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Non-selective beta-blockers enhance resolution of induced infections in animals and healthcare-associated infections in humans

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Healthcare-associated infections (HAIs) pose significant risks, leading to increased morbidity, mortality, and costs, exacerbated by multi-drug-resistant microorganisms. This study aimed to evaluate pharmacological prophylaxis targeting sympathetic reflex control of immunity to mitigate systemic infections, offering a novel approach to combating HAIs. The study included animal experiments and a retrospective analysis of orthopedic surgery patients in Romagna, Italy. Young female pigs were intravenously inoculated with *Escherichia coli* (*E. coli*) and divided into two groups: propranolol-treated (non-selective β -blocker; 3 mg/kg; 3x/day orally) and vehicle-treated, starting two days before infection. Parameters such as bacteraemia, serum cytokines, biochemical profile, blood count, lactate, glycemia, and flow cytometry were assessed. Additionally, a retrospective analysis of 92,649 orthopedic surgery hospitalizations (2017–2022) examined the association of non-selective and selective β 1-blockers with HAI development using conditional logistic regression. Propranolol-treated pigs exhibited a disinhibited immune response to systemic infection, clearing circulating bacteria much earlier than vehicle-treated animals. The retrospective analysis showed that patients on non-selective beta-blockers had a 71.7% reduced risk of developing HAIs, while those on selective β 1-blockers had an 18% higher risk. These findings suggest that targeting sympathetic reflex control of immunity via pharmacological prophylaxis may reduce HAIs in surgical patients.

Keywords Infections, β -blockers, Healthcare-associated infections, Sympathetic nervous system

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Abbreviations

AR	Adrenergic receptor
CBC	Complete Blood Count
CEROM	Ethics Committee of Romagna Health Authority
CFUs	Colony Forming Units
<i>E. coli</i>	<i>Escherichia coli</i>
FSC-A	Forward Scatter-Area
FSC-H	Forward Scatter-Height
HAIs	Healthcare-associated infections
ICD	International Classification Disease
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
IL-10	Interleukin-10
INF- γ	Interferon- γ
i.v.	Intravenously
IR	Inflammatory reflex
MFI	Mean Fluorescence Intensity
MPM	Mean platelet mass
MPV	Mean platelet volume
OR	Odds ratio
SEM	Standard error of the mean
SD	Standard deviation
SSC-A	Side Scatter-Area
SSC-H	Side Scatter-Height
TNF	Tumour necrosis factor- α

Healthcare-associated infections (HAIs) are a public health priority due to their frequency and severity. Their impact on populations and healthcare systems in terms of morbidity, mortality and attributable costs is considerable. Due to the shortage of new antibiotics and the rapid spread of multidrug-resistant microorganisms, HAIs continue to worsen. In a European study carried out in 2018, an estimated 6.5% (95% CI 5.4–7.8%) of patients in acute care hospitals developed HAIs¹. In a recent meta-analysis of 400 papers², the estimated rate of HAIs was 14% (95% CI 12–15%). Among various pathogens, *Escherichia coli* (*E. coli*) stood out as a more frequent infectious agent than others, such as coagulase-negative staphylococci, *Staphylococcus spp.*, and *Pseudomonas aeruginosa*².

Our study builds on the research demonstrating the substantial influence that the sympathetic nervous system exerts on immune responses^{3,4}. The immune system detects the presence of an infection, via specific receptors present on various leukocytes, and develops its own innate response, which, if successful, leads to the eradication of the pathogen from the host and the development of an immune memory. The regulation of this response has long been viewed as autonomous, mediated by interactions between immune cells in a largely self-regulated system. However, recent studies have provided substantial evidence that the nervous system exerts an active regulatory role in controlling the innate immune response, especially the resulting inflammation, via a mechanism that has been termed *the inflammatory reflex* (IR)⁵. The efferent arm of the IR travels in the splanchnic sympathetic nerves⁶ and is activated in different species (i.e. rats, mice and sheep) in response to several different types of immune challenges recognized by various Toll-like receptors (TLRs), mimicking bacterial (both Gram-positive and Gram-negative) and viral infections⁷. We, recently, showed that disabling the IR by bilateral surgical section of the splanchnic nerves resulted in an exaggerated inflammatory response in sheep infected by intravenous injection of *E. coli*⁸. Interestingly, without the inhibitory brake exerted by the reflex, the disinhibited innate immune system eradicated any circulating bacteria in less than 90 min from the administration of the *E. coli* bolus. In contrast, control animals, with a functioning IR, continued to present live circulating bacteria in the bloodstream even 48 h later⁸. The present study stems from these remarkable results.

Most of the literature dealing with the sympathetic effects on immunity focuses on the β 2-adrenergic receptor (AR)-mediated effects⁹; β 2 are the most expressed ARs on leukocytes³. The canonical view is that β 2AR-mediated effects on immunity are inhibitory. Moreover, several independent laboratories described strong anti-inflammatory effects of catecholamines via activation of β 2ARs, both in vitro and in vivo^{10,11}.

The aim of the study was to investigate the effects of a pharmacological prophylaxis with non-selective β -blockers (which would target β 2 as well as β 1ARs), on the ability of the immune response to resolve an infection. The pharmacological prophylaxis with non-selective β -blockers should, at least in part, impair the sympathetic inhibition and, thereby, disinhibit the innate immune response. In particular, we tested two scientific hypotheses: 1) animals treated with propranolol (non-selective β -blocker) have a greater ability to fight and resolve an *E. coli* systemic infection; 2) patients undergoing orthopedic surgery and treated with non-selective β -blockers should have a disinhibited innate immune system due to impairment of β 2-mediated sympathetic inhibition. As a result, even if their true risk of developing HAIs were similar to that of other patients, these infections would likely resolve more rapidly and thus remain undetected in hospital records. In contrast, patients treated with selective β 1-blockers—without any effect on β 2-mediated immune inhibition—should show no difference in HAI occurrence compared with untreated patients.

Results

Animal experiments

We designed an *in vivo* trial on non-anaesthetised pigs to directly assess the effects of pharmacological blockade of β -receptors on systemic bacterial infection (see the detailed experimental plan in Figure S1). Of the available pre-clinical laboratory animal species, pigs were chosen on the basis of their physiological similarity to humans and on the feasibility of chronic procedures and repeated blood samplings.

To test whether pigs treated with propranolol responded more effectively to a systemic infection with *E. coli* we analysed the level of bacteraemia of our animals (Fig. 1a). Both experimental groups, propranolol-treated ($n=4$) and vehicle-treated (control, $n=4$), showed a rise in bacterial counts in blood after the i.v. injection of *E. coli*. Initially, the level of bacteraemia rose comparably in both groups during the first three days following the challenge. However, from the fourth day onwards, a notable difference emerged ($H=11.298$, $p<0.001$), with propranolol-treated animals exhibiting significantly lower levels of bacteraemia compared with controls ($p=0.038$ on the fourth day post-injection). This disparity peaked on the sixth day, when propranolol-treated animals exhibited complete absence of circulating bacteria ($p=0.047$), contrasting with controls that achieved bacterial clearance only on the tenth day post-challenge, four days later than the propranolol-treated pigs (Fig. 1a).

To ascertain whether propranolol treatment bolstered the innate immune response to infection, we conducted a series of analyses on haematological parameters and cytokine levels.

While the overall number of circulating white blood cells showed no significant difference between groups, there was a notable increase in monocytes (particularly in the later stages of the trial; $F_{(1,126)}=8.185$, $p=0.005$) and basophils ($F_{(1,126)}=31.089$, $p<0.001$) in propranolol-treated and control animals, respectively (Figure S2–S3). Although neutrophil counts did not exhibit significant variation, flow cytometry evaluation revealed heightened FSC-A values in neutrophils from propranolol-treated pigs, particularly towards the trial's end ($F_{(1,38)}=4.827$, $p=0.034$; Fig. 1b), concurrent with increased aggregation ($F_{(1,38)}=7.066$, $p=0.011$; Fig. 1c), both being parameters of neutrophil activation. Moreover, the MFI, indicative of phagocytic capacity, tended to increase in neutrophils from propranolol-treated pigs, especially one to two weeks post-infection (Figure S4), although this did not reach statistical significance. Platelet indices differed notably, with propranolol-treated pigs displaying a significant increase in mean platelet volume (MPV; $F_{(1,126)}=68.355$, $p<0.001$) and mass (MPM; $F_{(1,126)}=66.330$, $p<0.001$) throughout the study duration (Fig. 2d–f).

Cytokine analysis revealed higher levels of the anti-inflammatory interleukin-10 (IL-10) in control animals compared with propranolol-treated pigs ($Z=-3.019$, $p=0.003$; Fig. 1d). Notably, other measured cytokines, including tumour necrosis factor- α (TNF), interferon- γ (INF- γ), interleukin-6 (IL-6), and interleukin-1 β (IL-1 β), remained below detectable thresholds (data not shown), indicating a mild infection in both groups.

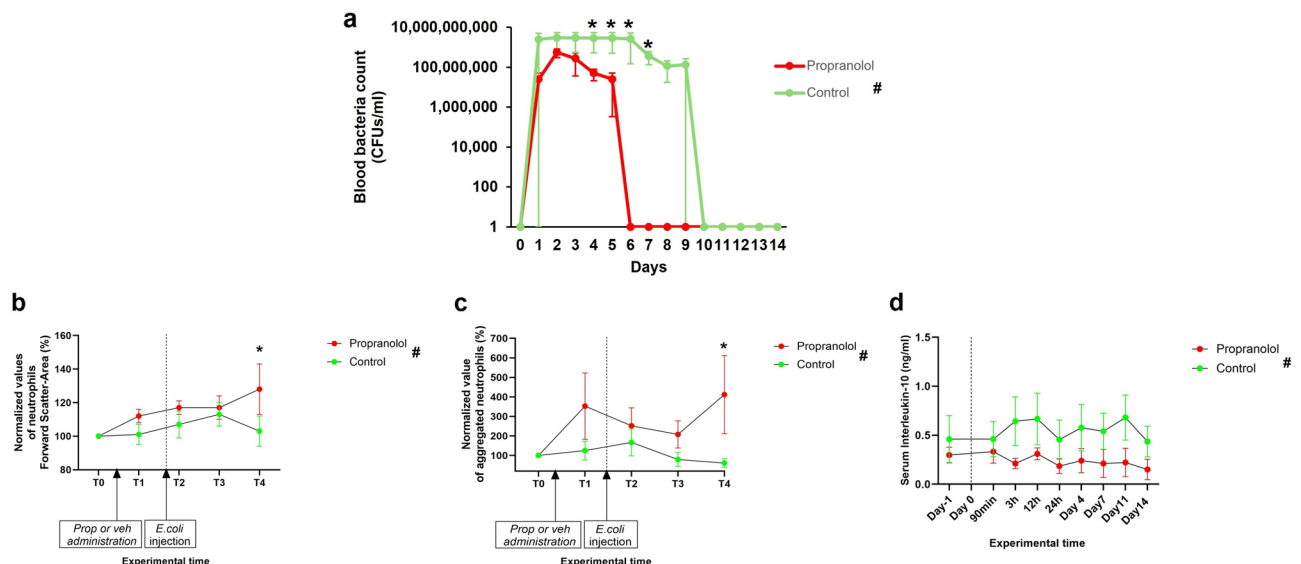


Fig. 1. Key findings upon *Escherichia coli* (*E. coli*) systemic infection in pigs. **(a)** Blood bacterial counts, expressed in colony forming units (CFUs) per milliliters of blood. Counts at 90 min, 6, 12, 24 h after i.v. *E. coli* injection are averaged on Day 1. **(b)** Flow cytometric evaluation of neutrophil Forward Scatter-Area (FSC-A) and **(c)** percentage of aggregated neutrophils. Data were normalized on the sample collected before propranolol/vehicle administration (T0). Non-normalized data are shown in the Supplementary (Figure S12–S13, p15–16). For panels B and C: T0 = before propranolol/vehicle administration; T1 = first day of propranolol/vehicle administration; T2 = 90 min after *E. coli* injection (dotted line); T3 = 7 days after *E. coli* injection; T4 = 14 days after *E. coli* injection. **(d)** Serum quantification of the anti-inflammatory cytokine interleukin 10 (IL-10). Day-1 indicated the day before *E. coli* infusion (represented by dotted line). Data are expressed as mean $\pm$ standard error of the mean (SEM). # indicates overall differences between propranolol-treated and control animals; * indicates differences between groups at specific timepoints ($p<0.05$).

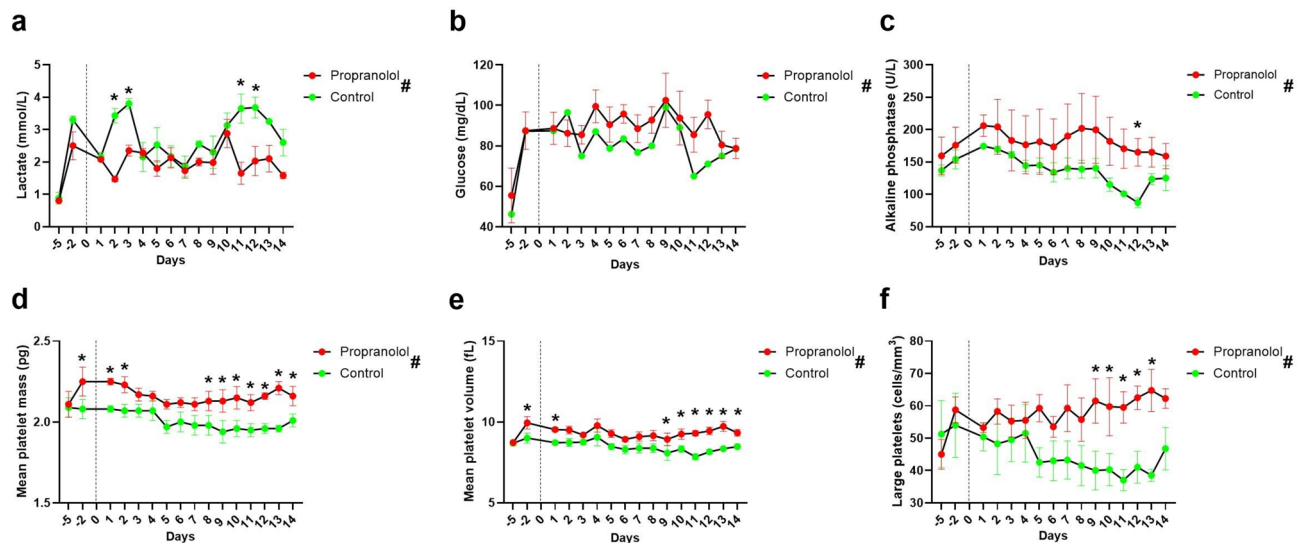


Fig. 2. Complete blood count (CBC) and clinical chemistry parameters upon *Escherichia coli* (*E.coli*) systemic infection in pigs. (a) Lactate, (b) glucose and (c) alkaline phosphatase blood concentrations. (d) Mean platelet mass (MPM), (e) mean platelet volume (MPV), and (f) large platelets concentrations. Day 0 (dotted line) represents the *E. coli* infusion. Values at 90 min, 6, 12, 24 h after infusion are averaged on Day 1. Data are expressed as mean $\pm$ standard error of the mean (SEM). # indicates overall differences between propranolol-treated and control animals; * indicates differences between groups at specific timepoints ($p < 0.05$).

Other clinical signs, including parameters indicative of renal and liver function did not differ between the propranolol-treated and control groups, and remained within the specific physiological ranges throughout the entire trial (Figure S5–S10, Appendix pp8–13). Nonetheless, lactate reached significantly higher concentrations in control pigs when compared with propranolol-treated animals ($F_{(1,126)} = 26.137$, $p < 0.001$; Fig. 2a), with an opposite effect recorded for glycaemia ($F_{(1,126)} = 6.691$, $p = 0.011$; Fig. 2b) and alkaline phosphatase ($F_{(1,126)} = 20.456$, $p < 0.001$; Fig. 2c).

Retrospective study on patients

We performed an observational study based on deterministic record linkage of hospital discharge records and drug prescriptions of people living in the catchment area of the Romagna local health authority (Italy). The study included people with hospital discharges in Romagna hospital facilities occurring between January 1st 2017 and December 1st 2022 with the following characteristics: i) age ≥ 18 years; ii) living in the catchment area of the Romagna local health authority; and iii) undergoing an orthopaedic procedure during hospitalisation (ICD-9 CM codes 76.xx–84.xx). Infections were searched in the index hospitalisation and in subsequent hospitalisations occurring for any reason within 14 days of discharge from the index hospitalisation (Table 1).

The study flow chart is shown in Figure S11. Of the 94,605 records initially identified, 621 were readmissions within 14 days (counted only once in the same episode of care), and 1335 were excluded because the infection was present on admission. A total of 90,893 hospitalisations without HAIs and 1756 (1.9%) with at least one recorded HAI were identified (Table 1 and Figure S11). The patient cohort included 77,826 individuals, of whom 12,312 (15.8%) had multiple hospitalisations (median = 2, range 2–16). The HAI rate (total number of infections/patients) was $2467/77,826 = 3.2\%$. Gram-negative and Gram-positive bacteria were the most common infectious agents ($N = 371$, 15.0% and $N = 236$, 9.6%, see Table 2). Among other infections in which the infectious agent was unknown, the most frequent ones were urinary (19.9%) and pulmonary (17.8%).

Impact of non-selective β -blockers (code C07AA) on infections.

Separate conditional logistic regression models were fit using as outcomes overall, Gram-positive and Gram-negative infections. We analysed 1756 cases (patients with at least one HAI) and 3512 matched controls (patients without HAIs) for sex, age group, and comorbidity. The characteristics of patients with at least one HAI and their matched controls are shown in Table 1 and Table S1. Figure 3 shows the odds ratio (OR) of HAIs in patients treated vs. those untreated with non-selective β -blockers using conditional logistic regression analyses. Patients treated with C07AA had a 72% reduced risk of overall HAI (OR = 0.28, 95% CI 0.09–0.87, $p = 0.03$; Fig. 3; Table 3a). When analyzed separately for different infections, patients treated with C07AA had a 74% reduced risk of Gram-negative infections, which however was not statistically significant due to the small number of events (OR = 0.26, 95% CI 0.03–2.16, $p = 0.21$; Table 3a). Similar results were obtained for Gram-positive infections, in which patients treated with C07AA had a non-significant 67% reduced risk of HAI (OR = 0.33, 95% CI 0.04–2.69, $p = 0.30$; Table 3a). After adjustment for antibiotic treatment, patients treated with C07AA still had a reduced risk of HAI by 71% (aOR = 0.29, 95% CI 0.10–0.90, $p = 0.03$) and a 74% reduced risk of Gram-negative infections was found, which however was not statistically significant due to the small number of events (aOR = 0.26, 95% CI

	No HAI controls (N = 3512)	Any HAI cases (N = 1756)	Test, p-value
Female, n (%)	1.904 (54.2%)	952 (54.2%)	1.000
Age, mean (SD)	77.2 (13.6)	77.3 (13.5)	0.878
Multimorbidity (Elixhauser index >= 2), n (%)	986 (28.1%)	493 (28.1%)	1.000
Non-selective beta-blockers, n (%)	35 (1.0%)	5 (0.3%)	0.004
Selective beta-blockers, n (%)	801 (22.8%)	451 (25.7%)	0.021
Other concomitant drugs by ATC, n (%)			
A Alimentary tract and metabolism	1.808 (51.5%)	892 (50.8%)	0.640
B Blood and blood forming organs	1.806 (51.4%)	905 (51.5%)	0.953
C Cardiovascular system	2.322 (66.1%)	1.186 (67.5%)	0.307
D Dermatologicals	18 (0.5%)	14 (0.8%)	0.258
G Genito urinary system and sex hormones	411 (11.7%)	234 (13.3%)	0.090
H Systemic hormonal preparations, excl. sex hormones and insulins	491 (14.0%)	299 (17.0%)	0.004
L Antineoplastic and immunomodulating agents	153 (4.4%)	108 (6.2%)	0.006
M Musculo-skeletal system	644 (18.3%)	380 (21.6%)	0.005
N Nervous system	1.187 (33.8%)	727 (41.4%)	<0.001
P Antiparasitic products, insecticides and repellents	35 (1.0%)	11 (0.6%)	0.209
R Respiratory system	289 (8.2%)	171 (9.7%)	0.070
S Sensory organs	271 (7.7%)	136 (7.7%)	1.000
V Various	65 (1.9%)	35 (2.0%)	0.748
J Anti-infectives for systemic use within the previous 10 days	406 (11.6%)	135 (7.7%)	<0.001

Table 1. Demographic characteristics and drug use by ATC code in patients with HAI and matched controls.

Infection Groups		N	%
Infections with known agent	Gram-negative	371	15.0%
	Gram-positive	236	9.6%
	Yeasts and fungi	8	0.3%
	Mycobacterium	0	0.0%
	Mixed	170	6.9%
	Virus	27	1.1%
	COVID-19	145	5.9%
Infections with unknown agent	Pneumonia	440	17.8%
	Meningitis	2	0.1%
	Sepsis	248	10.1%
	Tissue infection	41	1.7%
	Endocarditis	9	0.4%
	Pyelonephritis	11	0.4%
	Osteomyelitis	58	2.4%
	Urinary tract infection	491	19.9%
	Other	189	7.7%
	Total	2467	

Table 2. List of infections with known and unknown agents.

0.03–2.17, $p=0.21$). Similar results were obtained for Gram-positive infections, in which patients treated with C07AA had a non-statistically significant 67% reduced risk of HAI (aOR = 0.33, 95% CI 0.04–2.69, $p=0.30$).

Impact of selective β 1-blockers (code C07AB) on infections.

When we investigated the risk of infection associated with the use of selective β 1-blockers (C07AB), we found an opposite effect with respect to C07AA. Patients treated with C07AB had an 18% higher risk of any hospital infection than untreated patients (OR = 1.18, 95% CI 1.00–1.39, $p=0.04$; Fig. 3; Table 3b), a 46% higher risk of Gram-negative infection (OR = 1.46, 95% CI 1.11–1.90, $p=0.01$; Table 3b) and a non-statistically significant 14% higher risk of Gram-positive infection (OR = 1.14, 95% CI 0.84–1.54, $p=0.41$; Table 3b). After adjustment for antibiotic treatment, patients treated with C07AB still had a 20% higher risk of any hospital infection than untreated patients (aOR = 1.20, 95% CI 1.02–1.41, $p=0.03$) and a 46% significantly higher risk of Gram-negative

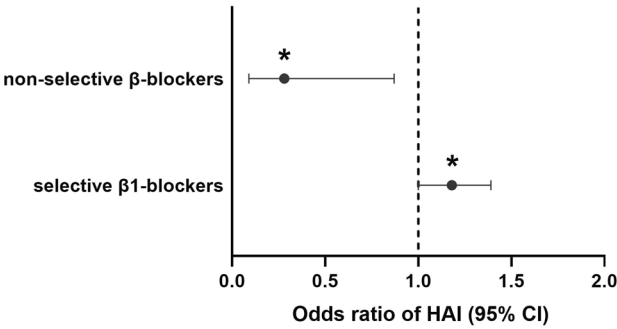


Fig. 3. Odds ratio of healthcare-associated infections (HAIs). The graph shows odds ratio (with 95% confidence interval (CI)) of postoperative infections in patients treated vs. those untreated with non-selective β -blockers and selective β 1-blockers. * $p < 0.05$ indicates significant difference from 1 (no effect of the treatment).

(a)		total	treated	non-treated	OR (95% CI)	P	aOR (95% CI)	P
All infections	cases	1756	5 (0.28%)	1751 (99.72%)	0.28 (0.09–0.87)	0.03	0.29 (0.10–0.90)	0.03
	controls	3512	35 (1.00%)	3477 (99.00%)				
Gram-negative	cases	371	1 (0.27%)	370 (99.73%)	0.26 (0.03–2.16)	0.21	0.26 (0.03–2.17)	0.21
	controls	1484	15 (1.01%)	1469 (98.99%)				
Gram-positive	cases	236	1 (0.42%)	235 (99.58%)	0.33 (0.04–2.69)	0.30	0.33 (0.04–2.69)	0.30
	controls	944	12 (1.27%)	932 (98.73%)				
(b)		total	treated	non-treated	OR (95% CI)	P	aOR (95% CI)	P
All infections	cases	1756	451 (25.68%)	1305 (74.32%)	1.18 (1.00–1.39)	0.04	1.20 (1.02–1.41)	0.03
	controls	3512	801 (22.81%)	2711 (77.19%)				
Gram-negative	cases	371	105 (28.30%)	266 (71.70%)	1.46 (1.11–1.90)	0.01	1.46 (1.12–1.90)	0.01
	controls	1484	321 (21.63%)	1163 (78.37%)				
Gram-positive	cases	236	57 (24.15%)	179 (75.85%)	1.14 (0.84–1.54)	0.41	1.13 (0.84–1.53)	0.42
	controls	944	208 (22.03%)	736 (77.97%)				

Table 3. Crude and adjusted (for antibiotic treatment) odds ratio of healthcare-associated infections (HAIs) in patients treated vs. those untreated with non-selective β -blockers (C07AA). Results of conditional logistic regression. Crude and adjusted (for antibiotic treatment) odds ratio of healthcare-associated infections (HAIs) in patients treated vs. those untreated with selective β 1-blockers (C07AB). Results of conditional logistic regression. OR = odds ratio; aOR = odds ratio adjusted for antibiotic treatment; CI = confidence interval.

infection (aOR = 1.46, 95% CI 1.12–1.90, $p = 0.01$), while a non-statistically significant 13% higher risk of Gram-positive infection (aOR = 1.13, 95% CI 0.84–1.53, $p = 0.42$) were found.

Discussion

HAIs pose a growing clinical challenge, particularly in the light of the emerging threat of antimicrobial resistance¹². Their prevalence in hospitalized patients is striking, with industrialized countries reporting rates of 6.5%¹, a figure that doubles in developing nations¹³. In response to this pressing issue, we propose an innovative approach: bolstering the innate immune system by counteracting the sympathetic-mediated immune suppression inherent in infections⁷. Our research unveils a promising avenue: pharmacological prophylaxis with non-selective β -blockers has the potential to disinhibit, ergo enhance, the immune response allowing it to combat infection and resolve or prevent the development of an infection in both pigs and humans.

In pigs, we provide evidence that propranolol treatment disinhibits the innate immune response during the course of an *E. coli* systemic infection. Pigs administered propranolol exhibited lower levels of the anti-inflammatory cytokine IL-10 (Fig. 1d), consistent with conclusions drawn from experiments in rodents and sheep subjected to surgical section of splanchnic sympathetic nerves^{8,9}. Moreover, signs of neutrophil (Figure S12–S13) and platelet activation in the propranolol-treated group, confirmed an enhanced innate immune response. Most importantly, propranolol treatment was associated with an improved ability to clear circulating bacteria (Fig. 1a). This confirms that impeding the sympathetic control of immunity, in our case with a non-selective antagonist of

β ARs, enhances the antimicrobial activity of the innate immune system. Such enhancement of innate immunity was previously shown to be present in β 2-AR-knock out mice¹⁴ and in sheep subjected to surgical section of the splanchnic sympathetic nerves⁸.

The main limitation of our animal study was the mild pathogenicity of the strain of *E. coli* with which our pigs were inoculated. Most of the biochemical and hematological results did not reach pathological levels (Supplementary Figures S2–S10). We were unable to assess detectable levels of the pro-inflammatory cytokines that normally increase during systemic immune challenges with Gram-negative bacteria as others have previously done using much higher concentration of *E. coli*¹⁵. Despite the infection being mild, we demonstrated that propranolol treatment strongly enhanced the ability to clear the circulating bacterial infection.

Our retrospective study involving patients undergoing orthopaedic surgery provides further encouraging evidence indicating the benefit of sympathetic inhibition for the prevention of life-threatening HAIs. We observed a strong association between the use of non-selective β -blockers and a beneficial protective outcome (Fig. 3). This study is based on the analysis of deterministic record linkage of hospital discharge records and pharmaceutical prescriptions, therefore we do not have any information of the dosage of the medications used nor can we be sure that the patients actually complied with the medication, we can only confirm that these drugs were prescribed. This would increase the variability of our results and make it more difficult to detect an association of non-selective β -blockers with reduced HAI risk. Despite that, the conditional logistic regression analysis confirmed a statistically significant protection from developing any HAIs, sufficiently severe to be recorded in the administrative hospital records, in patients treated with non-selective β -blockers (Fig. 3). This association persisted even when accounting for antibiotic treatments (Table 3a). Most surprisingly, the analysis with selective β 1-blockers as the treatment of interest demonstrated that these drugs, in contradistinction to non-selective β -blockers, facilitated the development of HAIs, especially from Gram-negative bacteria (Fig. 3; Table 3b). This result should be taken into account when assessing the effect observed with the non-selective β -blockers, given that these drugs antagonize both β 1 and β 2ARs. These findings indicate that the negative association between selective β 1-blocker use and increased HAI risk will partly counter the benefit obtained by blockade of β 2ARs.

There is a long history of the possible beneficial effects that β -blockers might have as preoperative treatment or to prevent life-threatening infections. Macchia and colleagues performed a retrospective analysis similar to ours and showed that prior prescription of β -blockers was associated with a decreased mortality in patients admitted to intensive care units for sepsis¹⁶. Another group recently confirmed this finding¹⁷. However, in both studies, the researchers did not specify whether the analysis was performed using selective or non-selective β -blockers as the treatment of interest^{16,17}. Additionally, non-selective β -blockers are commonly used in clinical settings to treat cirrhotic patients, protecting against spontaneous bacterial peritonitis and improving survival^{18,19}. Further human studies assessed the effectiveness of β -blockers to treat sepsis, as an intervention not a prophylaxis, but focusing mainly on the effects of selective β 1-blockers²⁰. Some studies have suggested that the use of selective β 1-blockers may be beneficial in sepsis^{21,22}.

Interestingly, the use of β 1-blockers for the prevention of postoperative adverse events in non-cardiac surgery has been extensively investigated in recent years. In these patients, selective β 1-blockers do not appear to have any beneficial effect, according to independent metanalysis^{23,24}, while a specific trial demonstrated that the use of metoprolol (selective β 1-blocker) increased mortality²⁵. More recently, Bouri and colleagues described the adverse effects of selective β 1-blockade treatment for the prevention of perioperative death in non-cardiac surgery²⁶.

In conclusion, we argue that the influence of the nervous system on the immune system may be underestimated. In fact, it is the power of the innate immune system that is underestimated because the intensity of the endogenous inhibitory action of the inflammatory reflex is not well appreciated. Indeed, our preclinical and clinical findings suggest that pharmacological inhibition of the inflammatory reflex allows an activated innate immune system to resolve many HAIs, potentially including multi drug resistant infections. However, the very low number of patients treated with non-selective β -blockers limits the generalizability of these findings. Confirmation in larger cohort studies is needed, ideally followed by randomized clinical trials comparing infection rates in patients receiving prophylactic non-selective β -blockers versus placebo. Future research is also needed to better understand the mechanism of action underlying the reflex sympathetic-mediated modulation of innate immunity, paving the way for optimization of pharmaceutical prophylactic strategies. Furthermore, in view of our recent finding that splanchnic nerve denervation improved bacterial clearance and clinical recovery in an already established ovine *E. coli* systemic infection²⁷, β 2-blockers (and not β 1) should be investigated as a possible pharmaceutical treatment to fight established infections not responding to conventional clinical interventions.

Methods

Animal experiments

Porcine intravenous bacteraemia

The experimental protocol for the pig trial was revised and approved by University of Bologna animal welfare body first, and then by the Italian Ministry of Health as dictated by the Legislative Decree 26/2014 implementing the European Directive 2010/63/EU (approval n° 594/2021-PR). All procedures were performed according to relevant guidelines and regulations, and methodologies are reported in accordance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Hybrid breed female pigs (n=8), weighing 44 ± 7 kg, were divided equally into two groups: propranolol-group and control-group (n=4 each). Five days (Day -5) before the immune challenge, pigs were implanted, under general anaesthesia, with a surgically-inserted double lumen central venous catheter allowing repeated blood samplings and bacterial infusion (Supplementary methods, page 2). Anaesthesia was achieved with an

intramuscular injection of tiletamine-zolazepam (5 mg/kg; Zoetel, Virbac S.r.l., Milan, Italy) followed by an intravenous (i.v.) bolus of propofol (2–4 mg/kg; Propofur, Boehringer Ingelheim Animal Health Italia S.p.a., Noventana, Italy), and maintained with Sevoflurane (2–4% in 1:1 air/oxygen mixture; SevoFlo, Zoetis S.r.l., Rome, Italy) upon oro-tracheal intubation. Animals in the propranolol-group were orally administered 3 mg/kg of DL-Propranolol hydrochloride, 99% (Fisher Scientific Hampton, NH, US), three times a day (8.00 AM; 1.00 PM; 10.00 PM), starting two days before the bacterial challenge (Day -2) until the end of the trial (Day 14). Control animals received vehicle (yogurt and biscuits to facilitate the oral administration of the drug). On the bacterial challenge day (Day 0) a bolus of *Escherichia coli* (*E. coli*, 1.4×10^4 CFUs/Kg; strain: ATCC 25,922) was intravenously (i.v.) administered over 15 min at 10.00 AM. Blood samples were collected on the day of the challenge at 1.5, 3, 6, 12 and 24 h after *E. coli* infusion and then daily (at 10.00 AM). Core body temperature was also measured (Anipill capsule, BodyCAP, Hérouville-Saint-Clair, France; Figure S14). Fourteen days after the immune challenge, animals were anaesthetized as previously described, euthanized by means of i.v. barbiturates overdose (sodium thiopental 40 mg/kg; Pentotal Sodium, MSD Animal Health S.r.l., Rahway, NJ, USA) and subjected to gross post-mortem necropsy (detailed timeline in Figure S1).

Blood analysis and flow cytometric assay

Blood samples were used for complete blood count (CBC), clinical chemistry, lactatemia, cytokine concentration (MILLIPLEX® Porcine Cytokine/Chemokine Magnetic Bead Panel, Merck Life Science S.r.l., Milan, Italy) and haemocultures (detailed protocol in supplementary methods, page 2).

A flow cytometric phagocytosis assay was performed on blood samples collected before the start of propranolol (or vehicle) treatment (T0), immediately before the bacterial challenge (T1) and then 90 min (T2), 1 and 2 weeks (respectively T3 and T4). Briefly, the blood samples collected with heparin as anticoagulant, were incubated, or not, with fluorescent *E. coli* Red BioParticles conjugated with a fluorogenic reagent that increases in fluorescence (emission filter 585/26) as the pH decreases in the surrounding environment (e.g. inside lysosomes; pHrodo™ BioParticles™ Conjugates for Phagocytosis and Phagocytosis Kit, Invitrogen™, Waltham, MA, US). The samples were then analysed on the flow cytometer (Attune NxT, ThermoFisher, equipped with a 488 nm laser, detailed protocol in supplementary methods, page 2). The white blood cells were identified through their typical localization in a Forward Scatter-Height (FSC-H) vs Side Scatter-Height (SSC-H) dot plot in order to exclude any fragments of the lysed red blood cells. Lymphocytic, granulocytic and monocytic populations were identified and appropriately gated to evaluate their specific physical and fluorescence characteristics. No further counterstaining was performed using conjugated antibodies towards specific cellular surface markers.

Granulocytes gated in negative control samples were analyzed for their light scattering properties: the forward scatter area (FSC-A) values referred to the size of cells were exploited to evaluate the state of activation of neutrophils²⁸. The side scatter high (SSC-H) and side scatter area (SSC-A) referred to the complexity of cells and were used to derive the percentage of disaggregated and aggregated neutrophils²⁹.

We measured the Mean Fluorescence Intensity (MFI) emitted by neutrophils and monocytes after the incubation with fluorescent *E. coli* Red BioParticles, as a mirror of the amount of BioParticles phagocytosed by phagocytes in blood samples (Figure S4 and S15). For each evaluated parameter, a normalization of all timepoints' value was done by the ratio of each value to the value of the sample collected before the start of pharmacological treatment (T0).

Statistical analysis – animal study

CBC, clinical chemistry, lactatemia and flow cytometry data were analysed using repeated measures ANOVA, with post-hoc comparisons between groups at specific time points using Holm's correction to the probability level to control for type I error. The assumption of homogeneity of variance was checked using the sphericity test. In case of violation of the assumption, Greenhouse–Geisser's correction was applied.

Blood bacterial counts, temperature and serum cytokine data were analysed using the non-parametric Kruskal–Wallis test because the frequency distribution of these variables was not normal. When Kruskal–Wallis test was significant, specific time-point comparisons between groups were performed using the Mann–Whitney test.

Data were expressed in the figures as mean $\pm$ standard error of the mean (SEM). Missing data were imputed. All analyses were carried out using SPSS software (28.0) The significance level was set to $p < 0.05$.

Retrospective study on patients

The FINDERS study protocol (#3473) was approved by the Ethics Committee of Romagna Health Authority (CEROM) on December 12, 2022, in accordance with the relevant guidelines. Data were collected in an aggregated and anonymous form. Due to the retrospective nature of the study, the Ethics Committee of Romagna Health Authority (CEROM) waived the need of obtaining informed consent. The datasets generated and/or analysed during the current study are not publicly available due to restrictions imposed by the Ethics Committee's policy on administrative databases but are available from the corresponding author on reasonable request.

Rehospitalisations with orthopaedic procedure codes ICD-9 CM 76.xx-84.xx occurring within 14 days of discharge from the first hospitalisation were considered as part of the same episode of care and therefore were counted only once.

Only infections not present on index admission were included in the analyses. The infection codes are listed in Table S2 in supplementary materials (Appendix pp 21). Prescriptions for β -blockers were identified by searching the anatomical therapeutic chemical classification system (ATC) codes C07AA (non-selective β -blockers) and C07AB (selective β_1 -blockers) in the pharmacological dispensing database, that includes retail pharmaceuticals dispensed through a pharmacy and drugs administered or dispensed by the hospital pharmacy at the end of an episode of hospital care. Data extraction was based on the following alternative criteria: i) at least 2 prescriptions

on different dates in the 90 days before hospital admission; ii) at least 1 prescription in the 30 days before hospital admission; iii) the sum of the total defined daily dosed in the 90 days before hospital admission was sufficient to encompass the admission date. Other drug prescriptions were classified by methods using the first letter of the ATC nomenclature (see Table S3). For group J “Anti-infectives For Systemic Use” we also determined whether dispensing of the drug had occurred within the 10 days prior to the index hospitalization.

Statistical analysis—patients

Age was summarized as mean $\pm$ standard deviation (SD) and compared between groups using the t-test. Categorical data were expressed as absolute and percentage frequencies and compared between groups using χ^2 test or Fisher’s exact test. The HAI rate was calculated as the sum of all HAIs divided by the number total number of patients.

Given our focus on non-selective β -blockers, all the primary analyses were conducted using C07AA as the treatment of interest. Conditional logistic regression was used to investigate the association of non-selective β -blockers with HAIs, by matching cases (patients with HAIs) with controls (patients without HAIs) for sex, age group (18–24, 25–29, ..., 90–94, > 94), and Elixhauser comorbidity index^{30,31}. This index is a comorbidity measure based on International Classification of Diseases (ICD) diagnosis codes, obtained as an unweighted count of comorbid conditions³². Matching was carried out with a ratio 1 case:2 controls using the SPSS procedure CSPLAN.

For descriptive purposes, we also dichotomized the index into three classes based on disease codes from hospitalisations in the previous two years: non-multimorbid (0–1), multimorbid (≥ 2), and not available (when information was missing in the hospital discharge records).

Three separate conditional logistic regression models were fitted: one for all infections (matching ratio 1:2; 1 case and 2 controls), one for Gram-negative infections (matching ratio 1:4) and one for Gram-positive infections (matching ratio 1:4). The matching ratio was 1:2 for the all-infection model because it was not possible to identify more than two controls (patients without HAIs) with the same sex, age group and Elixhauser index category for each case (patients with HAIs). For Gram-negative and Gram-positive infections, the matching ratio was 1:4 due to the fewer number of cases. To adjust for the potential effect of antibiotic treatment, a dichotomous variable indicating whether an antibiotic treatment was prescribed in the 10 days prior to the index hospitalization was included in all the models.

To investigate whether the association was specific for non-selective β -blockers, we replicated the above-mentioned analyses using selective β_1 -blockers as the treatment of interest. This was done under the assumption that the use of the latter does not modify the risk of infection. The analyses were carried out using SPSS 28.0.1.1 and the Stata/SE 17.0 procedure clogit.

Data availability

The animal datasets generated and/or analysed during the current study are available in the AmsActa institutional repository: <https://doi.org/10.6092/unibo/amsacta/7720>. The human datasets generated and/or analysed during the retrospective human study are not publicly available due to restrictions imposed by the Ethics Committee’s policy on administrative databases but are available from the corresponding author on reasonable request.

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Author contributions

AO, DV, SR, PR and DM wrote the manuscript. AO, DV, SR, LA, RA, MC, TH, DT, AC, SR, AE, AZ, SF, DG, ML, YRL, CNM, RMM, MJM, PP, EP, LT, MG, PV, MLB, PR, TL and DM contributed to the manuscript. AO, DV, AE, EP, LT, MLB performed in-vivo animal experiments. AC, AZ, SF and TL performed ex vivo animal experiments. SR, DG and PR performed the statistical analysis on patients. RMM, MJM, MLB, PR, TL and DM contributed devising the work. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Declaration

Competing interests

The authors declare no competing interests.

Additional information

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