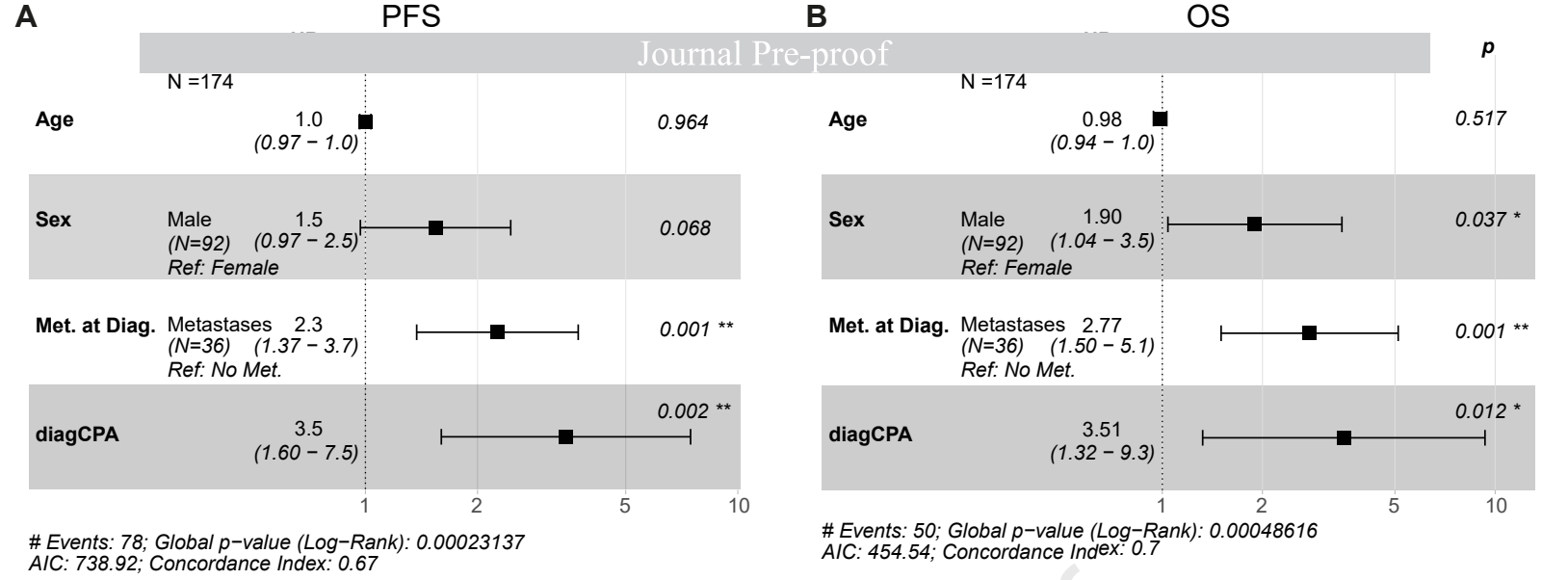
Table 1. Patient characteristics		ctDNA	OS2006	<i>p</i>
Age (years)	Median (range)	14.7 (4.71 - 50.41)	15.3 (4.62 - 67.06)	0.037
Sex	Female	86 (47%)	276 (45%)	0.54
	Male	97 (53%)	338 (55%)	
Puberty	Prepuberty	47 (26%)	137 (22%)	0.12
	Intrapuberty	43 (24%)	131 (22%)	
	Postpuberty	85 (46%)	310 (50%)	
	NA	8 (4%)	36 (6%)	
Metastases at diagnostic	No	142 (78%)	494 (80%)	0.15
	Yes	37 (20%)	102 (17%)	
	NA	4 (2%)	18 (3%)	
Upfront chemotherapy	API-AI	20 (11%)	111 (18%)	0.003
	MTX	158 (86%)	487 (79%)	
	NA	5 (3%)	16 (3%)	
Histological response	Good response	98 (53%)	335 (54%)	0.34
	Poor response	69 (38%)	206 (34%)	
	NA	16 (9%)	73 (12%)	
Relapse	Yes	78 (43 %)	251 (41%)	0.9
	No	105 (57%)	363 (59%)	
Status	Alive	131 (72%)	429 (70%)	0.8
	Dead	52 (28%)	185 (30%)	

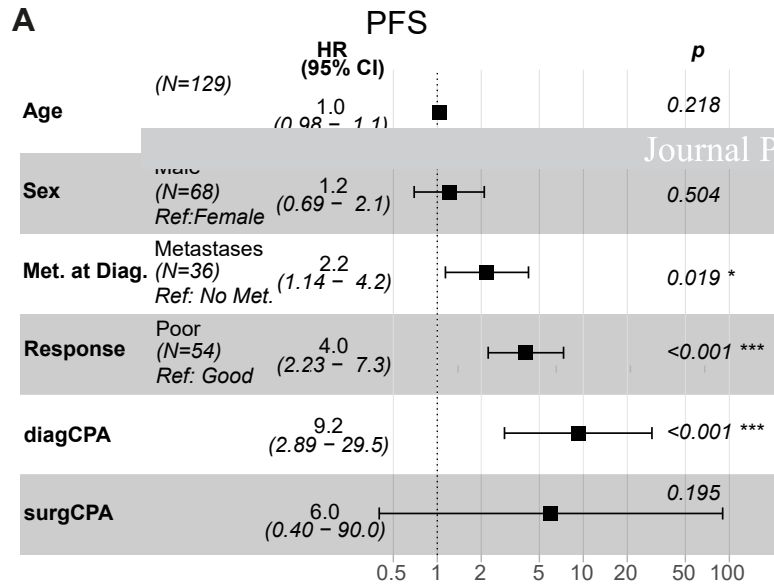
Wilcoxon was used for age, Log rank was calculated from PFS and OS comparison for relapse and status comparison respectively and Fisher Exact Test for other parameters.

Table 2. Impact of CPA score, metastatic status and histological response on DFS and OS

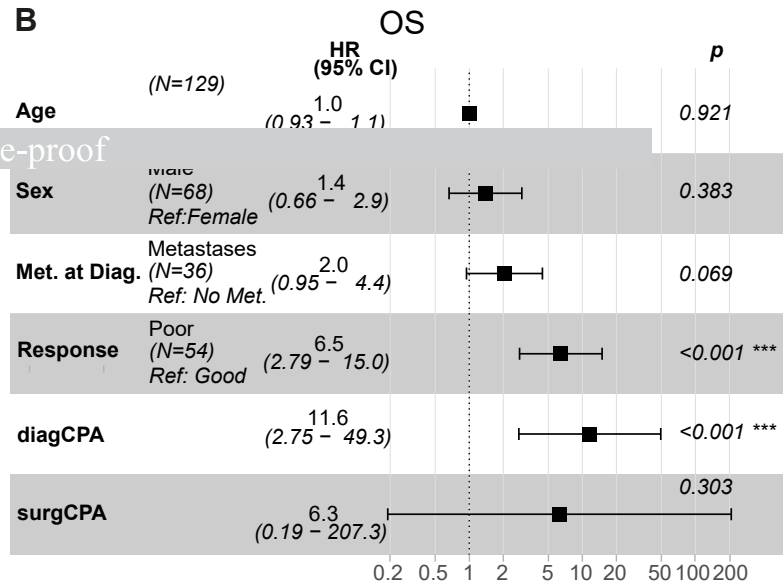
Patient and disease characteristics	5-year PFS (95% CI)	5-year OS (95% CI)
<i>Modelization at diagnosis</i>		
Low CPA score and localized disease	68% (59 – 79)	89.3% (80 – 1)
High CPA score and localized disease	46% (34 – 63)	76.3% (61 – 96)
Low CPA score and metastatic disease	47.6% (29 – 80)	64.2% (48 – 85)
High CPA score and metastatic disease	16.7% (6 – 47)	24.7% (10 – 60)
<i>Modelization at the time of surgery*</i>		
Low CPA score and good response	82.7% (73 – 94)	89.3% (80 – 1)
High CPA score and good response	60.4% (46 – 80)	76.3 (61 – 96)
Low CPA score and poor response	43% (30 – 61)	64.2% (48 – 85)
High CPA score and poor response	21.7% (10 – 47)	24.7% (10 – 60)
<i>Modelization at the end of treatment*</i>		
Low CPA score and good response	82.4% (73 – 94)	89.4% (80 – 1)
High CPA score and good response	60.4% (46 – 80)	76.3 (61 – 96)
Low CPA score and poor response	43.3% (31 – 61)	64.4% (49 – 86)
High CPA score and poor response	18.2 (8 – 44)	22.3% (9 – 67)

* The modelization uses CPA score at diagnosis and clinical data from surgery or end of treatment
PFS: Progression free survival; OS: overall survival

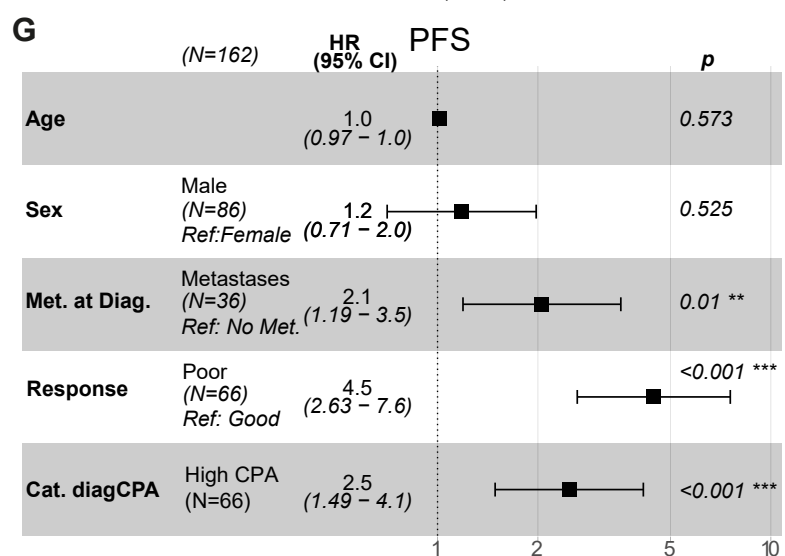
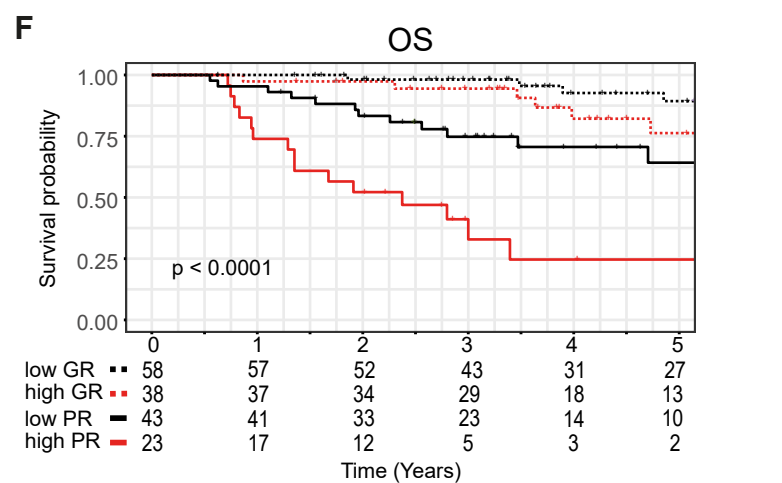
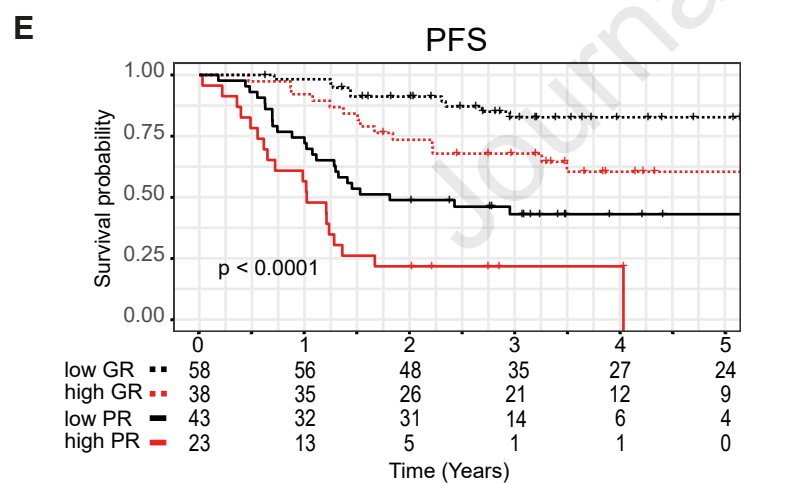
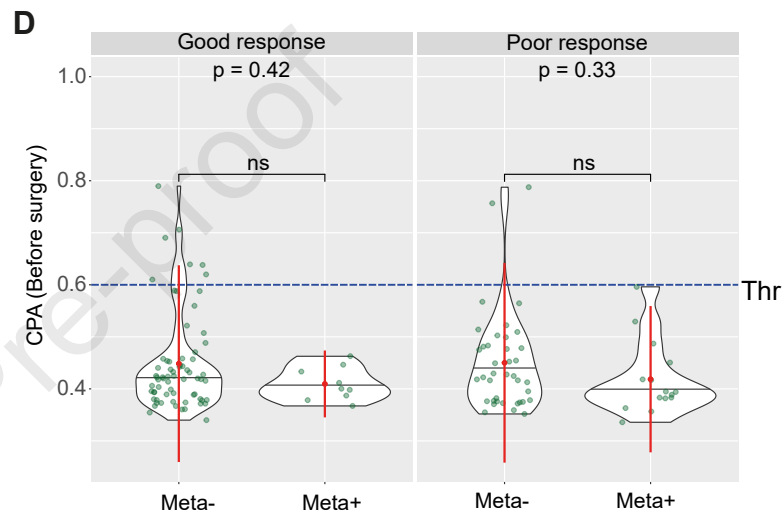
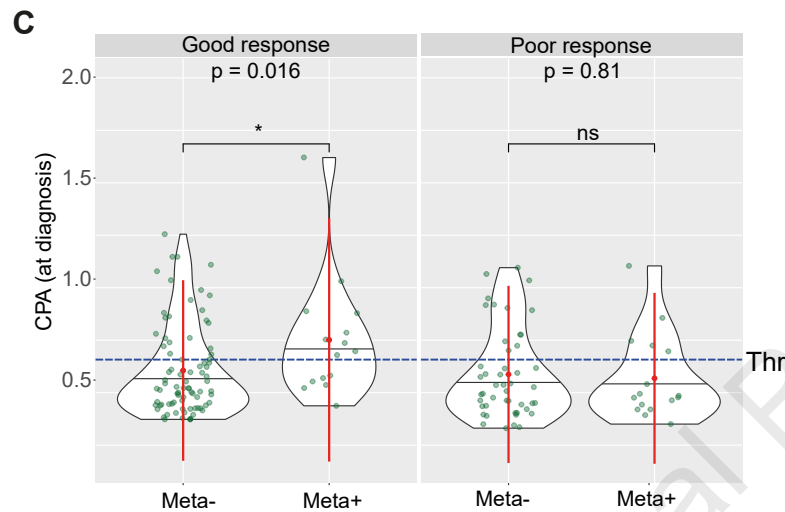




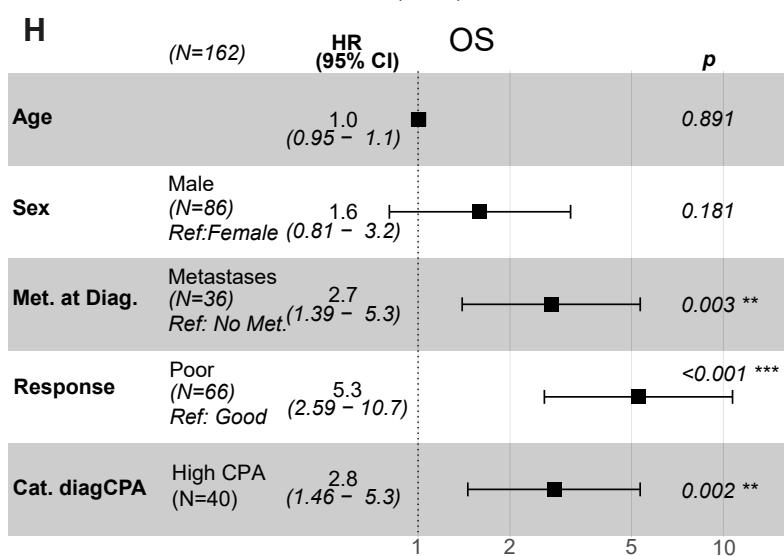
Events: 54; Global p-value (Log-Rank): 9.4152e-08
AIC: 460.31; Concordance Index: 0.77



Events: 35; Global p-value (Log-Rank): 1.3051e-06
AIC: 274.85; Concordance Index: 0.81



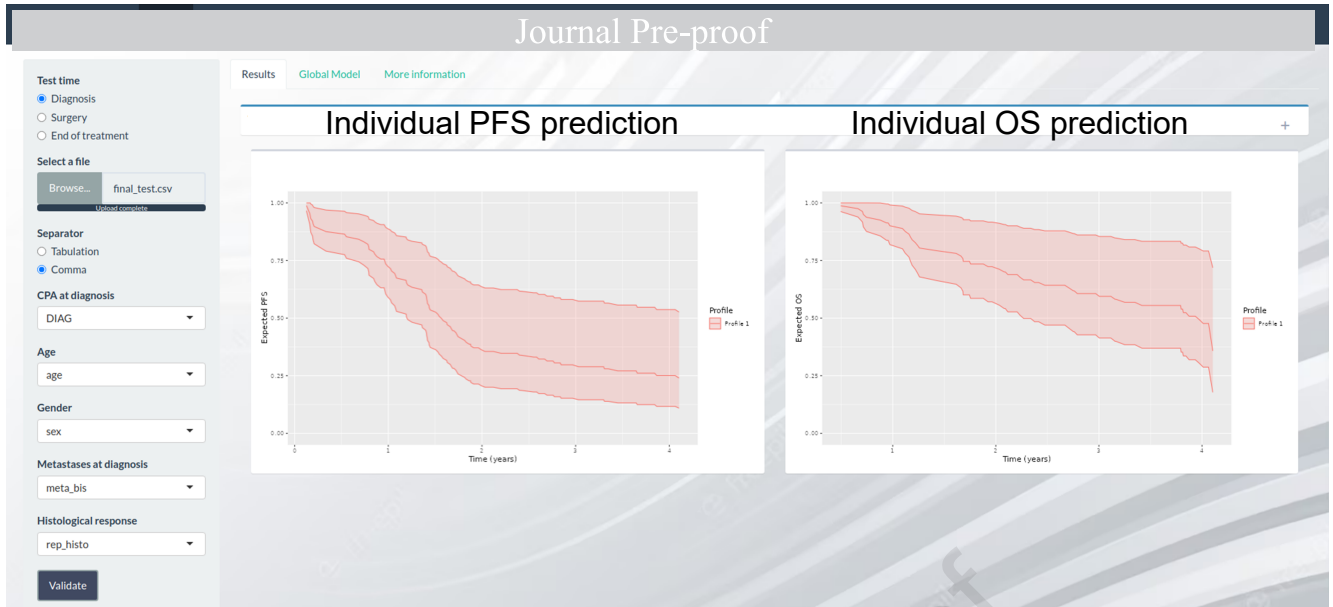
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AIC: 590.22; Concordance Index: 0.76



Events: 40; Global p-value (Log-Rank): 8.3151e-08
AIC: 332.56; Concordance Index: 0.81

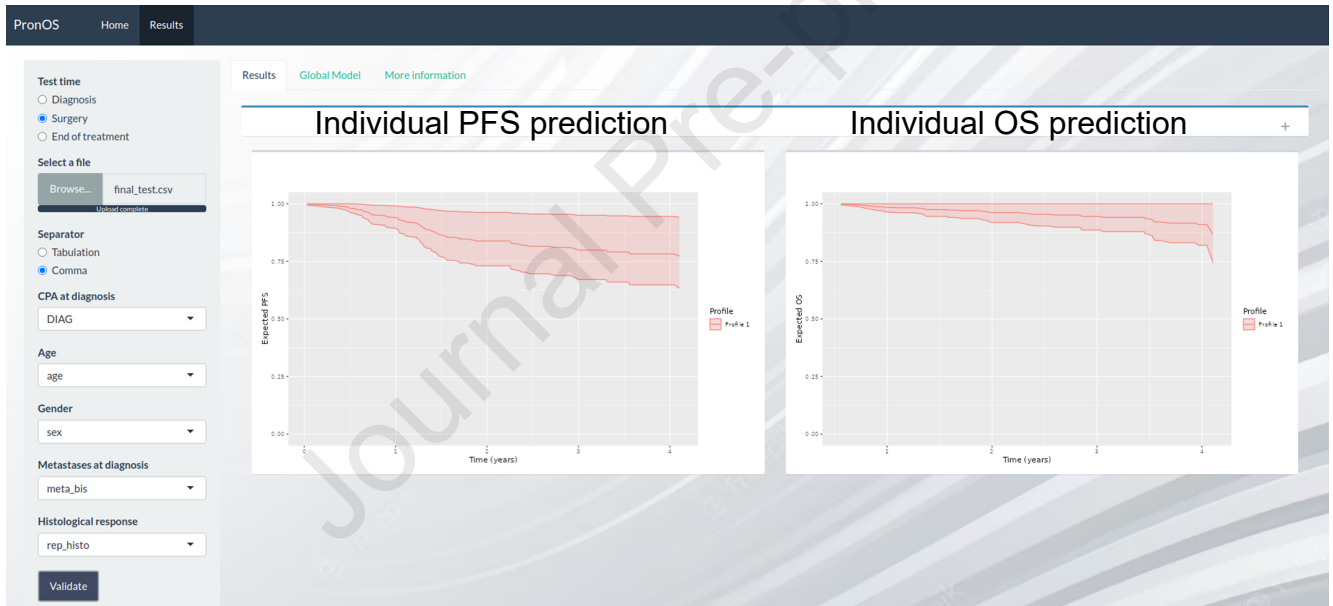
A

Test patient 1 risk at diagnosis



B

Test patient 2 risk at surgery



C

Test patient 3 risk at the end of treatment

