Bacterial lipopolysaccharide as a negative predictor of adjuvant gemcitabine efficacy in pancreatic cancer

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ABSTRACT

Adjuvant gemcitabine is one standard of care after pancreatic ductal adenocarcinoma (PDAC) resection. No biomarker for its efficacy is established. As bacteria mediate gemcitabine resistance, we analyzed whether lipopolysaccharide (LPS) as surrogate for bacterial colonization is prognostic in PDAC patients treated with (aGC) or without (naGC) adjuvant gemcitabine. We detected LPS in 86 tumors from 376 patients, which defined a specific microbiome as revealed by 16s-rRNA-sequencing. In the 230 aGC patients, LPS conferred worse disease free survival (8.3 vs 13.7 months; hazard ratio = 1.75, 95% confidence interval = 1.22 - 2.49, log-rank P = .002) and overall survival (21.7 vs 28.5 months; hazard ratio = 1.80, 95% confidence interval = 1.23 - 2.57, log-rank P = .001), but not in the 146 naGC patients, which was confirmed in an independent validation cohort (n=178). LPS may serve as negative predictor for adjuvant gemcitabine efficacy in PDAC, which suggests a role for microbiome modification to overcome bacteria-mediated chemotherapy resistance.

The dismal prognosis of PDAC is improved by adjuvant chemotherapy, for which gemcitabine remains a therapeutic mainstay in the clinically unfit patient[1], however, no biomarker for efficacy prediction is established. The tumor microbiome in PDAC affects patients´ prognosis [2] as well as response to gemcitabine-based chemotherapy in pre-clinical models [3] and advanced disease stages [4], in which single-agent gemcitabine is replaced by more efficient regimens [1]. However, the effect of the tumor microbiome on adjuvant gemcitabine efficacy has not been examined to date.

We retrieved formalin-fixed, paraffin-embedded (FFPE) primary tumor tissue from PDAC resections from the archives of the Institute of Pathology LMU. Clinicopathological, outcome and treatment data were derived from the databases of the Institute of Pathology, the Munich Cancer Registry and the LMU University Hospital. We updated each cases´ TNM classification to the current UICC staging system [5]. The ethics committee at medical faculty LMU approved the study (20-081). Tissue microarray (TMA) construction, LPS immunohistochemistry (IHC) and staining were described previously [4]. To evaluate the association of LPS detection with DFS and OS independent of other clinicopathologic factors, we employed log-rank-statistics, univariate and multivariate Cox regression models. DFS was calculated from adjuvant therapy initiation or surgery (in the patients without adjuvant therapy) to clinically apparent disease relapse. OS was calculated from surgery to death by disease excluding patients deceased within 30 days post-surgery. Statistical significance was indicated by $P < .05$. All statistical tests were 2-sided where appropriate. We examined the intratumoral microbiome by sequencing of the bacterial 16s rRNA locus (16s rRNA-Seq) using tumor DNA extracted from FFPE tissue [6] (Supplementary methods). Propensity score matching was conducted using pymatch (https://github.com/benmiroglio/pymatch) for Python (Anaconda Inc., Austin, TX, USA). Normalized abundance microbial data was downloaded from the data repository of The Cancer Genome Atlas (TCGA) as described [7]. Corresponding clinical patient information was downloaded from Broad GDAC Firehose and NCI Genomic Data Commons (GDC Data Release v29.0, Supplementary methods).

The study cohort comprised 197 men and 179 women (median age 66 years, range 41 – 83 years) of which 230 (61.2%) received adjuvant gemcitabine (aGC) and 146 (38.8%) received either non-gemcitabine based (n=29) or no adjuvant (n=117) treatment (naGC, Supplementary table 1). The median follow-up was 88.02 months (95% CI 72.2 – 103.8 months). Adjuvant gemcitabine therapy conferred superior DFS and OS over no or non-gemcitabine based adjuvant treatment (DFS 12.7 vs 6.9 months, HR 0.65, 95%CI 0.52 - 0.83, log-rank P< .001; OS 25.8 vs 15.6 months, HR 0.59, 95% CI 0.46 - 0.74, log-rank P<.001). We detected intratumoral LPS at similar rates in both cohorts (aGC cohort: 20.9 %, naGC cohort 26.0 %, Pearson χ^2 P=.25, Supplementary table 1). LPS positivity conferred reduced DFS (8.3 vs 13.7 months, HR 1.75, 95% CI 1.22 - 2.49, log-rank P = .002, figure 1A) and OS (21.7 vs 28.5 months, HR 1.80, 95% CI, 1.23 - 2.57, log-rank P = .001, figure 1B) in the aGC cohort, but not in the naGC cohort (DFS 5.6 vs 7.4 months, HR = 1.20, 95% CI 0.79 - 1.82, log-rank P = .39; OS 13.3 vs 18.7 months, log-rank P = .06, HR = 1.45, 95% CI 0.98 - 2.16, figure 1C, 1D). LPS positivity also reflected on five-years-survival rates of 21.1 % vs. 2.4% (LPS negative vs LPS positive tumors, Pearson χ^2 p=0.004) in patients of the aGC cohort, whereas no differences in the naGC cohort were detected (8.7% vs 5.7%; Pearson χ^2 p=0.58). LPS did not correlate to pre-operative antibiotic treatment, bile duct intervention or diabetes (Pearson χ^2 P>0.2 each). Multivariate analyses confirmed LPS as negative predictor for DFS (HR 1.83, 95%CI 1.26 - 2.65, Cox P=.001) and OS (HR 1.82, 95%CI 1.26 - 2.62, Cox P=.001) in the aGC cohort. Propensity score matching compensated imbalances between the cohorts (Supplementary table 1), resulted in balanced subgroups (n=100 each) and confirmed the findings evidently (DFS in the aGC cohort 9.4 vs. 15.1 months, HR 2.3, 95%CI 1.37 – 3.88, log-rank P=.001; in the naGC cohort 5.8 vs 7.4 months, HR 0.94, 95% CI 0.57 – 1.68, log-rank P=.94). For validation, we determined whether the abundance of gram-negative bacteria affects outcome dependent on adjuvant chemotherapy in the TCGA dataset (n=178, Supplementary table 2). Abundant intratumoral gram-negative bacteria conferred inferior DFS and OS in aGC patients (n=77), whereas in naGC patients (n=101) we observed no effect on outcome (figure 1 E-H). 16s rRNA-seq from nine advanced PDAC tumors[4, 8] revealed that LPS

positivity defined a specific tumor microbiome correlating with the relative abundance of the genera Comamonas, Diaphorobacter and Acinetobacter within the phylum of Proteobacteria as well as Weeksellaceae and Cloacibacterium within the phylum of Bacteriodetes. The phylum proteobacteria, to which the vast majority of bacteria belong that express the long isoform of cytidine deaminase (CDD_L), has been shown to cause gemcitabine resistance *in vitro* and *in vivo* [3] (figure 2 A-C).

Here we show that intratumoral LPS is associated with inferior DFS and OS in PDAC patients treated with gemcitabine-based adjuvant chemotherapy and that it defines a specific tumor microbiome. In patients receiving either no or non-gemcitabine-based adjuvant chemotherapy, LPS had no prognostic impact. This correlation was even more pronounced after propensity-score-matching and we confirmed this association in a validation dataset. Thus, in line with previously published data on gemcitabine resistance mediated by CDD_L – expressing bacteria [3], we reason that an LPS-positive tumor microbiome serves as negative predictor of adjuvant gemcitabine efficacy. Our observations are limited by the retrospective nature of this single-center study and lacking information on the tumor microbiome during disease progression, as we examined primary tumor tissue only. However, as most patients relapse eventually, PDAC is considered a systemic disease upon diagnosis [9] , which explains the negative predictive effect of LPS in the primary tumor. The non-significant trend towards decreased OS in LPS positive naGC cases may be due to the negative predictive effect of LPS on palliative gemcitabine-based therapy [4], which many patients received after relapse. Adjuvant gemcitabine is partly replaced by more efficient regimens in selected patients [10]. However, it is still widely used and recommended for patients with ECOG > 1 by NCCN guidelines[11], as many cannot receive more toxic adjuvant therapies due to their limited condition. Further studies on the tumor microbiomes´ impact on outcome in adjuvant treatment randomized controlled trials are required to verify our findings and to clarify whether they are limited to gemcitabine-based therapies. Our results offer a potential predictive biomarker for clinical decisions on adjuvant treatment. Additionally, they provide a rationale to address the tumor microbiome as therapeutic target in PDAC as it may be modified by antibiotics or

microbiome transplantation, which has already been established for gastrointestinal diseases [12] and in the context of immune therapy [13].

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Author contributions:

Conceptualization: MG, SO; Data curation: MG, LG, MAP, JE, SO. Resources: MG, LG, MAP, SAS, VH, MBB, JM, JE, JW, SB, SO; Formal Analysis: MG, SAS; Investigation: MG, SAS, LG, MAP, SO; Methodology, software and visualization: MG, SAS, SO; Project administration, supervision and validation: MG, SO; Writing – original draft: SO, SB; Writing – review & editing: SO

DATA AVAILABILITY

The study´s raw data can be obtained from the corresponding author upon reasonable request.

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FIGURE LEGENDS

Figure 1 – Intratumoral LPS detection and the abundance of gram negative bacteria are associated with poor disease free survival and overall survival in pancreatic cancer patients treated with adjuvant gemcitabine.

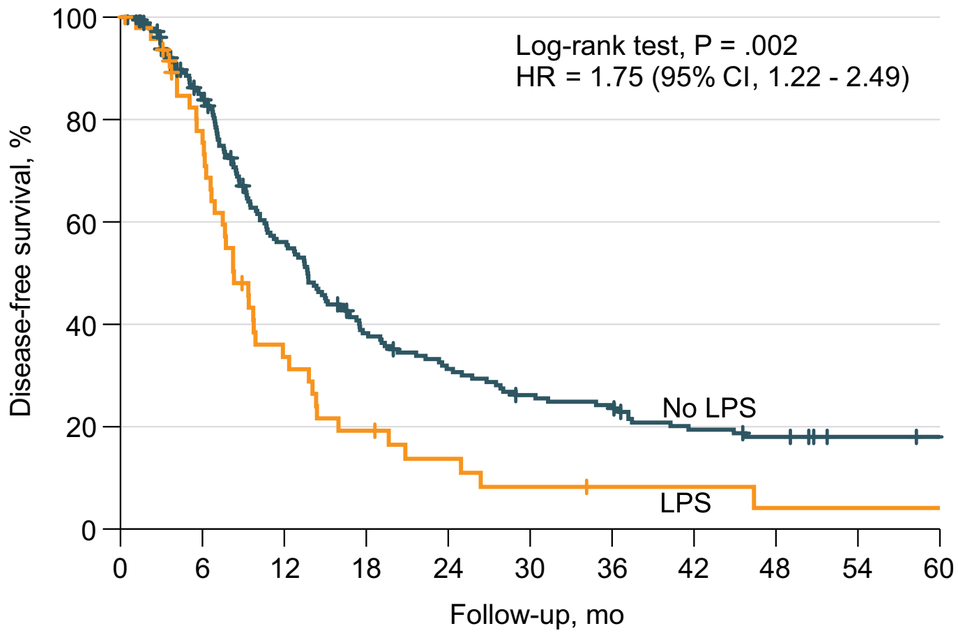
Intratumoral LPS detection and the abundance of gram-negative bacteria is negatively associated to disease-free survival and overall survival in **A**, **B** the aGC study cohort (n=230), **C**, **D** the naGC study cohort (n=146) as well as in **E**, **F** the aGC validation cohort (n= 101) and **G,H** the naGC validation cohort (n=77). All statistical tests were 2-sided.

Figure 2 – LPS positivity defines a specific tumor microbiome as determined by 16s rRNA sequencing.

A Phylogenetic distance tree calculated by generalized UniFrac distances, grouped by hierarchical clustering and taxonomic composition on family level based on the relative sequence abundances (colored bar plots). **B** Multidimensional scaling shows a significant clustering according to LPS positivity and a high level of dissimilarity between LPS positive and LPS negative samples (beta-diversity) based on generalized UniFrac distances. **C** Relative abundances of the five main differentially detected species between LPS positive and LPS negative samples by systematic testing of all available operational taxonomic units (OTU) using the non-parametric Kruskal-Wallis Rank Sum Test correcting the calculated pairwise test significance values for multiple testing using the Benjamini-Hochberg method. All statistical tests were 2-sided.

Figure 1

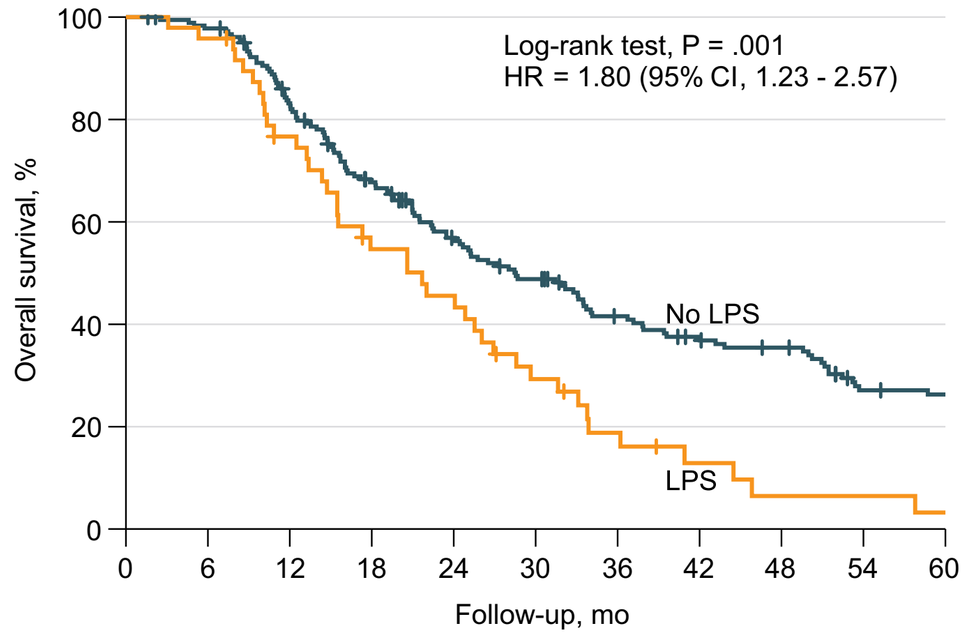
A



No. at risk

No LPS	182	142	92	61	49	40	37	28	25	21	20
LPS	48	34	14	8	5	3	2	2	1	1	1

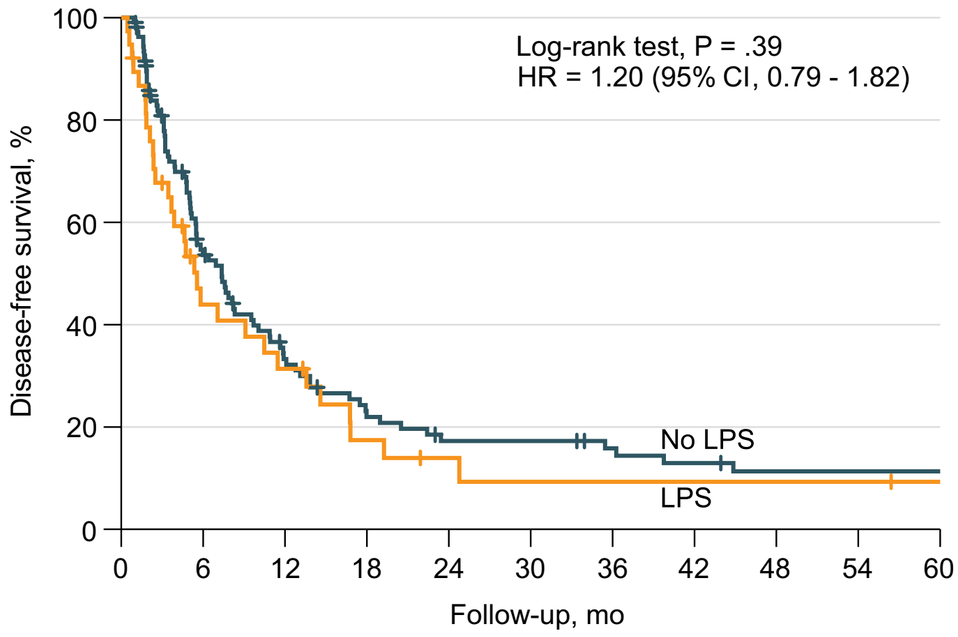
B



No. at risk

No LPS	182	176	147	116	92	78	62	54	49	34	32
LPS	46	46	35	24	20	12	7	4	2	2	1

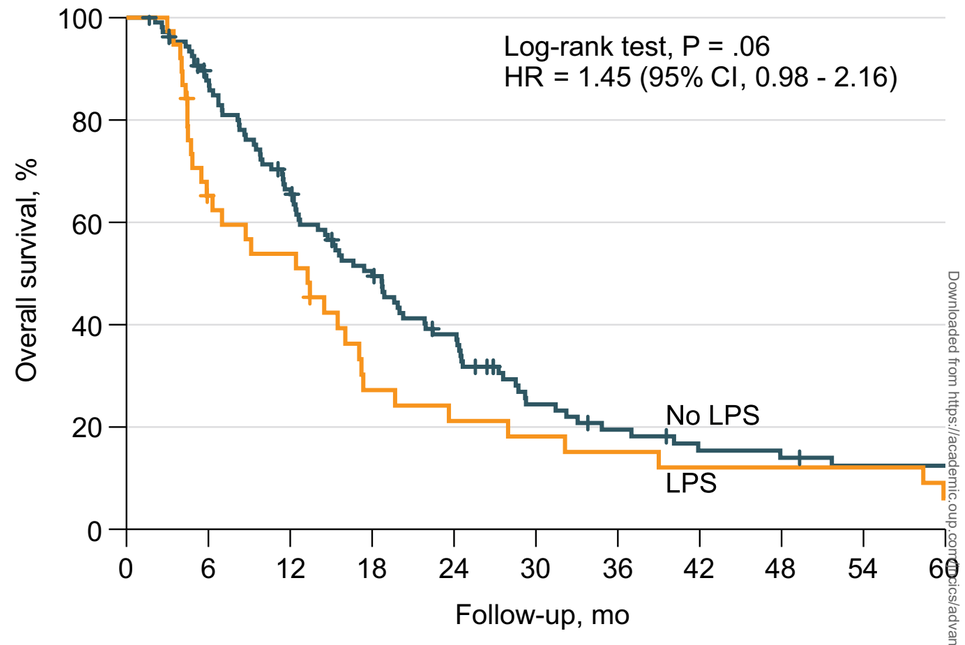
C



No. at risk

No LPS	108	52	30	19	14	14	11	9	7	7	7
LPS	38	14	10	5	3	2	2	2	2	2	1

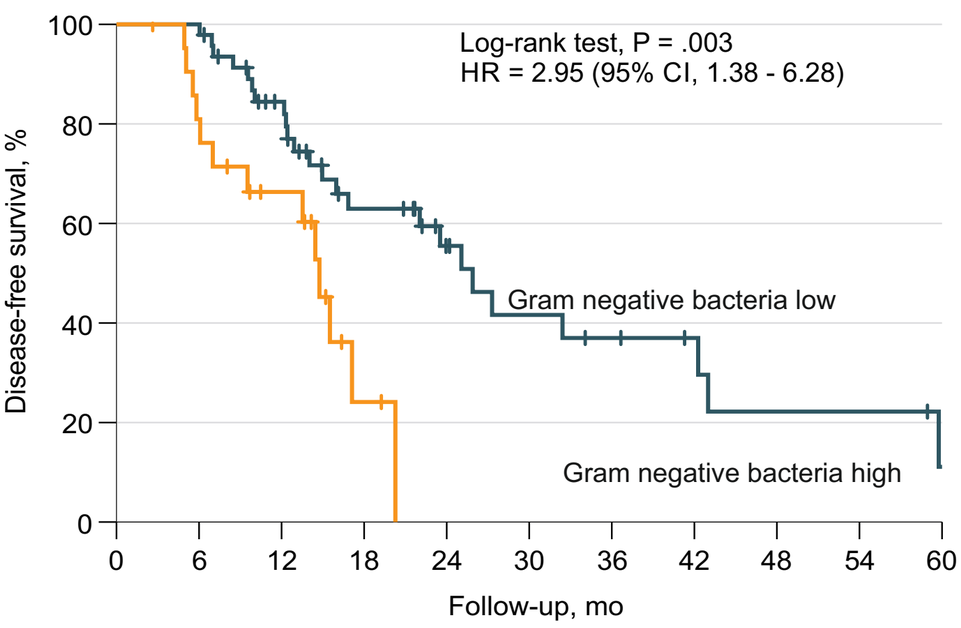
D



No. at risk

No LPS	108	91	68	50	36	20	15	11	10	8	8
LPS	38	23	19	9	7	6	5	4	4	4	2

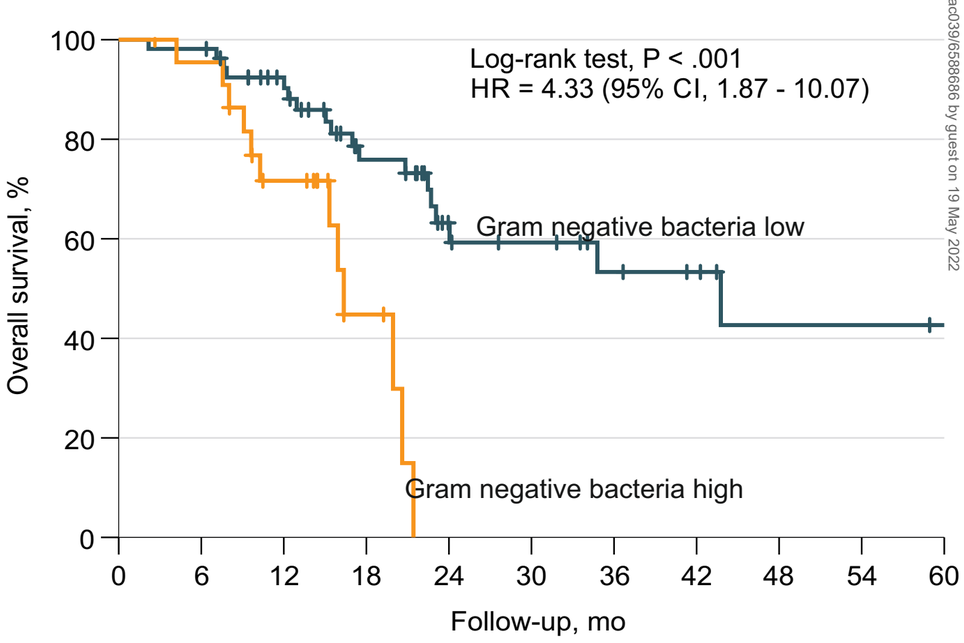
E



No. at risk

Gram negative bacteria low	47	47	34	21	13	9	7	5	3	3	1
Gram negative bacteria high	22	17	11	2	0	0	0	0	0	0	0

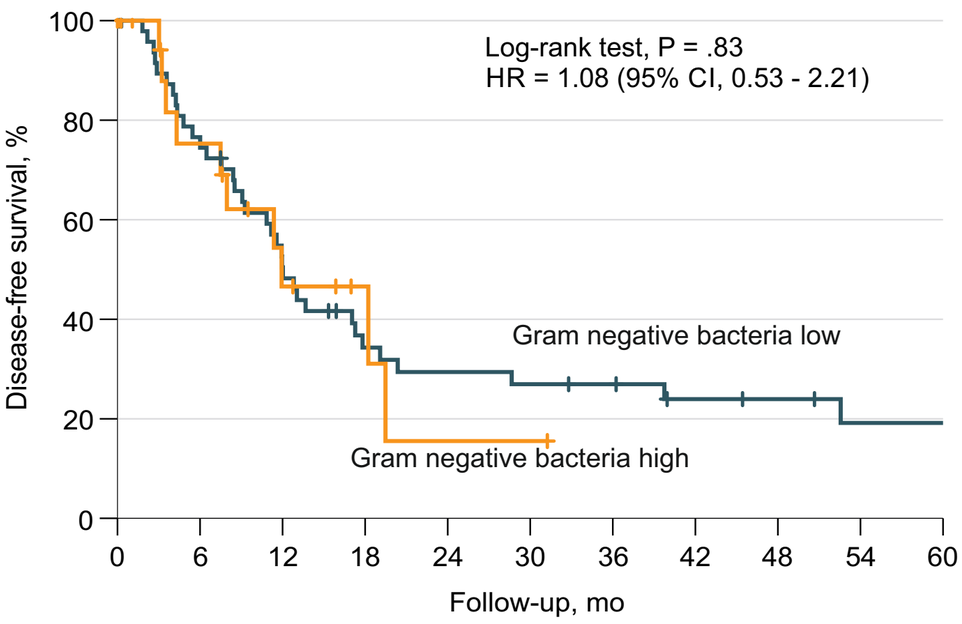
F



No. at risk

Gram negative bacteria low	54	53	43	28	16	13	9	7	4	4	3
Gram negative bacteria high	23	21	13	4	0	0	0	0	0	0	0

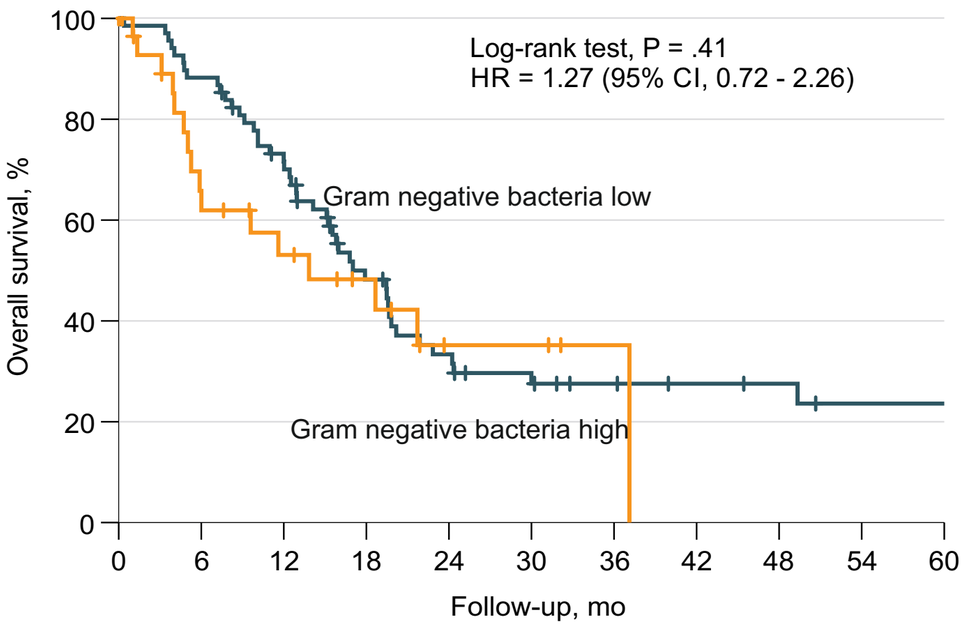
G



No. at risk

Gram negative bacteria low	49	36	23	14	12	11	10	7	6	4	4
Gram negative bacteria high	21	12	6	3	1	1	0	0	0	0	0

H



No. at risk

Gram negative bacteria low	70	60	46	27	18	13	10	8	7	5	5
Gram negative bacteria high	31	17	12	8	3	3	1	0	0	0	0

Figure 2