The efficacy and safety of thymosin α1 for sepsis (TESTS): multicentre, double blinded, randomised, placebo controlled, phase 3 trial

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Additional material is published online only. To view please visit the journal online.

Cite this as: *BMJ* 2025;388:e082583 http://dx.doi.org/10.1136/bmj-2024-082583

Accepted: 02 December 2024

ABSTRACT

OBJECTIVE

To evaluate whether the immunomodulatory drug thymosin α1 reduces mortality in adults with sepsis.

DESIGN

Multicentre, double blinded, placebo controlled phase 3 trial.

SETTING

22 centres in China, September 2016 to December 2020.

PARTICIPANTS

1106 adults aged 18-85 years with a diagnosis of sepsis according to sepsis-3 criteria and randomly assigned in a 1:1 ratio to receive thymosin α1 (n=552) or placebo (n=554). A stratified block method was used for randomisation, and participants were stratified by age (<60 and ≥60 years) and centre.

INTERVENTIONS

Subcutaneous injection of thymosin α1 or placebo every 12 hours for seven days unless discontinued owing to discharge from the intensive care unit, death, or withdrawal of consent.

MAIN OUTCOME MEASURE

The primary outcome was 28 day all cause mortality after randomisation. All analyses were based on a modified intention-to-treat set, including participants who received at least one dose of study drug.

RESULTS

Of 1106 adults with sepsis enrolled in the study, 1089 were included in the modified intention-to-treat analyses (thymosin α1 group n=542, placebo group n=547). 28 day all cause mortality occurred in 127 participants (23.4%) in the thymosin α1 group and 132 (24.1%) in the placebo group (hazard ratio 0.99, 95% confidence interval 0.77 to 1.27; P=0.93 with log-rank test). No secondary or safety outcome differed statistically significantly between the two groups. The prespecified subgroup analysis showed a potential differential effect of thymosin α1 on the primary outcome based on age (<60 years: hazard ratio 1.67, 1.04 to 2.67; ≥60 years: 0.81, 0.61 to 1.09; P for interaction=0.01) and diabetes (diabetes: 0.58, 0.35 to 0.99; no diabetes: 1.16, 0.87 to 1.53; P for interaction=0.04).

CONCLUSIONS

This trial found no clear evidence to suggest that thymosin α1 decreases 28 day all cause mortality in adults with sepsis.

TRIAL REGISTRATION

ClinicalTrials.gov NCT02867267.

WHAT IS ALREADY KNOWN ON THIS TOPIC

The dysregulated response to infection in sepsis plays a crucial pathophysiological mechanism, making immunomodulatory treatment an appealing option

Thymosin α1, a peptide with pleiotropic immune effects during infections, is a promising immunomodulatory agent

Studies suggest thymosin α1 might reduce 28 day mortality in patients with sepsis, but the quality of evidence is low owing to small sample sizes and methodological issues

WHAT THIS STUDY ADDS

This study found no conclusive evidence that thymosin α1 reduces 28 day mortality in adults with sepsis

Thymosin α1 did, however, show good safety in the treatment of sepsis

Future research on thymosin α1 in sepsis should address the heterogeneity of the disease, particularly focusing on patients aged 60 and older and those with chronic conditions

Introduction

Sepsis is a life threatening syndrome of organ dysfunction caused by a dysregulated response to infection.[1] The immune system serves as a natural barrier against invasion by pathogenic microorganisms. Sepsis induced immunosuppression, however, leads to a reduction in the number and function of immune cells, such as T cell exhaustion, which weakens the immune system's ability to eliminate pathogens.[2-5]

Immunomodulation treatment, an important adjunct to use of antibiotics, has become a focus of clinical trials aimed at managing sepsis induced immunosuppression, with several alternative drugs, including thymosin α1, currently under investigation.[6-9] Thymosin α1, a peptide primarily secreted by the thymus, attenuates chemotherapy induced immune damage to exert a synergistic effect,[10] modulates dendritic cells to enhance the antifungal effect of T helper 1 cells,[11] and reduces T cell exhaustion to improve lymphopenia.[12 13] Given its multiple immune effects, thymosin α1 has been used in the treatment of non-small cell lung cancer,[14 15]

melanoma,[16] chronic hepatitis,[17-19] sepsis,[20] AIDS,[21] and covid-19.[13]

Previous studies with small patient numbers showed that thymosin α1 improved immune function and reduced mortality in patients with sepsis. A meta-analysis of 10 randomised controlled trials with a total of 530 patients suggested that thymosin α1 might offer a 41% relative reduction in 28 day mortality (22% *v* 38%, relative risk 0.59, 95% confidence interval (CI) 0.45 to 0.77), but the quality of evidence was low owing to the small sample sizes of the included trials.[22] Subsequently, an expert consensus on sepsis immunosuppression recommended the use of thymosin α1 in patients with sepsis, albeit based on limited evidence.[23]

We conducted a multicentre, randomised, double blinded, placebo controlled clinical trial (TESTS, The Efficacy and Safety of Thymosin α1 for Sepsis) to evaluate the efficacy and safety of thymosin α1 for the treatment of sepsis.

Methods

Study design and oversight

Our multicentre trial involved 22 centres in nine provinces across all five geographical regions of China: eastern (Shanghai, Shandong, Jiangsu, Zhejiang), southern (Guangdong), western (Sichuan), northern (Beijing, Shaanxi), and central (Hubei). The trial took place between September 2016 and December 2020 (see supplementary Protocol and SAP file, p3). Written informed consent was obtained from all participants or their legally authorised representatives. An independent data and safety monitoring board oversaw the safety of the trial.

Participants

We included participants aged 18 to 85 years with a diagnosis of sepsis according to Sepsis-3 criteria.[1] Patients were excluded if they met any of the following criteria: were pregnant or lactating; had haematological malignancies; had undergone organ or bone marrow transplantation; had an acute phase autoimmune disease or glomerulonephritis; had an allergy or intolerance to thymosin α1; had a history of cardiopulmonary resuscitation within 72 hours before signing the consent form, with incomplete neurological recovery (Glasgow coma scale score ≤8); had a history of radiotherapy, chemotherapy, treatment with immunosuppressive drugs, or continuous treatment with prednisolone >10 mg/day (or an equivalent dose of other steroids) in the past 30 days; had participated in clinical trials related to immunity in the past 30 days; had undrained foci of infection (eg, intra-abdominal infections that cannot be managed through surgery or drainage); had an underlying disease estimated to result in death within 28 days; or the immediate family expressed a wish for life sustaining treatment to be discontinued or end-of-life care instigated. The supplementary appendix lists the inclusion and exclusion criteria.

Randomisation and masking

Using a computer generated block randomisation protocol with a block size of 8, we randomly assigned (1:1) eligible participants to receive either thymosin α1 or matched placebo. Randomised numbering and treatment allocation were done through an interactive web response system, and random assignment was stratified by centre and age (<60 and ≥60 years).

Statisticians not connected with the trial provided the random number tables for drug randomisation, using SAS version 9.4 software based on fixed seed number. They were also responsible for masking of the drug and preparing emergency letters containing individual treatment assignments, which could be opened for medical emergencies when knowledge of treatment allocation was required. The investigators, participants, care providers, and statisticians were all blinded to the assigned treatment.

Procedures

Participants in the intervention group received a subcutaneous injection of 1.6 mg of lyophilised thymosin α1 powder dissolved in 1 mL of sterilised water every 12 hours. The control group received placebo (lyophilised saline) in the same manner. The trial drugs were administered for seven days, unless discontinued owing to discharge from the intensive care unit (ICU), death, or withdrawal of consent. In both study arms, standardised treatment was provided according to the Surviving Sepsis Campaign.[24] After enrolment, participants were evaluated according to the study procedures (see supplementary Protocol and SAP file, pp 98-105). A validated electronic clinical data management system was used to collect and manage data from the trial, with data entered on an electronic case report form.

Outcomes

The primary outcome was 28 day all cause mortality after randomisation. Secondary outcomes included incidence of new onset infections within 28 days, 28 day clearance rate of pathogenic microorganisms, duration of ICU and hospital stays, readmission to hospital within 28 days, changes in the sequential organ failure assessment score on day 7, 90 day all cause mortality, mortality on the ICU, mechanical ventilation-free days within 28 days, ICU-free days within 28 days, continuous renal replacement therapy-free days within 28 days, vasopressors-free days within 28 days, and score on the 36-item short form (SF-36) quality of life scale within 90 days. Exploratory outcomes included change in monocyte human leucocyte antigen-DR, neutrophil to lymphocyte ratio, regulatory T cell percentage, and lymphocyte count at 7, 14, and 28 days, compared with baseline values obtained at screening. We monitored adverse events and serious adverse events for safety signals. See supplementary Protocol and SAP file, pp 89 and 90, for details and definitions of all outcomes.

Statistical analysis

We determined the sample size to detect superiority in the primary outcome on the basis of data from a previous trial and assumed a 27% 28 day mortality rate for the intervention group and 35% for the control group, with a one sided type I error of 0.025 and power of 80%.[20] Considering a dropout rate of 5%, we determined that 553 participants (1106 in total) would be required in each group. Continuous variables were analysed using either an independent sample *t* test or the Wilcoxon rank-sum test between two groups. Paired group comparisons were made using a paired *t* test or the Wilcoxon signed-rank test. Categorical variables were analysed using the χ^2 test or Fisher's exact test, and the Cochran-Mantel-Haenszel test was used to calculate the between group difference according to stratified factors. Kaplan-Meier estimates were used for time-to-event analyses, and log-rank tests were used for comparisons. The primary outcome was analysed using a Cox proportional hazards model adjusted for centre and age and assessed based on Schoenfeld residuals. The mixed effects model for repeated measures was used to analyse the dynamic changes in immune markers. Subgroup analyses were conducted by age (a stratified factor), sex, and chronic conditions (hypertension, coronary heart disease, chronic obstructive pulmonary disease, diabetes, chronic kidney disease, and solid malignant tumour). Tests for interaction between treatment and subgroups were conducted by including multiplicative interaction terms in the Cox proportional hazards models. Confidence intervals and P values were not adjusted for multiple testing, except for interaction P values of subgroup analyses. We used Bonferroni correction to adjust for multiple testing in subgroup analyses. All outcomes were analysed in the modified intention-to-treat set, which included participants who were randomised and received at least one dose of study drug. To assess the superiority of the primary outcome, we examined whether the upper limits of the confidence intervals exceeded zero. P values were two sided, and we considered P<0.05 to indicate statistical significance, except for the primary outcome. The primary outcome was not imputed because no data were missing after study completion. Missing data for other outcomes were replaced using regression multiple imputation under the assumption of monotonic patterns. All statistical analyses were prespecified in the statistical analysis plan and independently conducted using SAS version 9.4 (see supplementary Protocol and SAP file, pp 139-177).

Patient and public involvement

Limitations in funding and expertise restricted our ability to conduct focus groups for patient and public involvement. However, our investigators' clinical experience with patients played an important role in informing the study's design and rationale. The involvement of patients in the later phases of the trial, as well as the statistical analysis after the trial's conclusion, was affected by the covid-19 pandemic.

Results

Baseline characteristics

From September 2016 to December 2020, a total of 1106 participants with sepsis were enrolled in

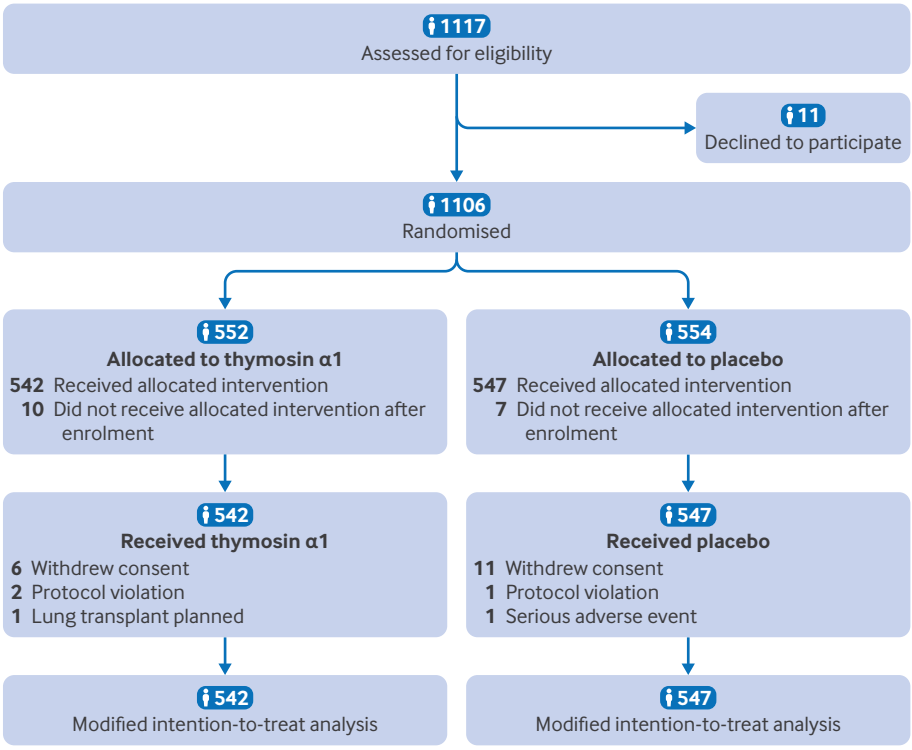

Fig 1 | Flow of participants through study

Table 1 | Baseline characteristics of participants with sepsis randomly assigned to receive thymosin α1 or placebo. Values are number (percentage) unless stated otherwise

Characteristics	Overall (n=1089)	Thymosin α1 group (n=542)	Placebo group (n=547)
Median (IQR) age (years)	65 (52-73)	65 (52-74)	65 (51-72)
Age group (years):			
<60	427 (39.2)	212 (39.1)	215 (39.3)
≥60	662 (60.8)	330 (60.9)	332 (60.7)
Sex:			
Men	750 (68.9)	360 (66.4)	390 (71.3)
Women	339 (31.1)	182 (33.6)	157 (28.7)
Surgical admission*:			
Overall	538 (49.4)	264 (48.7)	274 (50.1)
Emergency surgery	227 (42.2)	113 (42.8)	114 (41.6)
Non-emergency surgery	311 (57.8)	151 (57.2)	160 (58.4)
Pre-existing conditions:			
Hypertension	421 (38.7)	221 (40.8)	200 (36.6)
Coronary heart disease	128 (11.8)	65 (12.0)	63 (11.5)
COPD	88 (8.1)	37 (6.8)	51 (9.3)
Diabetes	277 (25.4)	152 (28.0)	125 (22.9)
Chronic kidney disease	64 (5.9)	28 (5.2)	36 (6.6)
Solid malignant tumours	121 (11.1)	59 (10.9)	62 (11.3)
Organ support before randomisation:			
Mechanical ventilation	836 (76.8)	423 (78.0)	413 (75.5)
Median (IQR) support (days)	8 (4-16)	9 (4-15)	8 (4-16)
Vasopressors	674 (61.9)	333 (61.4)	341 (62.3)
Median (IQR) support (days)	5 (2-10)	4 (2-11)	5 (3-10)
CRRT	184 (16.9)	90 (16.6)	94 (17.2)
Median (IQR) support (days)	6 (3-14)	7 (3-14)	6 (3-14)
Antibiotic use at screening	1046 (96.1)	524 (96.7)	522 (95.4)
Infection sites†:			
Lung	343 (31.6)	177 (32.8)	166 (30.4)
Abdomen	108 (10.0)	49 (9.1)	59 (10.8)
Bloodstream	86 (7.9)	46 (8.5)	40 (7.3)
Urinary tract	24 (2.2)	13 (2.4)	11 (2.0)
Biliary tract	26 (2.4)	9 (1.7)	17 (3.1)
Skin and soft tissue	9 (0.8)	6 (1.1)	3 (0.6)
Others	42 (3.9)	24 (4.5)	18 (3.3)
Multiple sites	216 (19.9)	107 (19.9)	109 (20.0)
Unknown	231 (21.3)	108 (20.0)	123 (22.5)
Microorganisms†:			
Gram negative	379 (34.9)	199 (36.9)	180 (33.0)
Gram positive	77 (7.1)	38 (7.1)	39 (7.1)
Fungi	100 (9.2)	52 (9.7)	48 (8.8)
Atypical pathogens	3 (0.3)	3 (0.6)	0
Mixed	293 (27.0)	138 (25.6)	155 (28.4)
Culture negative	233 (21.5)	109 (20.2)	124 (22.7)
Median (IQR) APACHE II score	14 (10-19)	15 (10-20)	14 (10-18)
Median (IQR) SOFA score	7 (5-10)	7 (5-10)	7 (5-10)
Median (IQR) immune markers‡:			
mHLA-DR (n=427/432)§	4185 (2413-7185)	3871 (2381-7280)	4381 (2423-7069)
Lymphocyte count	0.70 (0.42-1.06)	0.71 (0.45-1.09)	0.70 (0.43-1.05)
NLR (n=541/547)§	13.9 (7.9-23.5)	14.1 (8.4-23.2)	13.7 (7.7-24.7)
Regulatory T cells % (n=431/430)§	9.0 (7.0-11.6)	9.2 (7.1-11.7)	8.8 (6.8-11.5)
Laboratory results, median (IQR):			
Leucocytes ($\times 10^9$/L) (n=541/546)§	12 (8-17)	12 (8-17)	11 (8-17)
Platelets ($\times 10^9$/L)	154 (86-240)	152 (89-243)	157 (83-234)
CRP (mg/L) (n=410/422)§	128 (71-189)	126 (66-181)	129 (76-196)
Procalcitonin (ng/mL) (n=446/463)§	3 (1-22)	3 (1-23)	3 (1-19)
Creatinine (μmol/L) (n=541/542)§	89 (58-153)	90 (60-152)	89 (57-156)
Total bilirubin (μmol/L) (n=528/540)§	19 (12-37)	19 (12-38)	18 (12-36)

APACHE=acute physiology and chronic health evaluation; COPD=chronic obstructive pulmonary disease; CRP=C reactive protein; CRRT=continuous renal replacement therapy; mHLA-DR=monocyte human leucocyte antigen-DR; NLR=neutrophil-to-lymphocyte ratio; SOFA=sequential organ failure assessment.
*Defined by admission to ICU after a surgical procedure.
†Microbiological cultures were not available for four patients.
‡The immune markers assessed were mHLA-DR expression (normal reference >15 000 antibodies per cell), lymphocyte count (normal reference >1.1×10^9/L), neutrophil to lymphocyte ratio (normal reference range 1-3), and regulatory T cell percentage (normal reference <7.7%).
§Number of patients in thymosin α1 group/number in placebo group.

Table 2 | Outcomes of modified intention-to-treat population in patients with sepsis randomised to receive thymosin α1 or placebo. Values are number (percentage) unless stated otherwise

Outcomes	Thymosin α1 group (n=542)	Placebo group (n=547)	Group difference (95% CI)	P value*
Primary outcome				
28 day all cause mortality	127 (23.4)	132 (24.1)	−0.7 (−5.8 to 4.4)	0.80
Secondary outcomes				
90 day all cause mortality	168 (31.0)	177 (32.4)	−1.4 (−6.9 to 4.2)	0.63
ICU mortality	46 (8.5)	54 (9.9)	−1.4 (−4.8 to 2.0)	0.40
New onset infection ‹28 days†	137 (25.3)	143 (26.1)	−0.9 (−6.1 to 4.3)	0.74
Pathogenic microorganism clearance rate ‹28 days‡	94 (20.3)	76 (16.3)	4.0 (−0.9 to 9.0)	0.13
Median (IQR) change in SOFA score (n=451/456)§:				
Day 7¶	4 (2-7)	4 (2-7)	0 (0 to 1)	0.50
Percentage change	38 (0-67)	40 (0-67)	0 (−3.3 to 7.7)	0.56
Hospital readmission to day 28	75 (13.8)	72 (13.2)	0.7 (−3.4 to 4.8)	0.71
Median (IQR) length of ICU stay ‹90 days	15 (8-31)	15 (8-28)	1 (−1 to 2)	0.28
Median (IQR) length of hospital stay ‹28 days	24 (13-28)	23 (14-28)	0 (0 to 0)	0.99
ICU-free days ‹28 days	13 (0-20)	13 (0-20)	0 (0 to 0)	0.30
Organ support before randomisation:				
Mechanical ventilation	301 (55.6)	298 (54.5)	1.1 (−4.9 to 7.0)	0.77
Median (IQR) mechanical ventilation-free days ‹28 days	19 (8-25)	21 (9-26)	−1 (−2 to 0)	0.15
Vasopressors	230 (42.4)	224 (41.0)	1.5 (−4.4 to 7.3)	0.60
Median (IQR) vasopressors-free days ‹28 days	23 (13-26)	23 (13-26)	0 (−1 to 1)	0.84
CRRT	75 (13.8)	72 (13.2)	0.7 (−3.4 to 4.7)	0.75
Median (IQR) CRRT-free days ‹28 days	17 (9-24)	19 (10-25)	0 (−3 to 2)	0.88
Median (IQR) SF-36 score to day 90 (n=278/274)§**:				
Physical component	62 (54-68)	61 (52-69)	0.5 (−1.5 to 2.5)	0.63
Mental component	63 (53-71)	60 (52-69)	1.5 (−0.8 to 4.0)	0.17
Safety outcomes				
Adverse events	360 (66.4)	370 (67.6)	−1.2 (−6.8 to 4.4)	0.70
Serious adverse events	145 (26.8)	160 (29.3)	−2.5 (−7.8 to 2.8)	0.38
Exploratory outcomes				
Median (IQR) mHLA-DR (n=360/356)§:				
Day 7¶	7494 (4375-12 648)	7518 (4497-11 983)	54 (−672 to 775)	0.89
Absolute change	2480 (−45-5731)	2678 (−16-5815)	5 (−710 to 728)	0.99
Percentage change	69 (−1-164)	60 (0-162)	1 (−15 to 18)	0.87
Median (IQR) lymphocyte counts (n=448/455)§:				
Day 7¶	0.96 (0.63-1.45)	0.91 (0.61-1.32)	0.07 (0 to 0.14)	0.07
Absolute change	0.20 (−0.06-0.57)	0.20 (−0.12-0.57)	0.04 (−0.03 to 0.10)	0.28
Percentage change	33 (−7-100)	31 (−15-99)	6 (−5 to 15)	0.33
Median (IQR) NLR (n=448/455)§:				
Day 7¶	8.0 (4.7-13.6)	8.7 (5.5-15.1)	−0.8 (−1.5 to 0)	0.05
Absolute change	−4.9 (−12.3-0)	−3.3 (−11.9-1.9)	−1.7 (−3.0 to −0.6)	0.02
Percentage change	40 (0-66)	31 (−28-60)	9 (2 to 15)	0.006
Median (IQR) T regulatory cells (%) (n=364/364)§:				
Day 7¶	9.6 (7.5-11.4)	9.2 (7.4-11.6)	0.2 (−0.3 to 0.7)	0.42
Absolute change	0.1 (−1.6-1.6)	−0.1 (−1.7-1.7)	0.1 (−0.3 to 0.5)	0.74
Percentage change	1 (−16-23)	−1 (−17-20)	1 (−3 to 6)	0.62

CI=confidence interval; CRRT=continuous renal replacement therapy; ICU=intensive care unit; IQR=interquartile range; mHLA-DR=monocyte human leucocyte antigen-DR; NLR=neutrophil to lymphocyte ratio; SOFA=sequential organ failure assessment.
*Adjusted for centre and age, but not for multiple testing.
†Defined as an irrefutably positive culture (no identified pathogen) from an initially unaffected site or a new organism cultured from the original infected site; with either combined with clinician declaration of a definite infection based on clinical symptoms, laboratory tests results, and imaging.
‡928 patients (462 in thymosin α1 group and 466 in placebo group) were evaluated in follow-up. The clearance of pathogenic microorganism is defined as the transition of culture specimens from the previously definite pathogenic microorganisms to a negative state, coupled with the investigator's comprehensive assessment of clinical symptoms, laboratory tests results, and imaging.
§Number of patients in thymosin α1 group/number in placebo group.
¶Defined as follow-up at end of trial treatment, which included day 7 for patients who completed the course of trial treatment and end of trial treatment for patients who did not complete the course of trial treatment. The absolute change of immune marker from screening period to follow-up (days 7, 14, and 28) was defined as follow-up values minus baseline values, and percentage change in immune markers was calculated as 100%×(follow-up values−baseline values)/baseline values.
**Proportion of patients alive and eligible for SF-36 follow-up at 90 day visit was 74.1% (n=552/745).

the trial, 552 of whom were randomised to the thymosin α1 group and 554 to the placebo group (fig 1 and supplementary appendix table S1). After randomisation, 17 participants (10 in the thymosin α1 group and seven in the placebo group) were excluded because they had not received any study drug before withdrawing consent, and 1089 participants were included in the modified intention-to-treat analysis (see supplementary appendix table S2). The daily number of participants who received the study drugs was similar between the two groups (see supplementary appendix table S3).

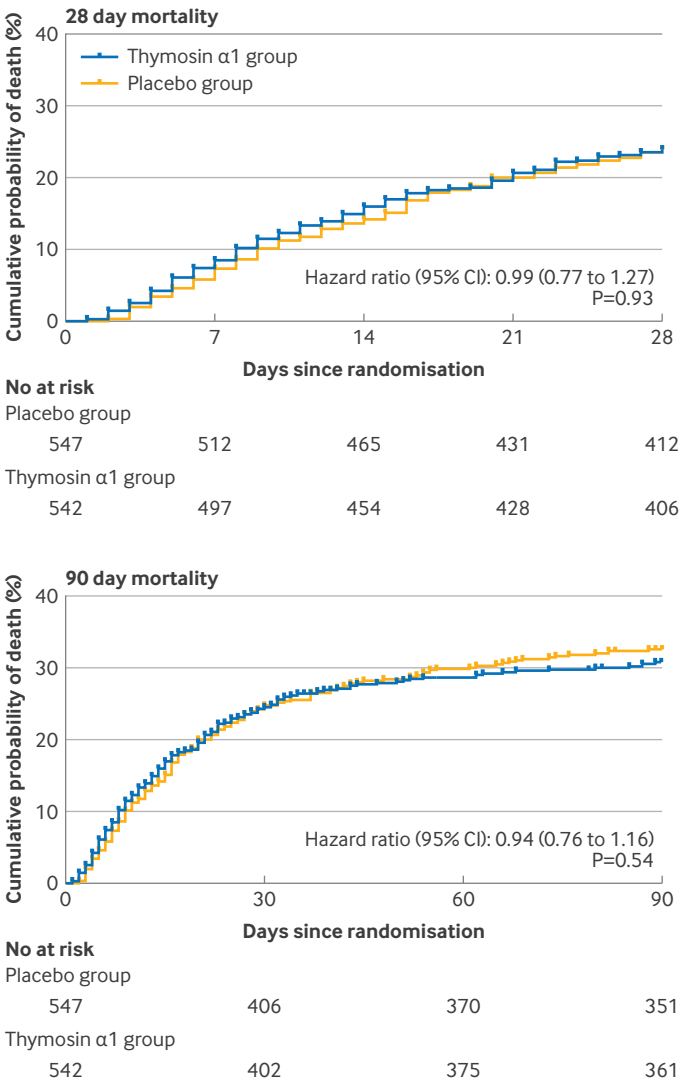

Fig 2 | Kaplan-Meier estimates of time-to-event for death at 28 days (primary outcome) and 90 days from randomisation. Log-rank tests were used for comparisons and the hazard ratios for relative risks of the primary outcome between the two groups, along with corresponding 95% CIs, were calculated using a Cox regression model adjusted for centre and age. The model assumption was assessed using Schoenfeld residuals. CI=confidence interval

Personal and clinical characteristics were well balanced between the two groups at baseline (table 1), including indicators for severity of illness and immune function. The median age was 65 years (interquartile range (IQR) 52-73), and 68.9% of participants (750/1089) were men. The most common site of infections was lung (31.6%). A total of 1046 patients (96.1%) received antibiotics at screening (table 1 and see supplementary appendix table S4). The median acute physiology and chronic health evaluation-II (APACHE-II) scores and sequential organ failure assessment (SOFA) scores at screening were 14 (IQR 10-19) and 7 (5-10), respectively, indicating disease severity. Before randomisation, 76.8% of participants (n=836) received mechanical ventilation for a median of eight (IQR 4-16) days, 61.9% (n=674) received vasopressors for a median of five (2-10) days, and 16.9% (n=184) received continuous renal replacement therapy for a median of six (3-14) days.

Primary outcome

In the modified intention-to-treat set, all cause mortality within 28 days of randomisation occurred in 127 participants (23.4%) in the thymosin α1 group and 132 (24.1%) in the placebo group (hazard ratio 0.99, 95% CI 0.77 to 1.27, P=0.93) (table 2 and fig 2). The 90 day all cause mortality was not significantly different between the thymosin α1 group and placebo group (31.0% *v* 32.4%, hazard ratio 0.94, 0.76 to 1.16, P=0.54) (table 2 and fig 2). Sensitivity analysis further confirmed the findings for the primary outcome (supplementary appendix table S5).

Subgroup analysis

Subgroup analysis was performed in eight subgroups (fig 3). Among these subgroups, we observed heterogeneity for the treatment effect by age: in the thymosin α1 group, 28 day mortality was higher in participants younger than 60 years (1.67, 1.04 to 2.67); for participants aged 60 and older, the hazard ratio was 0.81 (0.61 to 1.09, P for interaction=0.01) (fig 3 and supplementary figure S1). Post hoc analysis suggested that thymosin α1 might not have increased 28 day mortality in participants younger than 60 (1.45, 0.89 to 2.36, P for interaction=0.04) after adjusting for organ support before randomisation (supplementary appendix table S6). For chronic conditions, participants with diabetes in the thymosin α1 group had a lower 28 day mortality than participants with diabetes in the placebo group (0.58, 0.35 to 0.99, P for interaction=0.04). Additionally, in participants with hypertension (0.71, 0.49 to 1.04, P for interaction=0.06) and coronary heart disease (0.47, 0.21 to 1.01, P for interaction=0.06) the trend was consistent with those aged 60 and older and those with diabetes.

Secondary outcomes

No statistically significant differences were found between the thymosin α1 group and placebo group for secondary outcomes, including change in SOFA score, new onset infections, mechanical ventilation-free days within 28 day, vasopressors-free days within 28 days, and continuous renal replacement therapy-free days within 28 days (table 2). These results were consistent with the original analysis accounting for missing data (supplementary appendix table S7).

At the time of enrolment, nearly all participants with sepsis (1088/1089, 99.9%) had at least one abnormality in an immune marker, and about seven out of 10 participants (747/1089, 68.6%) had three or more (supplementary appendix table S8). Exploratory analyses of immune markers showed that after a week of treatment both study arms showed a similar change in monocyte human leucocyte antigen-DR, lymphocyte count, and regulatory T cell percentage (table 2). However, the absolute change (group difference −1.7, 95% CI −3.0 to −0.6, P=0.02) and percentage

Subgroup	Thymosin α1 group: No of patients	Thymosin α1 group: 28 day mortality	Placebo group: No of patients	Placebo group: 28 day mortality	Hazard ratio (95% CI)	P for interaction	Hazard ratio (95% CI) for interaction
Age (years)						0.01	0.49 (0.29 to 0.86)
<60	212	20.7 (15.3 to 26.2)	215	13.5 (8.9 to 18.1)	1.67 (1.04 to 2.67)		
≥60	330	25.2 (20.5 to 29.8)	332	31.0 (26.1 to 36.0)	0.81 (0.61 to 1.09)		
Sex						0.97	1.01 (0.58 to 1.75)
Female	182	20.9 (15.0 to 26.8)	157	21.7 (15.2 to 28.1)	1.06 (0.65 to 1.73)		
Male	360	24.7 (20.3 to 29.2)	390	25.1 (20.8 to 29.4)	0.99 (0.74 to 1.32)		
Hypertension						0.06	0.62 (0.37 to 1.01)
No	321	23.7 (19.0 to 28.3)	347	20.5 (16.2 to 24.7)	1.22 (0.88 to 1.69)		
Yes	221	23.1 (17.5 to 28.6)	200	30.5 (24.1 to 36.9)	0.71 (0.49 to 1.04)		
Diabetes mellitus						0.04	0.54 (0.30 to 0.96)
No	390	25.6 (21.3 to 30.0)	422	23.0 (19.0 to 27.0)	1.16 (0.87 to 1.53)		
Yes	152	17.8 (11.7 to 23.8)	125	28.0 (20.1 to 35.9)	0.58 (0.35 to 0.99)		
Solid malignant tumours						0.60	0.83 (0.42 to 1.64)
No	483	22.6 (18.8 to 26.3)	485	22.5 (18.8 to 26.2)	1.00 (0.77 to 1.31)		
Yes	59	30.5 (18.8 to 42.3)	62	37.1 (25.1 to 49.1)	0.83 (0.42 to 1.60)		
Coronary heart disease						0.06	0.49 (0.24 to 1.03)
No	477	23.7 (19.9 to 27.5)	484	22.7 (19.0 to 26.5)	1.08 (0.83 to 1.41)		
Yes	65	21.5 (11.5 to 31.5)	63	34.9 (23.2 to 46.7)	0.47 (0.21 to 1.01)		
COPD						0.60	0.80 (0.37 to 1.81)
No	505	23.0 (19.3 to 26.6)	496	23.2 (19.5 to 26.9)	1.00 (0.77 to 1.30)		
Yes	37	29.7 (15.0 to 44.5)	51	33.3 (20.4 to 46.3)	0.84 (0.35 to 2.04)		
Chronic kidney disease						0.76	0.87 (0.36 to 2.12)
No	514	23.0 (19.3 to 26.6)	511	23.1 (19.4 to 26.8)	0.99 (0.77 to 1.29)		
Yes	28	32.1 (14.8 to 49.4)	36	38.9 (23.0 to 54.8)	0.70 (0.27 to 1.81)		
Total	542	23.4 (19.9 to 27.2)	547	24.1 (20.6 to 27.9)	0.97 (0.76 to 1.24)		

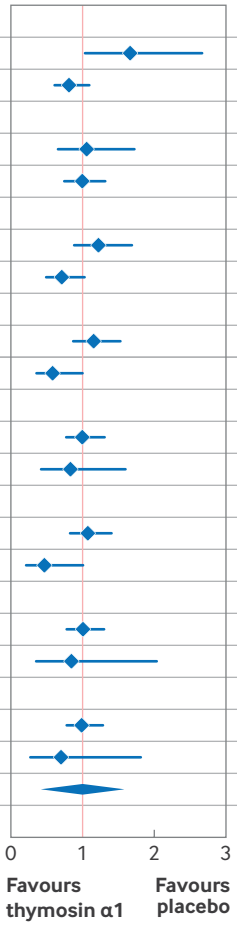

Fig 3 | Subgroup analyses. A Bonferroni threshold for significance for overall type I error of 0.05 was P=0.006. The HRs for relative risk of primary outcome of the two groups and associated 95% CIs were calculated with a Cox regression model adjusting for centre and age. An interaction term was added between treatment and subgroup in the Cox regression model. CI=confidence interval; COPD=chronic obstructive pulmonary disease

change (group difference 9%, 2% to 15%, P=0.006) of neutrophil to lymphocyte ratio in the thymosin α1 group were greater than in the placebo group (table 2). This result was consistent when missing data were infilled (supplementary appendix table S7). As for longitudinal dynamic change of these markers, an ancillary finding was a significant improvement in monocyte human leucocyte antigen-DR, lymphocyte count, and neutrophil to lymphocyte ratio over the 28 day follow-up period, but no between group differences between thymosin α1 and placebo (fig 4 and supplementary appendix figure S2).

Safety

Overall, 730 participants (67.0%) experienced at least one adverse event (360 participants (66.4%) in the thymosin α1 group and 370 (67.6%) in the placebo group; group difference −1.2%, 95% CI −6.8% to 4.4%, P=0.70), and 305 participants (28.0%) experienced at least one serious adverse event (145 participants (26.8%) in the thymosin α1 group and 160 (29.3%) in the placebo group; group difference −2.5%, −7.8% to 2.8%, P=0.38) within 90 days of follow-up (table 2 and supplementary appendix tables S9 and S10). The most common adverse effects were anaemia (10.7%), followed by fever (9.6%), abdominal distension (5.4%), and coagulation disorders (4.8%). No unexpected serious adverse events related to thymosin α1 occurred during the study.

Discussion

This multicentre trial found no conclusive evidence that thymosin α1 reduces 28 day mortality in adults with sepsis. The drug's good safety profile was, however, validated. Additionally, thymosin α1 might have beneficial effects in patients aged 60 years and older and those with chronic conditions.

Comparison with other studies

The immunomodulatory drug thymosin α1 reduces T cell exhaustion and preserves the number and function of effector T cells, thereby exerting a sustained effect in eliminating pathogenic microorganisms.[12 13] A meta-analysis of previous trials of thymosin α1 showed a

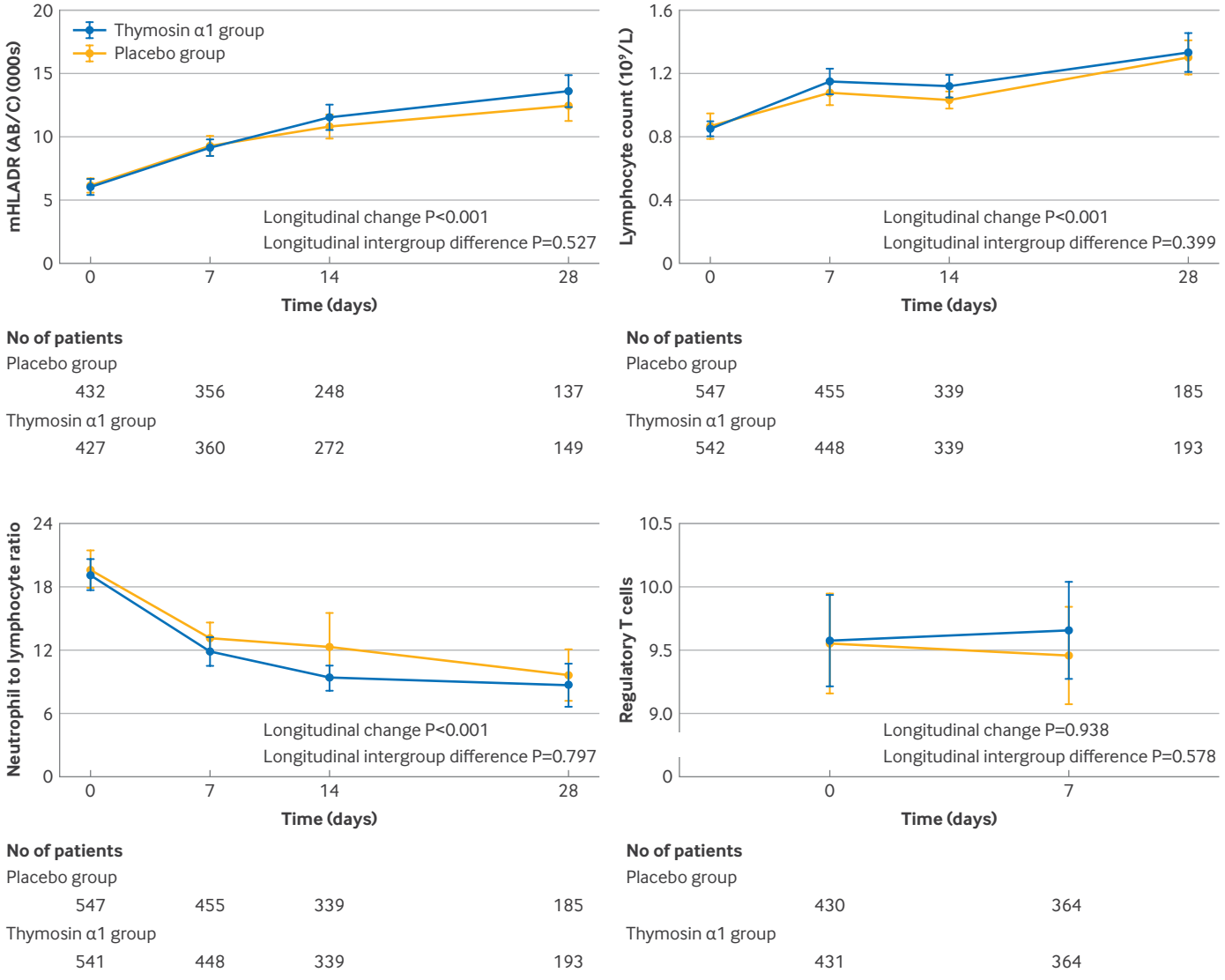

Fig 4 | Dynamic change of immune markers in participants with sepsis. Mixed effects model for repeated measures was used to analyse the absolute changes in immune markers: mHLA-DR, lymphocyte count, neutrophil to lymphocyte ratio, and regulatory T cells from screening period to 28 day follow-up. This model included measurements of immune markers on days 7, 14, and 28 as dependent variables (the proportion of regulatory T cells was only measured on day 7), whereas immune marker levels at screening, centre, and age group served as independent variables. mHLA-DR=monocyte human leucocyte antigen-DR

41% reduction in 28 day mortality, but these trials had important limitations.[22] In the current study, the failure to observe efficacy from thymosin α1 use can be explained in several ways. Firstly, previous studies were small and positive results might have been chance findings. Methodological issues were also present, including lack of masking and imbalance between trial arms in key patient characteristics. Secondly, sepsis is a highly heterogeneous disease, with complex and diverse pathophysiological processes, as well as diverse immune states. Therefore, improving the overall survival rate of all patients with sepsis may be unrealistic with a single agent. Thirdly, despite the sample size of the TESTS trial being nearly twice that of the combined total from the previous 10 trials, a modest reduction in mortality may still be possible. The broader confidence intervals include values that could be statistically significant, highlighting the need for further research. Finally, it is possible that the dosage of thymosin α1 was inappropriate. In the treatment of patients with cancers and viral hepatitis, the conventional therapeutic dose of thymosin α1 is 1.6 mg twice weekly, although the half life of the drug is 1.5 hours.[25] In patients with sepsis, a previous study reported that treatment with 3.2 mg of thymosin α1 daily for one week in combination with anti-inflammatory drugs reduced morbidity and mortality, whereas 1.6 mg daily was ineffective.[26] Therefore, in the current study we chose 1.6 mg twice daily for one week.

Clinical implications and rationale

Age is an important factor influencing immune system function, particularly in patients with sepsis when age

related changes can affect treatment responses.[27 28] Our findings indicate that thymosin α1 use may be more effective in patients aged 60 years and older and in those with chronic conditions such as diabetes, hypertension, and coronary heart disease. As a multifunctional immunomodulatory drug, thymosin α1 has shown efficacy in alleviating T cell exhaustion, a key phenotype associated with impaired immune function in patients aged 60 years and older and those with chronic conditions.[12 29 30] This T cell exhaustion compromises the ability of patients with sepsis to clear pathogens, resulting in poor prognoses. Thus, the rationale for administering thymosin α1 to these patients is well supported. Conversely, we did not observe similar efficacy in patients younger than 60 years with sepsis. Although younger patients seemed to experience worse outcomes, part of this difference in mortality may be due to discrepancies in baseline clinical characteristics between the thymosin α1 group and placebo group. It may be worthwhile for future studies on thymosin α1 for the treatment of sepsis to focus on patients aged 60 years and older and those with chronic conditions.

Monocyte human leucocyte antigen-DR expression, regulatory T cell percentage, lymphocyte count, and neutrophil to lymphocyte ratio are commonly used as markers of immune status in patients with sepsis.[31-34] Based on these markers, almost all patients in this study had immune dysregulation at enrolment, and improvement in these markers was observed in both groups after treatment. Monocyte human leucocyte antigen-DR are rarely assessed in multicentre studies because their detection requires the use of flow cytometry. This study initially aimed to reduce heterogeneity between laboratories through standardised quantification. Despite this effort, unlike in previous studies using a single laboratory, we did not observe an increase in monocyte human leucocyte antigen-DR levels in response to thymosin α1 treatment.[35] This lack of observed increase may partly be attributed to the high variability among different laboratories, which may have masked any true treatment related differences between groups. The neutrophil to lymphocyte ratio reflects the balance between innate and adaptive immunity and is a cheap biomarker and easy to obtain.[36 37] A meta-analysis showed that a high neutrophil to lymphocyte ratio was a reliable immune biomarker for predicting poor prognosis in patients with sepsis.[38] Consequently, a more pronounced reduction in the ratio after thymosin α1 treatment is supportive of a role of thymosin α1 in maintaining immune homoeostasis of sepsis.

Limitations of this study

Several limitations should be considered when interpreting our findings. Firstly, owing to the high heterogeneity of sepsis, it is difficult to achieve consistent efficacy with a single treatment. Although we attempted to identify potential study populations during the trial design, previous studies lacked relevant evidence. To deal with this issue, we predefined subgroups, hoping to identify potential beneficiary subpopulations for thymosin α1 treatment through this trial. Secondly, our study may have been underpowered. Our sample size calculation was guided by data from the largest published trial at the time we initiated the current study. The former found outcome rates of 26% and 35% in the thymosin α1 and placebo groups, respectively. We powered our study based on a difference of 8% (27% *v* 35%).[20] In retrospect, this might have been too ambitious. In the event, the observed effect was close to 1, with a wide confidence interval (hazard ratio 0.99, 95% CI 0.77 to 1.27). Therefore, further research might still be warranted, although based on the results from this trial there is no strong evidence to support the presence of an effect. Thirdly, this study was not able to determine the precise times from onset of sepsis to diagnosis because of individual variability and disease progression. To estimate the interval from sepsis diagnosis to randomisation, we collected data on the duration of organ support before randomisation. This suggests that most enrolled patients were likely not in the early stages of sepsis.

Conclusions

This multicentre trial found no conclusive evidence that thymosin α1 reduces 28 day mortality in adults with sepsis. The drug's good safety profile in the treatment of sepsis was, however, validated. Additionally, thymosin α1 might have beneficial effects in patients aged 60 and older and those with chronic conditions. Future research might consider focusing on these patients to clarify potential therapeutic advantages.

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Contributors: JW, FP, and XG conceived the study. JW, FP, and XG designed the study, with input from all other authors. JW, FP, LZ, WL, RS, Y Li, ZW, ZH, XZ, XJ, Y Long, WC, CW, EC, JZ, JY, QL, FZ, LH, YS, MD, WZ, DZ, CY, and SP contributed equally to this work and are joint first authors. FP and JW wrote the original manuscript. JW, FP, LZ, WL, RS, Y Li, ZW, ZH, XZ, XJ, WC, Y Long, CW, EC, JZ, JY, QL, FZ, LH, YS, MD, WZ, DZ, QK, and XG contributed to data acquisition and review of the final manuscript. SZ and Y Liu coordinated the laboratory component. CY, SP, MS, and QZ contributed to the statistical analysis of the protocol and manuscript. JW, FP and KC revised the manuscript. FP, JW, and XG are joint corresponding authors and the guarantors. All authors interpreted the data and critically reviewed the manuscript. All authors revised the report and read and approved the final version before submission. The corresponding authors attest that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

Funding: The trial was funded by the Sun Yat-sen University Clinical Research Program 5010 (grant No 2019002 and No 2024006), the Guangdong Clinical Research Center for Critical Care Medicine (grant No 2020B1111170005), and SciClone Pharmaceuticals. The funders were not involved in the trial's design, data collection, interpretation, or manuscript preparation.

Competing interests: All authors have completed the ICMJE uniform disclosure form at https://www.icmje.org/disclosureof-interest/ and declare: support from the Sun Yat-sen University Clinical Research Program 5010, the Guangdong Clinical Research Center for Critical Care Medicine, and SciClone Pharmaceuticals for the submitted work; XG, JW, WL, ZH, Y Li, DZ, MZ, JY, WC, MD, ZW, and QL received grants from SciClone Pharmaceuticals; WL, JZ, and LK received consultancy fees from SciClone Pharmaceuticals, Jiangsu Nhwa Pharmaceutical, and Nutrici; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Ethical approval: This study was approved by the ethics committee of The First Affiliated Hospital of Sun Yat-sen University (2016007). The study protocol received approval from the ethics committees of all participating centres, adhering to local laws and the Declaration of Helsinki.

Data sharing: Requests for data sharing can be sent to peif3@mail.sysu.edu.cn, wujianf@mail.sysu.edu.cn, or guanxd@mail.sysu.edu.cn. Only deidentified data will be made available to academic researchers upon reasonable request. Data will be available once all planned analyses have been completed and published or presented.

Transparency: The lead authors (the manuscript's guarantors) confirm that this manuscript accurately, honestly, and transparently represents the study being described. It ensures that no critical aspects of the study have been left out, and any deviations from the original study plan (and, if applicable, registration) have been clarified.

Dissemination to participants and related patient and public communities: Results were communicated to patients who expressed an interest during visits. Outcomes will be disseminated through press releases, academic conferences, and social media.

Provenance and peer review: Not commissioned; externally peer reviewed.

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Supplementary information: Additional tables S1-12 and figures S1-S3