



Translocation microbienne, insuffisance gastro-intestinale et microbiote intestinal au cours du choc septique : une étude observationnelle prospective

Thomas Lancry

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UNIVERSITE DE MONTPELLIER
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THESE

Pour obtenir le titre de
DOCTEUR EN MEDECINE

Présentée et soutenue publiquement
Par
Thomas LANCERY

le 14/10/2021

TITRE

**Translocation microbienne, insuffisance gastro-intestinale
et microbiote intestinal au cours du choc septique :
une étude observationnelle prospective**

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JURY

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UNIVERSITY OF MONTPELLIER
UNIVERSITY OF MEDICINE MONTPELLIER-NIMES

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To obtain the title of
DOCTOR OF MEDICINE

Presented and publicly defended
By
Thomas LANCERY

on 10/14/2021

TITLE

**Bacterial translocation, gastrointestinal failure
and intestinal microbiota during septic shock :
a prospective observational study**

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RESUME

Introduction : Plusieurs théories présentent la dysfonction intestinale comme moteur de la défaillance d'organe dans le choc septique. Notre hypothèse est que le choc septique entraîne une insuffisance intestinale aiguë entraînant des altérations de la fonction barrière de l'intestin et du microbiote intestinal favorisant la translocation bactérienne.

Matériel et méthodes : Il s'agit d'une étude observationnelle prospective, monocentrique, chez 60 patients durant les 7 premiers jours du choc septique. L'évaluation clinique de la fonction digestive reposait sur les critères de l'ESICM. La translocation bactérienne était évaluée par la qPCR ADNr 16S, le LBP et le sCD14. L'altération de la paroi intestinale était évaluée par l'I-FABP et la Claudine-3. Le microbiote fécal était étudié à J0 et J7.

Résultats : 90% des patients en choc septique ont une PCR ADNr 16S positive (>12.25 copies/ μ L) au moins une fois durant les 7 premiers jours du choc septique et plusieurs patients ont eu ce critère à plusieurs reprises.

La cinétique des différents biomarqueurs n'était pas associée aux caractéristiques du choc septique (foyer infectieux, bactériémie...), la symptomatologie digestive ou le support nutritionnel à J3. Aucune corrélation forte entre ces biomarqueurs n'a été montrée.

Le taux d'I-FABP était associé à la positivité de la PCR ADNr 16S ($> 12,25$ copies/ μ L) et à la mortalité à J7.

La diversité du microbiote intestinal diminuait au cours des 7 premiers jours du choc septique.

Conclusion : La translocation bactérienne semble fréquente au cours du choc septique. La PCR ADNr 16S est un marqueur prometteur pour lequel un seuil ayant un intérêt clinique doit être défini.

LISTE DES ABREVIATIONS

rDNA	Ribosomal deoxyribonucleic acid
ANOVA	Analysis of variance
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
GI	Gastro-intestinal
IAP	Intra-abdominal pressure
IBD	Intestinal bowel disease
ICU	Intensive Care Unit
I-FABP	Intestinal fatty acid-binding protein
AGI	Acute Gastrointestinal Insufficiency
IGS II	Simplified Severity Index II
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MODS	Multiple organ dysfunction syndrome
OTU	Taxonomic Operational Units
PCR	Polymerase chain reaction
qPCR	quantitative chain reaction polymerase
sCD14	Differentiation cluster 14 (soluble form) / Presepsin
SOFA	Sequential Organ Failure Assessment
BT	Bacterial translocation
MT	Microbial translocation
HIV	Human immunodeficiency virus

INTRODUCTION

The gut was considered metabolically inactive for a long time, until the end of the 1990s, when functions other than the absorption of nutrients were highlighted (1) : defense against infection (barrier effect and immunity) or metabolism (fermentation of sugars) as an example. Impairment of these functions alters the patient's prognosis as patients with clinical digestive signs exhibit a higher mortality rate (2,3).

The concept of bowel dysfunction was elaborated at that time, with the desire to create a clinically relevant definition and a system of quantification. The European Society of Intensive Care Medicine (ESICM) has defined acute gastrointestinal failure (AGI) as GI dysfunction secondary to acute illness and has proposed a classification based on intolerance to enteral nutrition, with 4 stages of severity (2). A recent meta-analysis using this classification showed that clinical AGI is common and its severity is associated with mortality (4). However, bowel dysfunction is underdiagnosed (5) because its definition is imprecise, its clinical signs are rarely in the foreground in the ICU and no biological marker has been validated.

Several hypotheses support the fact that, after aggression, the gut could be the motor, which maintains organ failure. Deitch's "three hit model" (6,7) is based on the fact that the initial aggression causes intestinal hypoperfusion (first hit) and that resuscitation generates ischemia-reperfusion phenomena releasing pro-inflammatory mediators (second hit), leading to a loss of the gut barrier function which favors the translocation of microorganisms. These elements are responsible for a systemic inflammatory response syndrome (third hit). Clark and Coopersmith have developed the theory of "intestinal crosstalk" (8), according to which three major elements of the gut (its epithelium, its immune system and its microbiota) interact with each other and with the rest of the body. Aggression could disrupt this dialogue and promote intestinal failure, as well as other organs failure. In 2008, Watkins suggested, with the theory of "gut-lymph induced MODS" (9), that the toxic mediators released by the failing gut reach other organs from the mesenteric lymphatic system rather than through the blood. Septic shock, common in the ICU (10), is a model of aggression that can lead to intestinal failure. Microbial translocation (MT) is defined as the paracellular or

transcellular passage of microorganisms from the lumen of an organ into sterile tissue. Digestive MT concerns the passage of pathogens from the digestive tract into the blood, whether they are bacteria (bacterial translocation, BT) or fungi (fungal translocation). The literature in humans is mainly concerned with the context of cirrhosis, where it has been shown that low-level MT can be physiological but that the intensity of MT is proportional to the severity of cirrhosis (11,12), HIV infection, acute pancreatitis and IBD. The diagnosis of certainty is based on the identification of microorganisms in nodes of the portal system or in the peritoneum when cultured (13,14).

As this method is very cumbersome and invasive, plasma markers are sought to indicate the presence of BT.

LPS is an endotoxin composing the wall of gram negative bacteria. It binds to the CD14 receptor expressed on the surface of myeloid cells, activating a TLR-dependent signaling pathway leading to the secretion of pro-inflammatory mediators.

LBP is a protein synthesized by the liver, playing a major role as a soluble pattern recognition molecule: it binds to endotoxins (such as LPS) and transports them to immune cells expressing the CD14 receptor.

CD14 is a glycoprotein, existing in membrane form (on the surface of various cells: monocytes, macrophage, neutrophils, dendritic cells...) or soluble (sCD14). CD14 has a high affinity for the LBP-LPS complex, and their binding activates the toll like receptor type 4 (TLR4) of immune cells starting a pro-inflammatory cascade against the infectious agent.

The LPS-LBP-CD14 complex is released into the circulation by detachment of CD14 from the membrane, creating sCD14 (15).

LPS and its co-receptors LPS-binding protein (LBP) and sCD14 have been extensively studied, but LPS has a very short half-life (16) and LBP and sCD14, which have a longer half-life (17), are proteins produced by the host in response to the presence of LPS (aggression by gram-negative bacteria) and may reflect the intensity of the host immune response rather than the intensity of BT (18). All of these markers are specific for gram-negative bacteria, possibly causing a translocation of gram-positive bacteria to be missed. (18)

Plasma quantification of bacterial DNA has been developed using real-time quantitative polymerase chain reaction (qPCR) targeting the 16S ribosomal gene (16S rDNA), which has the particularity of being a direct marker (not dependent on the immune response) and highly conserved in bacteria (gram-negative or positive). The 16S rDNA has a constant region, common to all bacteria, and a variable region, specific to a bacterial species (allowing its identification by sequencing). 16S rDNA PCR has been studied to measure BT during pancreatitis (19,20) or in HIV-infected patients (21–23) but in a qualitative manner (rDNA detected or not). No studies have investigated BT quantitatively during septic shock.

The gut barrier function is provided by enterocytes, which are held together by tight junctions (24,25). Several proteins are involved in this barrier function. FABPs are intracytoplasmic proteins present in different organs, used to detect tissue damage in these organs (26). I-FABP (Intestinal Fatty Acid Binding Protein), is a subtype of FABP found exclusively in enterocytes (27). I-FABP increases in case of direct enterocyte injury (27) – regardless of the cause of the injury (28–32). Several studies (33–35) have shown the prognostic importance of I-FABP, the elevation of which is associated with mortality in the ICU, with a threshold value of 355 pg/mL described by Piton as being best associated with mortality at D28 (34).

Claudins are epithelial transmembrane proteins expressed in the tight junctions of many epithelia. Only claudin-3 is highly expressed in the jejunum, ileum and colon (36). In the case of intestinal damage, the loss of tight junctions (i.e. the increase in permeability) results in a decrease in tissue claudin-3 as early as 30 minutes after the onset of the attack (37) and an increase in urine claudin-3 (38,39).

In animals, Thuijls has shown that this increase in permeability favours MT because mesenteric lymph node cultures are more often positive when claudin-3 levels indicate a loss of tight junctions (37).

In the few studies that have looked at plasma claudin-3 concentration, it is higher in subjects at risk of epithelial damage (40,41).

The intestinal microbiota corresponds to the endogenous flora of the small intestine and the colon, it is composed of more than 300 trillion bacteria of more than 1000 different species.

The intestinal microbiota has several roles : defense against pathogens (barrier effect supporting the epithelial barrier); immunity by participating in the maturation of intestinal lymphoid organs (42,43); metabolic functions (44). Intestinal dysbiosis is rapidly established in the ICU, manifested by a decrease in diversity sometimes associated with the predominance of one species (45). Dysbiosis can be favored by many elements in the ICU : the initial aggression, therapeutics such as vasopressors, broad spectrum antibiotics, artificial nutrition... (46–49). Its alteration favors MT (50) and is associated with an increase in infectious complications and mortality in the ICU (51,52).

Our hypothesis is that some patients in septic shock present an acute intestinal failure leading to alterations in the gut barrier function (I-FABP, claudin 3) and a modification of the intestinal microbiota favoring BT assessed by 16S rDNA qPCR.

The main objective of the study is to investigate BT and its predictive factors by measuring 16S rDNA levels in patients in septic shock.

The secondary objectives are to describe the kinetics of biomarkers of microbial translocation (16S rDNA, LBP, sCD14), intestinal failure (I-FABP) and digestive permeability (claudin-3), to search for a correlation between these biomarkers, to describe the evolution of the intestinal microbiota during septic shock, and to study the relationship between these biological elements and digestive symptomatology.

MATERIALS AND METHODS

Design of the study

This is a prospective, single-center, observational study.

This study followed the STROBE guidelines for observational studies (53).

The study was registered under the number 2018-A02192-53 and approved by the ethics committee (West III Committee for the Protection of Persons) on December 15, 2018. Written informed consent was not required due to the observational nature of the study. A letter of information and no-objection was sent to the patients or their legal representatives.

Study population

Eligible patients for the cohort were adults admitted to one of the three ICUs at the Nîmes University Hospital between April 2019 and September 2020 for a diagnosis of septic shock.

Inclusion criteria were age greater than or equal to 18 years and the presence of septic shock according to the SEPSIS-3 definition (54) :

- Suspected or documented infection
- AND an acute increase of ≥ 2 SOFA points
- AND hypotension requiring vasopressors to maintain MAP ≥ 65 mmHg and having a serum lactate level > 2 mmol/L despite vascular filling.

Non-inclusion criteria were: pregnancy or breastfeeding; inflammatory bowel disease; HIV infection; Child C liver cirrhosis; digestive neoplasia or lymphoma; low life expectancy (moribund or therapy-limited); digestive surgery (recent or upcoming, urgent or scheduled); patient under judicial protection, inability to provide information to the patient or relatives, patient in an exclusion period determined by a previous study, and patient receiving norepinephrine for more than 12 hours at inclusion.

The primary endpoint was plasma 16S rDNA level.

Secondary endpoints were fecal microbiota diversity, clinical stage of intestinal failure, and plasma levels of biomarkers of intestinal barrier alteration (I-FABP and claudin-3) and microbial translocation (LBP and sCD14).

Clinical data

Data were collected prospectively at different time points defined as follows : admission (Day 0 = D0), 12 hours after admission (H12), 1 day after admission (D1), 2 days after admission (D2), 3 days after admission (D3), 7 days after admission (D7).

Baseline characteristics were recorded at inclusion: age, gender, body mass index (BMI), comorbidities, IGS II score, infected site, and immunosuppressive or healthcare-related context.

Data were collected according to the following schedule.

	D0	H12	D1	D2	D3	D7
Blood sample	✗	✗	✗	✗	✗	✗
Stool sample	✗					✗
Clinical data	✗		✗	✗	✗	✗
Nutritional status					✗	✗
Vital status						✗

Clinical data were recorded at 5 time points (D0 ,D1, D2, D3, D7) with the following variables: SOFA score; intra-abdominal pressure measured in the bladder; mechanical ventilation; continuous veno-venous hemofiltration; antibiotic treatment; digestive symptoms (vomiting, gastric residual volume > 200mL in a single measurement, diarrhea > 250 mL/day, gastrointestinal bleeding, paralytic ileus, intestinal dilation on imaging, or abnormal bowel sounds < 5/min or > 35/min). The definition of paralytic ileus was the absence of bowel movements for three days or more. Criteria for bowel dilatation were established according to ESICM guidelines (55): small bowel diameter > 3 cm, colon diameter > 6 cm, cecum diameter > 9 cm.

Nutritional status was assessed by the type of nutrition at D3 and D7 (enteral or parenteral). At D3, if enteral nutrition had not been started, the reason was recorded. At D7, the caloric intake was quantified and if it did not reach 80% of the objectives, the reason was requested.

Vital status was assessed at D7.

To assess bowel function, the ESICM 4-stage AGI classification (55) was planned, but could not be applied due to a lack of precision regarding the cause of failure to achieve caloric targets. We had to use a modified ESICM score, non-validated, based on objective criteria mentioned in the original ESICM score.

The score is dynamic according to the time elapsed since admission with the following criteria : at D0 and D1, it considers intra-abdominal pressure and major digestive symptoms. From D2 onwards, it considers the absence of transit for two days or more, in addition to the previous criteria. From Day 3 onwards, it uses the same criteria, adding tolerance to enteral nutrition (oral or nasogastric tube) without quantification. This non-validated score is called in this study "local AGI score" and is presented in **Appendix 1**.

Biological data

Blood samples were collected by 2 swabs (1 EDTA tube and 1 dry tube) at 6 time points of interest in the patients (D0, H12, D1, D2, D3, D7) to collect 16S rDNA (copies/ μ L) and the following intestinal markers : I-FABP (pg/mL), sCD14 (μ g/mL), LBP (μ g/mL) and Claudin-3 (ng/mL).

Fecal samples were collected at two time points of interest in the patients: D0 and D7 to assess the composition and diversity of the microbiota. In anticipation of the difficulty of collecting stools exactly at D7, a window between D5 and D9 was allowed.

Laboratory analyses were performed by a biologist not involved in the collection of clinical data and blinded to the clinical results.

- 16S rDNA qPCR : 16S rDNA was extracted from plasma samples using the QIAcube® automatic extractors (Qiagen, Courtaboeuf, France) of the microbiology department of the Nîmes University Hospital, using the QIAmp MinElute ccfDNA® kit (Qiagen).

In order to control the absence of bacterial elements foreign to the translocation process, a negative control (pure water necessary for molecular biology) was systematically extracted in parallel. Real-time quantitative PCR was performed in Taqman technique using Lightcycler 480 II® thermal cycler (Roche diagnostics, Meylan, France) and FastStart LC DNA Master^{PLUS}HybProbe® master mix (Roche diagnostics) in a volume of 20 µL, in 96-well plates.

The samples were analyzed in duplicate in two different manipulations (four measurements) and a negative PCR control (water of sufficient purity for molecular biology) was systematically performed. The absolute quantification analysis will be performed with the LightCycler® 480 software (Roche diagnostics), using a standard curve performed with a previously established standard range. This method has been published previously (56).

16S rDNA qPCR was considered positive for a threshold >12.25 copies/µL. This threshold was determined previously on a cohort of 100 healthy subjects so that 95% of healthy subjects were negative (57).

- I-FABP was measured by ELISA with the Human i-FABP ELISA kit (Hycult biotech, Uden, Netherlands).
- LBP was measured by ELISA with the Enzyme Immunoassay for Quantification of free human LBP® kit (Enzo Life Sciences, Villeurbanne, France).
- sCD14 was measured by ELISA using the CD14, soluble (human) ELISA kit (Enzo Life Sciences, Villeurbanne, France).
- Claudin-3 was measured by ELISA with the Human CLDN3 ELISA Kit (Elabscience, Chicago, USA).

Statistical analysis

Statistical analyses were performed with R software (version 4.1.0).

As this was a pilot study, no power and sample size calculations were performed. In case of missing data for a given test, the patient concerned was removed from the analyses. All tests were two-sided and the first-species risk was set at 5%.

Categorical variables are presented as frequencies and percentages, while continuous variables are presented as median and quartiles. For univariate analyses, Student's, Welch's or Wilcoxon-Mann-Whitney's tests were used depending on the conditions of application (normality, heteroscedasticity, etc.). For multivariate analyses, analyses of variance (ANOVA) or Kruskal-Wallis (depending on normality) were performed. Given the longitudinal nature of the study, mixed ANOVAs (for repeated measures) were also conducted in the study.

The analysis of the microbiota was performed using the R software packages phyloseq and vegan.

To represent the pattern of evolution of severity and digestive conditions, patients were grouped into clusters for the SOFA score and the local AGI score.

The clustering method is presented in **Appendix 2**.

Different subgroups of patients were identified post hoc for exploratory analysis based on severity (assessed by IGS II and SOFA at D0 or by cluster during the stay), infection characteristics (infected site and bacteriemia at initial infection), digestive conditions (assessed by digestive symptomatology at D0 or D3, IAP at D0 or D3, local AGI score at D0, D3 or by cluster during the stay) and nutritional status.

The composition of the subgroups is described in **Appendix 3**.

The evolution of microbiota was analyzed with the same subgroups as well as :

- enterocyte destruction at D0 (I-FABP > 355 pg/mL or not) and alteration of tight junctions (claudin 3) at D0 and D3.
- microbial translocation assessed by 16S rDNA (> 12.25 copies/μL or not) at D0 and D3.
- the type of antibiotic therapy at D0 and D3.

RESULTS

Description of the population

Patients were recruited between April 2, 2019 and September 2, 2020, 536 patients with septic shock were prospectively screened for eligibility resulting 60 patients finally enrolled into the study (See Figure 3. Flow chart).

Baseline patient characteristics are summarized in **Table 1**.

Mortality at D7 was 10% (6/60).

Prevalence of bacterial translocation assessed by 16S rDNA PCR (>12.25)

Within the first 7 days of septic shock, 54 patients (90%) had a 16S qPCR > 12.25 copies/ μ L at at least one time point of interest.

At each time point of interest, approximately 60-70% of patients had 16S rDNA levels >12.25 copies/ μ L (see **Table 2**).

Evolution of biomarkers of bacterial translocation

16S DNA qPCR

In univariate analysis, the 16S rDNA level did not change significantly during the first 7 days of septic shock ($p= 0.292$), see **figure 2**. The evolution of the 16S rDNA PCR was highly variable between patients. See **appendix 4**.

43.3% of patients (26/60) had positive blood cultures and 91.5% (54/59) of patients had 16S rDNA levels above threshold.

30 of 34 patients (88.2%) with negative blood cultures had 16S rDNA above threshold. 24 of 26 patients (92.3%) with positive blood cultures had 16S rDNA above the cut-off.

In multivariate analysis, the repeated measures ANOVA showed no association ($p > 0.05$) between 16S rDNA level and severity (assessed by IGS II score or SOFA score at D0 or cluster during the stay), infected site, bacteremia associated with the initial infection, digestive symptomatology (assessed by local AGI score at D0, D3 or by cluster during the stay, by IAP at D0 or D3 or by the presence of digestive signs at D0 or D3) or nutritional support at D3.

sCD14

In univariate analysis, sCD14 levels decrease during the first 7 days of septic shock ($p = 0.013$). See **Figure 3A**.

In multivariate analysis, repeated measures ANOVA showed that sCD14 is not associated ($p > 0.05$) with severity (assessed by IGS II score or SOFA score at D0 or cluster during the stay), infected site, bacteremia associated with the initial infection, digestive symptomatology (assessed by local AGI score at D0, D3 or by cluster during the stay, by the IAP at D0 or D3 or by the presence of digestive signs at D0 or D3), nutritional support at D3 or with 16S rRNA qPCR positivity (based on the proposed threshold of 12.25 copies/ μ L) at D0 or D3.

LBP

In univariate analysis, LBP levels decrease during the first 7 days of septic shock ($p < 0.001$). See **Figure 3B**.

In the multivariate analysis, the repeated measures ANOVA showed no association ($p > 0.05$) between LBP rate and severity (assessed by IGS II score or SOFA score at D0 or cluster during the stay), infected site, bacteremia associated with the initial infection, GI symptomatology (assessed by local AGI score at D0, at D3 or by cluster during the stay, by IAP at D0 or D3, or by the presence of digestive signs at D0 or D3), nutritional support at D3 or 16S rDNA qPCR positivity (according to the threshold of 12.25 copies/ μ L) at D0 or D3.

Microbiota evolution

Alpha-diversity was decreased in patients with septic shock ($p < 0.05$). See **Figure 4**.

The decrease in diversity was not related ($p > 0.05$) to (assessed by IGS II score or SOFA score at D0 or cluster during the stay), infected site, bacteremic nature of initial infection, GI symptomatology at D3 (local AGI score, IAP, GI symptoms), nutritional support at D3, intestinal permeability at D0 (defined as IFABP > 355 pg/mL), nor 16S rDNA qPCR positivity (based on the proposed threshold of 12.25 copies/ μ L) at D0 or D3. See **Table 3**.

The evolution of alpha-diversity has been influenced by :

- the local AGI score at D0 or during the stay (cluster). See **Figure 5**.
- the number of antibiotics at D0 ($p < 0.05$ for the number of OTUs and the Chao1 index) but not at D3 .

Evolution of intestinal barrier biomarkers

I-FABP

In univariate analysis the I-FABP level varied significantly during the first 7 days of septic shock ($p = 0.007$) : it decreased from D0 to D3 ($p = 0.039$) and then seemed to increase again between D3 and D7 but the difference between D3 and D7 did not reach significance ($p = 0.092$). See **Figure 6A**.

In multivariate analysis, the repeated measures ANOVA showed that the level of IFABP

- was associated with severity assessed by IGS II score ($p = 0.008$) and SOFA cluster during stay ($p = 0.004$).
- was associated with 16S rDNA qPCR positivity (based on the proposed threshold of 12.25 copies/ μ L) at D0 ($p = 0.049$) and D3 ($p = 0.047$).
- was not associated ($p > 0.05$) with SOFA score at D0 at the infected site, bacteremia associated with the initial infection, digestive symptomatology (assessed by local AGI score at D0, at D3, or by cluster of evolution during the

stay, by IAP at D0 or at D3 or by the presence of digestive signs at D0 or at D3), nutritional support at D3.

Using the I-FABP D0 threshold of 355 pg/mL, there was no significant difference regarding 16S rDNA qPCR positivity (at the proposed threshold of 12.25 copies/ μ L) at D0 ($p= 0.79$) and D3 ($p= 0.56$).

Claudin-3

In univariate analysis, Claudin-3 decreased during the first 7 days of septic shock ($p< 0.001$). See **Figure 6B**.

In multivariate analysis, the repeated measures ANOVA shows that the claudin-3 level:

- was associated with SOFA D0 ($p= 0.036$) with a higher claudin-3 level in patients with high SOFA
- was associated with local AGI score at D3 ($p= 0.022$).
- was not associated ($p> 0.05$) with other markers of severity (IGS II score, SOFA cluster during the stay), with the infected site, with the bacteremia associated with the initial infection, with the digestive symptomatology (local AGI score at D0 or by evolution cluster during the stay, IAP at D0 or D3, presence of digestive signs at D0 or D3), nutritional support at D3 or 16S rDNA qPCR positivity (according to the proposed threshold of 12.25 copies/ μ L) at D0 or D3.

Correlations between 16 rDNA and other bacterial translocation markers

There was no strong correlation between biomarkers according to Spearman's correlation coefficient (**rs**). See details in **appendix 5**.

Digestive symptomatology

There was no difference in survival according to the log-rank test ($p > 0.05$) depending on the IAP at D0 or at D3, the local AGI score at D0, the infected site.

D7 mortality

There was no association between mortality at D7 and the evolution of microbiota or biomarkers ($p < 0,05$), except for I-FABP. There was a significant association between mortality at D7 and a I-FABP value >355 pg/mL at D0 ($p = 0.033$, CI 95 0.00 - 1.07) and the absolute value during the stay ($p < 0,001$ in repeated measures ANOVA).

DISCUSSION

Based on the 16S rDNA threshold defined in our study, BT is frequent in patients in septic shock as 90% of these patients translocate at least once in the first 7 days of septic shock.

This rate of BT is consistent with earlier studies that evaluated BT in rats in septic shock by lymph node culture (58). No study have used 16S rDNA quantification to assess BT during septic shock, most studies use 16S rDNA qualitatively with gene sequencing to determine microbiota composition (59–62).

The 16S rDNA level does not change significantly during the first 7 days of septic shock, but the kinetics are highly variable between patients and 60 to 70% of patients have a 16S rDNA level above the threshold at each time point of interest, suggesting that BT is a discontinuous and dynamic phenomenon, with intermittent releases, requiring iterative sampling for its detection.

Wiest has shown that there is a certain degree of physiological MT in cirrhotic patients (12), but without a quantitative threshold being described. Similarly, subclinical MT may happen in septic shock, which may have frequent MT, but the correlation with worse prognosis is unclear.

The frequency of this event raises questions about its clinical impact and further studies seem necessary to evaluate the threshold of 12.25 copies/ μ L in order to assess its relevance or to look for a threshold for recognizing BT episodes with prognostic impact.

In our study, the 16S rDNA level was not associated with a significant difference in the severity of septic shock, in contrast to the results of Bloos et al (63) who showed that patients with a positive 16S rDNA PCR had a significantly higher SOFA score and to the results of Kirkbright, which showed that bacteremic patients with 16S rDNA levels above 1237 copies/mL were more likely to progress to septic shock had a higher risk of developing septic shock within 48 h

Bloos *et al*/ (63) showed that in patients with severe sepsis, 16S rDNA PCR was about twice as often positive as blood cultures, a result that is consistent with our study. The literature concerning patients with severe sepsis shows 30 to 57 % of false negatives (63,64).. In our study, 92% of the positive blood cultures were confirmed by 16S rDNA qPCR above the threshold. One explanation for these results is that the efficacy of the PCR may differ depending of the type of organism since lysis may be more difficult in gram-positive bacteria and fungi and could be responsible for the lack of detection by the PCR despite a positive blood culture. We are not able to state that the blood culture and 16S rDNA qPCR positivity were related to the same pathogen (since we did not identify the bacterium responsible for the 16S rDNA positivity) or that these bacteremias did not originate from the initial outbreak rather than from a translocation from the gastrointestinal tract.

The levels of sCD14 and LBP decrease during the first 7 days of septic shock. These biomarkers rather represent the intensity of the inflammatory response secondary to the presence of LPS (18,65) than direct markers of TM.

Their decrease seems to reflect control of the inflammatory syndrome and a favorable evolution, as several studies have shown that the absence of a decrease in LBP after several days of treatment of septic shock was associated with an increase in mortality (66,67)

In our study, there was no relationship between the severity of septic shock and the level of these biomarkers.

In our study, the diversity of the gut microbiota decreases during septic shock. This result is consistent with the literature. In 2017, Lankelma (68) had shown that gut microbiota diversity was lower in critically ill patients than in healthy patients, whether septic or not, but this study only assessed the microbiota at a given time and not dynamically as in our study and Ojima's study (60). Yeh (69) showed that alpha-diversity was significantly lower in critically ill patients than in healthy patients, up to more than 10 days after admission. Several studies have shown that dysbiosis, characterized by a decrease in diversity and a change in composition (with a decrease

in *Firmicutes* and *Bacteroidetes* and an increase in *Proteobacteria*), worsens with time in the ICU (45,70).

In this study, the decrease in diversity was greater when the local AGI score is high at D0 or globally during the ICU stay or when initial antibiotic therapy included more than two antibiotics. This result is consistent with Lankelma's study published in 2016 (47), showing that exposure of healthy subjects to broad-spectrum antibiotics decreased microbiota diversity very early. The change in diversity was not influenced by the other components of our subgroup analysis. This result is in conflict with that of Schneider who showed a greater decrease in diversity in the absence of enteral nutrition (48), but consistent with the study of Shimizu (71) who studied the microbiota of patients admitted to the ICU for SIRS (mainly septic, but also post-traumatic), without finding a significant difference concerning the type of artificial nutrition. Evoquer Ojima 2021

The I-FABP level varies significantly during the first 7 days of septic shock : it decreases significantly until D3 and then does not change significantly. This evolutionary profile is similar to that shown by Timmermans in the trauma patient (35) and is pathophysiologically consistent since the control of septic shock allows the gut to suffer less.

The I-FABP level is associated with the severity of septic shock in our study, consistent with the results of Sekino (72) and especially Piton (34), whose threshold of 355 pg/mL is also associated with mortality in our study. Other studies did not find an association with mortality in septic shock (4,31).

Piton had suggested the usefulness of this biomarker to identify patients at risk of MT (29). In our study, there is an association between 16S rDNA qPCR positivity at D0 and D3 and I-FABP in absolute value during the stay, but this is not found using the the I-FABP D0 threshold of 355 pg/mL defined by Piton.

This association deserves to be explored by further studies, as it supports the pathophysiological rationale that alteration of the enterocyte barrier promotes BT.

The lack of significance using the I-FABP threshold at D0 could be related to a lack of power in our study.

Claudin-3 levels decrease during the first 7 days of septic shock.

The decrease in claudin-3 could reflect the fact that the initial aggression is responsible for a sudden increase in permeability, but that its management allows the gut to gradually restore its barrier function.

No other study has investigated the kinetics of claudin-3 to our knowledge.

The multivariate analysis for claudin-3 shows that it is higher in patients with the most critical septic shock (high SOFA score at admission) and with the most marked digestive symptomatology (high local AGI score) at D3, but not at the other times or for the other digestive symptomatology criteria.

These discrepancies could be related to this biomarker, whose plasma determination is not well documented in the literature, since it is usually determined in urine or directly at the tissue level (36,38). Only few study has investigated plasma claudin-3 to our knowledge (40,41,73).

There is no correlation between the different biomarkers, whether they assess microbial translocation or intestinal permeability.

Abad-Fernandez et al. evaluated MT markers in HIV patients: they found a good correlation between LBP and sCD14, but not between these biomarkers and 16S rRNA levels (18). The Donnadieu-Rigole team studied the same biomarkers in patients undergoing alcohol withdrawal, without finding any correlation between them either before or after withdrawal (56).

The site of infection does not influence the level of biomarkers. To our knowledge, no study has investigated biomarker levels according to the origin of the infection.

The bacteremic nature of the initial infection did not influence the biomarker levels in our study. One might have thought that bacteremic patients have a higher bacterial load that could more significantly alter intestinal permeability and promote microbial translocation. This study does not support this hypothesis and no other study has looked at the level of these biomarkers in relation to the existence of initial bacteremia.

Digestive symptomatology (symptoms, IAP, AGI score) does not influence biomarker levels in our study, except for the association between local AGI score at D3 and claudin-3. The literature is poor on this subject :

- Strang (74) showed that IFABP does not predict which patients will develop intra-abdominal hypertension or abdominal compartment syndrome.
- Wang had shown in resuscitation patients that the level of I-IFABP increased when the ESICM AGI score increased. (75)

The type of nutritional support at D3 does not influence the level of biomarkers. MacFie showed that patients on exclusive parenteral nutrition, and therefore probably with the most advanced intestinal failure, had more MT, assessed by lymph node culture (14).

Strengths of the study

- Our study is, to our knowledge, the first to use 16s rDNA quantification to assess MT in the ICU, in patients with septic shock, whereas it has been done for other pathologies outside the ICU.
- Longitudinal study allowing a dynamic approach of the intestinal microbiota and its evolution during the septic shock

Limitations of the study

- Risk of selection bias as many eligible patients were not included without justification.
- Lack of a cohort of healthy subjects to assess biomarker levels and trends. We reviewed the literature regarding the level of these biomarkers in healthy subjects :
 - The 16S rDNA levels described in the literature for healthy subjects are lower than those in our cohort. Jiang (21) showed an undetectable 16S rDNA level in healthy subjects, whereas for Donnadieu-Rigole (57) it averaged 9.2 copies/ μ L (+/- 1.9).

- The levels of sCD14 and LBP measured in our patients in septic shock are higher than those published in the literature for healthy subjects, which are close to 5 µg/mL on average for sCD14 and 2 to 10 µg/mL on average for LBP (15,56).
- The level of I-FABP in healthy subjects is variable in the literature : often <100 pg/mL (29,34,76,77), but sometimes up to 300 pg/mL (56).
- Plasma claudin-3 levels are lower in our septic shock patients than those described in the literature in healthy subjects which averaged 43.3 ng/mL (40).
- Use of a non-validated clinical score of digestive symptomatology, created within the framework of this study, because the best known score was not applicable with our data. Recently, Asrani (78) had underlined the lack of applicability to critical patients of the multiple scores described in gastrointestinal dysfunction and the need to develop an objective score, usable for research but also in daily practice, in order to promote the diagnosis and management of gastrointestinal failure. This score will have to be evaluated by dedicated studies.
- The 16S rDNA reflects BT, but in the absence of identification of the species concerned, it seems difficult to affirm that this translocation is of digestive origin, and not related to the initial infectious focus (pulmonary...). It would be interesting to identify the bacteria concerned by sequencing the plasma 16S rDNA when it is above the translocation threshold.
- 16S rDNA PCR is very sensitive and may reflect the passage of bacterial fragments (endotoxins or others), which are capable of triggering SIRS, but are not cultivable since they are not viable bacteria. This could explain the difference in sensitivity between blood cultures and 16S rDNA PCR.
- No study of the fungal microbiome, which is also disrupted under different pathological conditions (79,80).
- No study of the prognostic impact (superinfection, length of stay, long term survival...) of BT, permeability, microbiota assessment.
- Our study lacked power to investigate the impact of different classes of antibiotics on the microbiota, which is variable as discussed by Ianiro (81).

CONCLUSION

This study shows that 90% of patients in septic shock have a positive 16S rDNA PCR (>12.25 copies/ μL) at least once during the first 7 days of septic shock and some patients had this criterion on several occasions.

This threshold of 12.25 copies/ μL is proposed to evoke BT but we cannot affirm that it reflects the translocation of digestive origin (and not of the initial focus) nor that it has a clinical significance.

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TABLEAUX

Table 1. Characteristics of the study population

Variable	Value (%) or Median (Q1-Q3)
Age	75 (67,7 – 80,2)
Males	37 (61,7%)
BMI	27,2 (23,4-30,1)
IGS II	59 (48,0-69,0)
SOFA	8,5 (7-10)
Catecholamine support	60 (100%)
Mechanical ventilation	36 (60%)
AKI KDIGO 3	16 (26.6%)
Diabetes	20 (33,3%)
Coronary artery disease	13 (21,7%)
Hypertension	41 (68,3%)
Chronic heart failure	7 (11,7%)
Chronic renal insufficiency	18 (30,0%)
- GFR < 60	11 (18,3%)
- GFR < 30	7 (11,7%)
Alcohol	4 (6,6%)
Cirrhosis (Child A or B)	1 (1,7%)
COPD	7 (11,7%)
Long term oxygen therapy	3 (5%)
Immunosuppression context	8 (13,4%)
- Hematological disease	4 (6,7%)
- Cancer	0 (0%)
- Immunosuppressive therapy	4 (6,7%)
Infected site	Lung : 21 (35%) Urinary tract : 16 (26,7%) Digestive tract : 8 (13,3%) Soft tissue : 5 (8,3%) Catheter : 1 (1,7%) Teeth : 1 (1,7%) Heart : 1 (1,7%) Bone : 1 (1,7%) Central nervous system : 2 (3,3%) Unknown : 4 (6,7%)
Healthcare-related	
- Nosocomial infection	20 (33,3%)
- Recent hospitalization	Yes : 21 (35%) / No : 13 (22%) / Unknown : 26 (43%)
- Recent antibiotic therapy	Yes : 14 (23%) / No : 27 (45%) / Unknown : 19 (31%)

Table 1. Characteristics of the study population

% : percent / SD : Standard-Deviation / BMI : body mass index / GFR : Glomerular Filtration Rate / AKI: Acute Kidney Injury assessed according to KDIGO guidelines
COPD : Chronic Obstructive Pulmonary Disease

Table 2. Percentage of patients with positive qPCR at each time of interest

	J0	H12	J1	J2	J3	J7
Positive (qPCR 16S rDNA < 12,245)	20.0	23.00	18.00	20.00	17.00	14.00
Negative (qPCR 16S rDNA > 12,245)	39.0	36.00	37.00	35.00	35.00	35.00
Positive %	66.1	61.02	67.27	63.64	67.31	71.43
Number of 16s PCR analysed	59	59	55	55	52	49
Number of patient still hospitalized	60	60	60	59	57	49
Number of missing values	1	1	5	4	5	0

Table 3 : Evolution of alpha diversity according to the subgroup

Evolution of alpha-diversity	<i>Number of OTUs</i>		<i>Shannon</i>		<i>Chao1</i>	
	Median (Q1; Q3)	p	Median (Q1-Q3)	p	Median (Q1-Q3)	p
Global	- 28.00 (-62.00 ; 1.00)	0.0002	-0.76 (-1.90 ; -0.02)	0.0003	- 28.00 (-62.0 ; 1.00)	0.0002
SOFA D0		0.520		0.923		0.520
Low	-44.00		-0.664		-44.00	
Moderate	-23.00		-0.772		-23.00	
High	-41.00		-0.666		-41.00	
SOFA clusters		0.709		0.964		0.709
1	-37.00		-0.613		-37.00	
2	-26.00		-0.719		-26.00	
3	-25.00		-0.765		-25.00	
IGS II		0.738		0.934		0.738
Low	-24.50		-0.527		-24.50	
Moderate	-38.00		-0.581		-38.00	
High	-28.00		-0.773		-28.00	
Infected site		0.711		0.329		0.711
Lung	-18.50		-0.33		-18.50	
Urine	-44.00		-1.15		-44.00	
Digestive tract	-27.00		-0.53		-27.00	
Other	-50.00		-1.19		-50.00	
Initial bacteremia		0.574		0.888		0.574
NO	-28.00		-0.533		-28.00	
YES	-23.00		-0.874		-23.00	
qPCR 16S rDNA D0		0.814		0.975		0.814
≥ 12,245 copies/mL	-44.00		-0.454		-44.00	
< 12,245 copies/mL	-26.50		-0.765		-26.50	
qPCR 16S rDNA D3		0.512		0.156		0.512
≥ 12,245 copies/mL	-5.00		-0.223		-5.00	
< 12,245 copies/mL	-29.00		-0.823		-29.00	
I-FABP D0		0.592		0.498		0.592
≥ 355 pg/mL	-26.00		-0.719		-26.00	
< 355 pg/mL	-36.00		-0.773		-36.00	

Evolution of alpha-diversity	<i>Number of OTUs</i>		<i>Shannon</i>		<i>Chao1</i>	
	Median (Q1; Q3)	p	Median (Q1-Q3)	p	Median (Q1-Q3)	p
AGI local score D0		0.009		0.007		0.009
0	14.00		0.238		14.00	
1	-27.50		-0.961		-27.50	
2	-44.00		-1.10		-44.00	
AGI local score D3		0.257		0.339		0.257
0	-13.00		-0.456		-13.00	
1	-27.00		-0.759		-27.00	
2	-44.00		-0.936		-44.00	
AGI local clusters		0.004		0.014		0.004
1	27.00		0.806		27.00	
2	-23.00		-0.759		-23.00	
3	-67.00		-1.70		-67.00	
Digestive signs D0		0.016		0.009		0.016
NO	11.00		0.186		11.00	
YES	-37.00		-0.989		-37.00	
Digestive signs D3		0.833		0.910		0.833
NO	-28.00		-0.874		-28.00	
YES	-27.50		-0.666		-27.50	
IAP D0 ≥ 12 mmHg		0.046		0.098		0.045
NO	-22.00		-0.443		-22.00	
YES	-46.50		-0.989		-46.50	
IAP D3 ≥ 12 mmHg		0.099		0.132		0.099
NO	-25.00		-0.765		-25.00	
YES	-49.00		-1.21		-49.00	
Enteral nutrition D3		0.528		0.774		0.528
NO						
YES						
Number of ATBs D0		0.007		0.052		0.007
1 (n=0)						
2 (n=16)						
> 2 (n = 22)						
Number of ATBs D3		0.209		0.343		0.209
1 (n = 15)						
2 (n = 16)						
> 2 (n = 8)						

ATB : Antibiotics

FIGURES

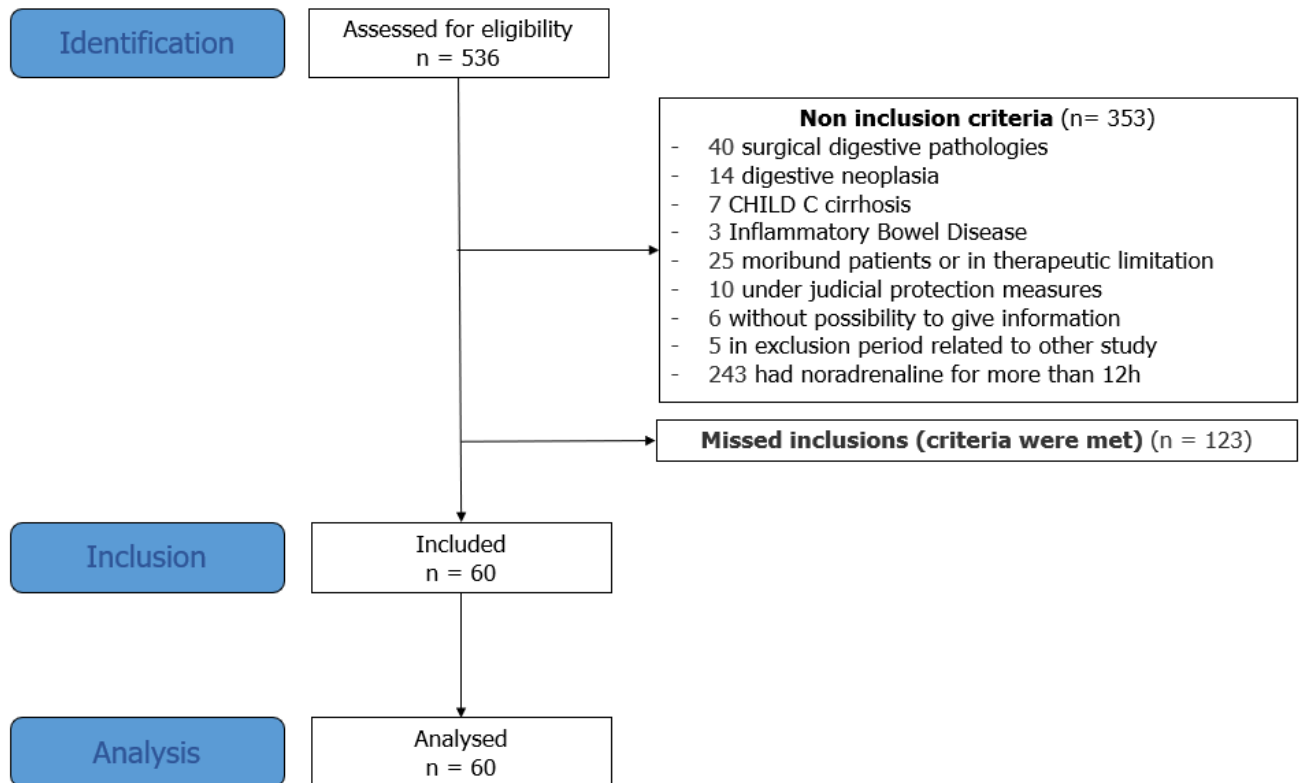


Figure 1. Flow-chart of the study

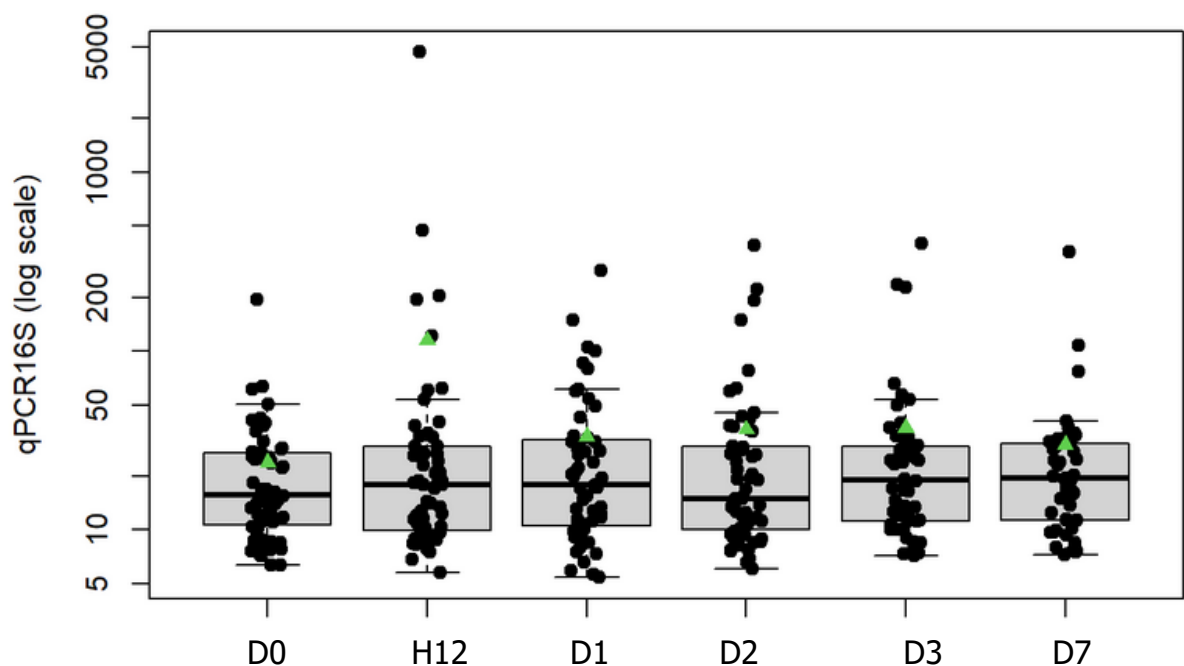


Figure 2. Evolution of qPCR 16S rDNA during the first 7 days of septic shock

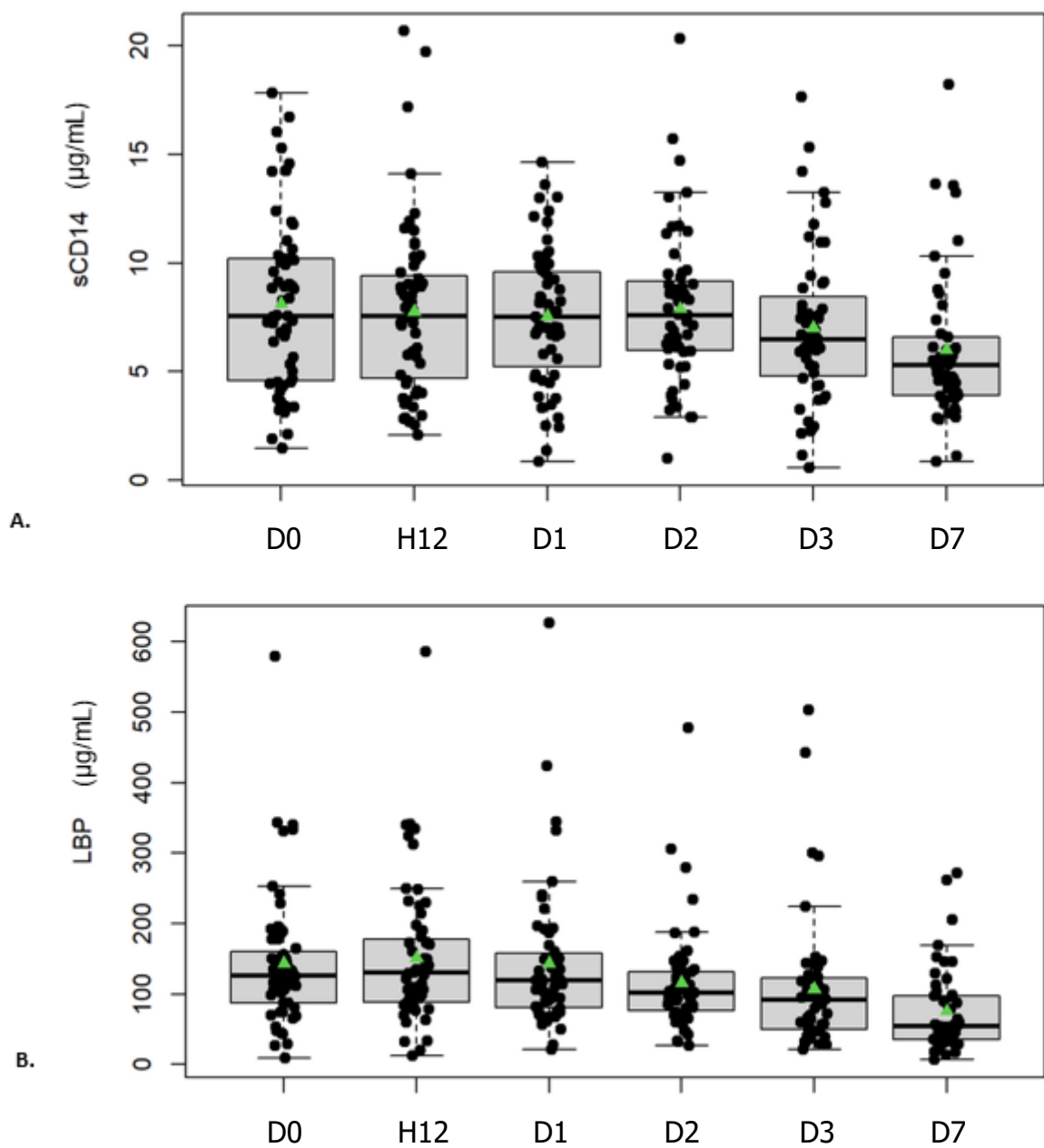


Figure 3. Evolution of sCD14 (fig 3A.) and LBP (fig 3B.) during the 7 first days of septic shock

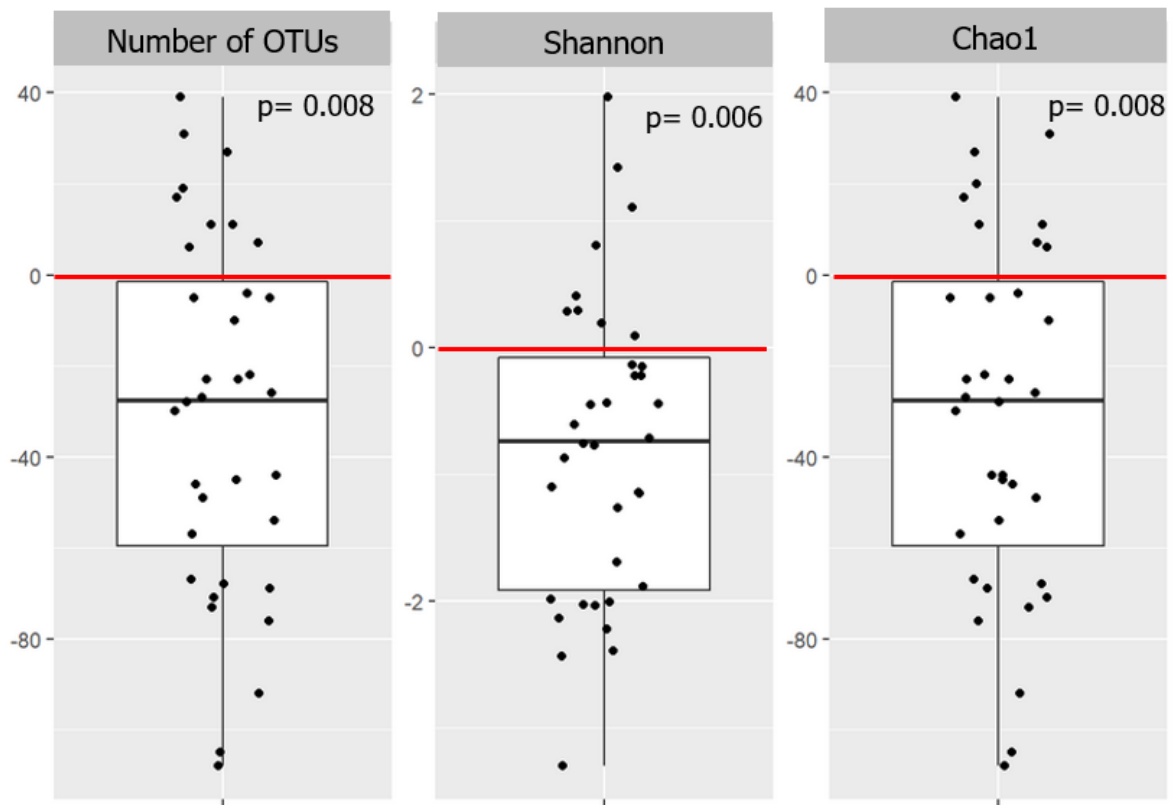


Figure 4. Evolution of alpha diversity during the 7 first days of septic shock. *The patient is his own control, the red line corresponds to the diversity at D0 and each point represents the variation of the diversity for each patient.*

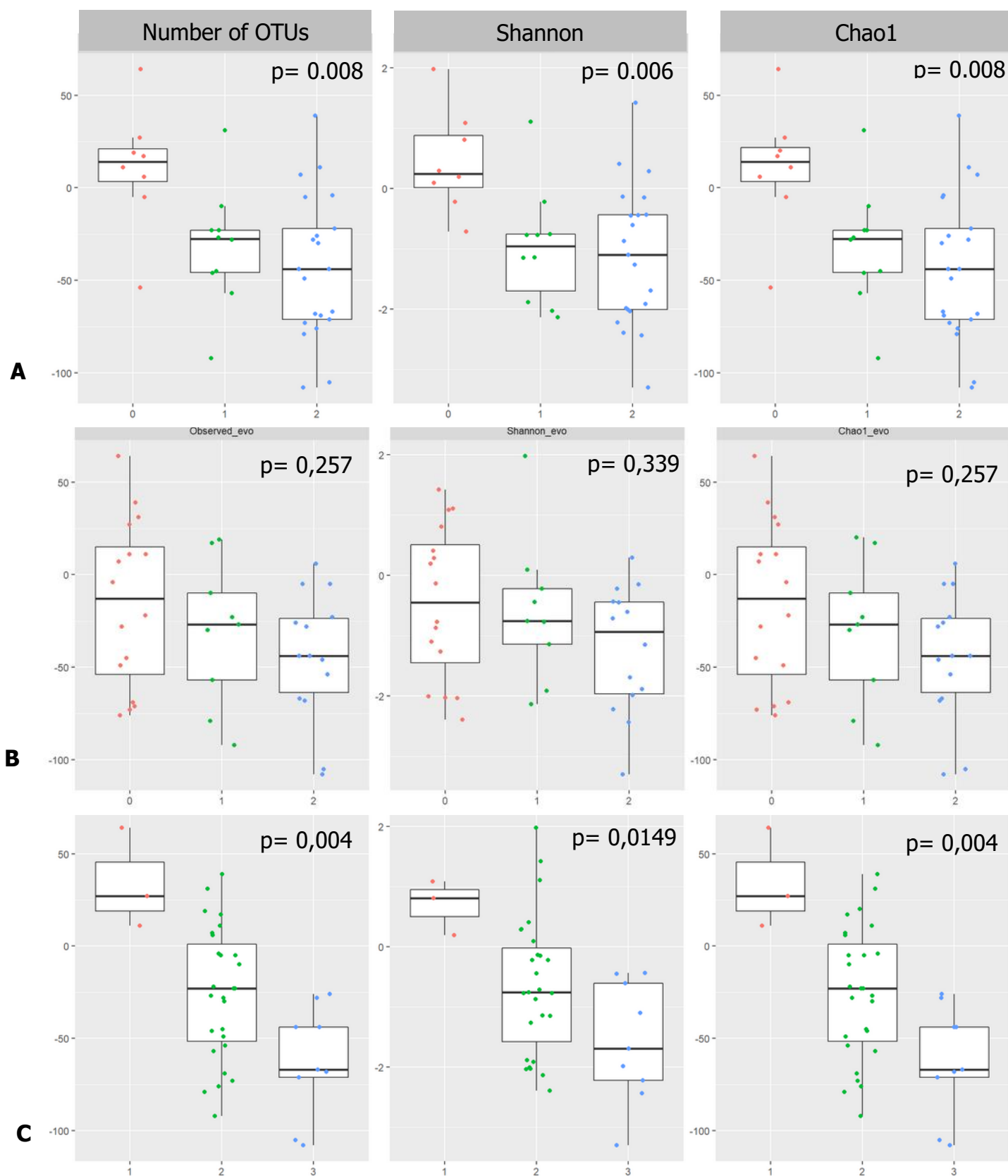


Figure 5. Evolution of the alpha-diversity according to the local AGI score during the 7 first days of septic shock. **5A.** at D0 **5B.** at D3 **5C.** during the stay (cluster)

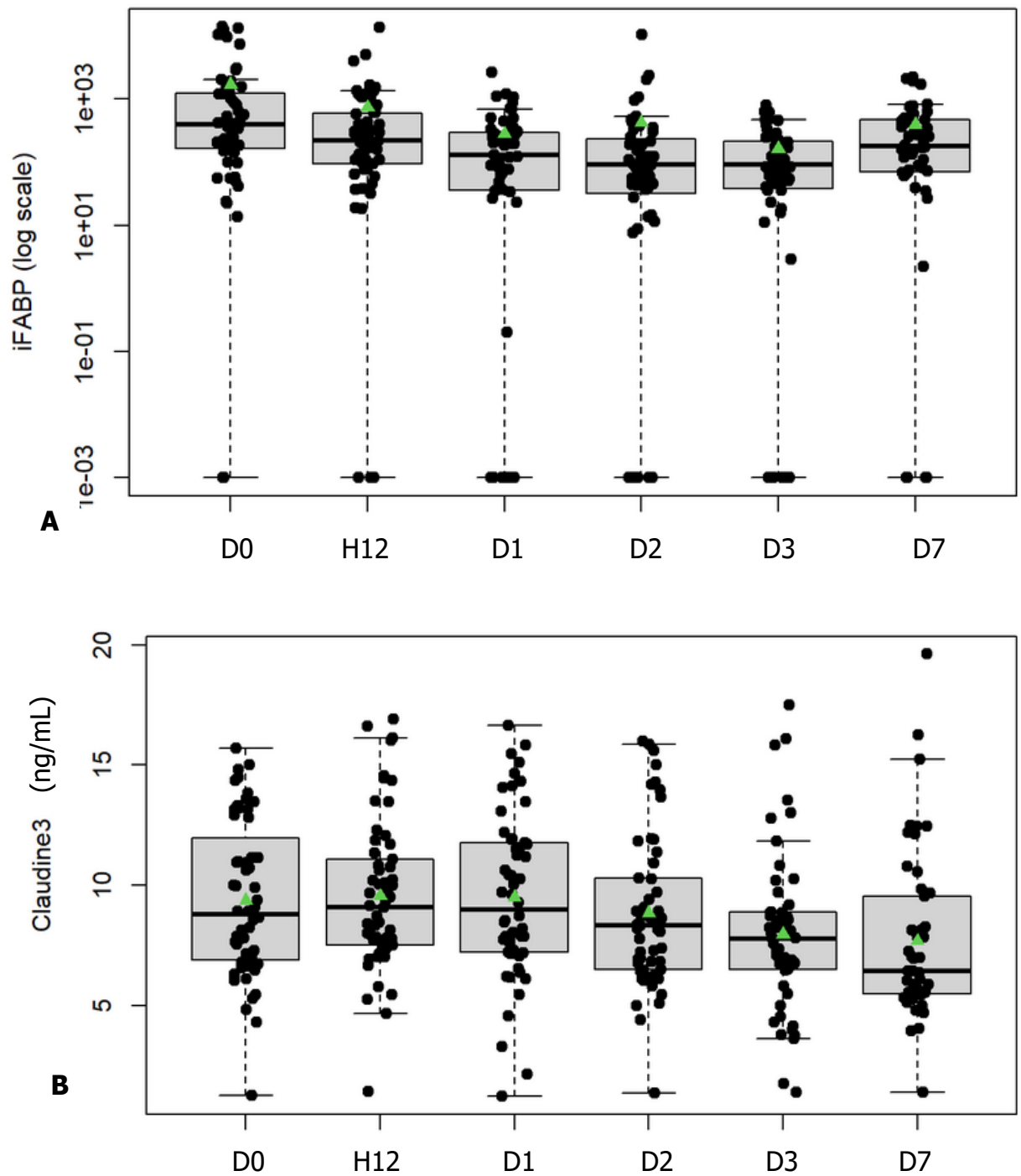
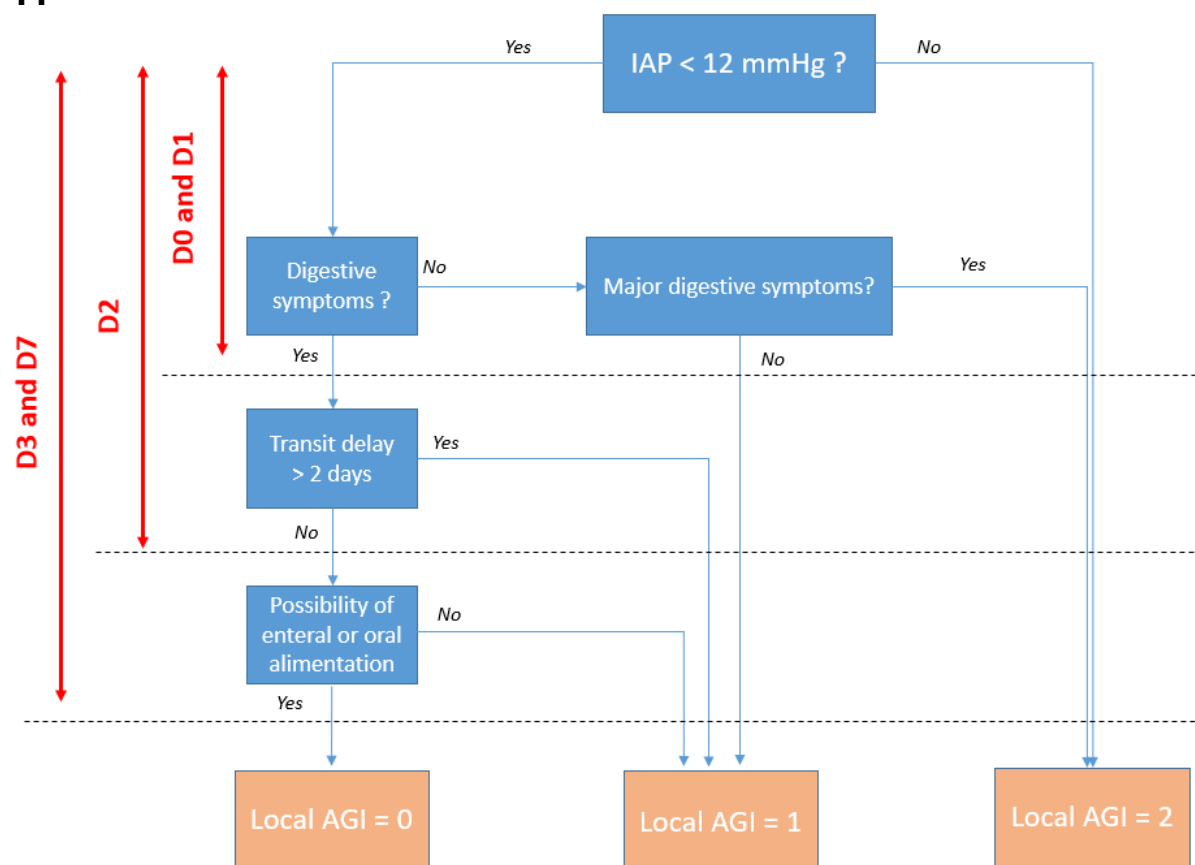


Figure 6. Evolution of intestinal barrier biomarkers during the 7 first days of septic shock: I-FABP (fig. 6A) and Claudin-3 (fig 6B.)

ANNEXES

Appendix 1. Local AGI score



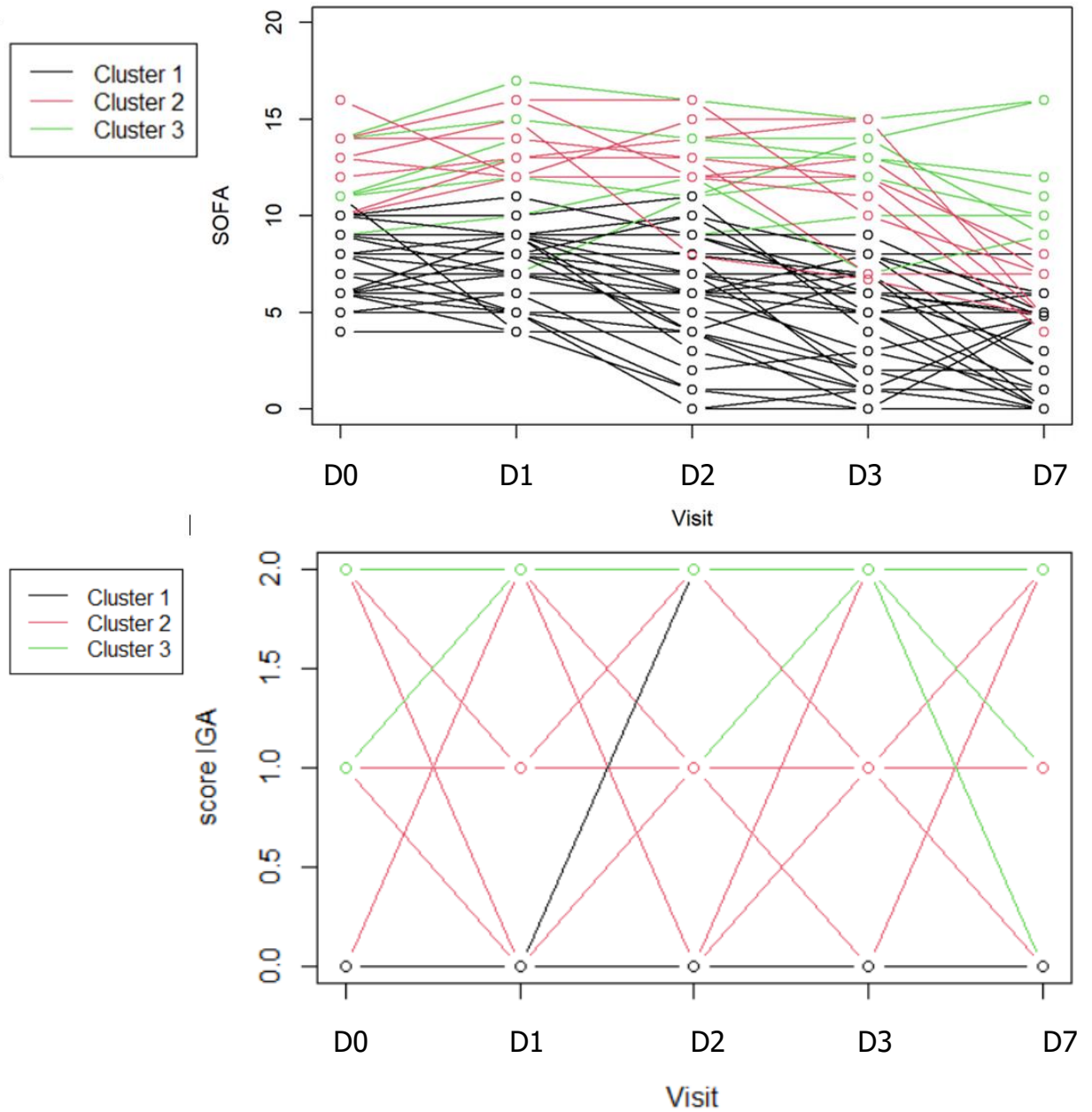
IAP = Intra-abdominal pressure

Digestive symptoms were : vomiting, gastric residual volume > 200mL in a single measurement, diarrhea > 250 mL/day, gastro-intestinal bleeding, intestinal dilatation on imaging, paralytic ileus or abnormal bowel sounds < 5/min or > 35/min.

Major digestive symptoms were the same except abnormal bowel sounds and transit delay.

Appendix 2. Clustering

A patient cluster corresponds to a grouping of patients with a similar evolution during the stay for a given criterion. Clustering was performed for SOFA score and for AGI local score with the K-MEANS method, giving 3 clusters for each criterion, with increasing severity.

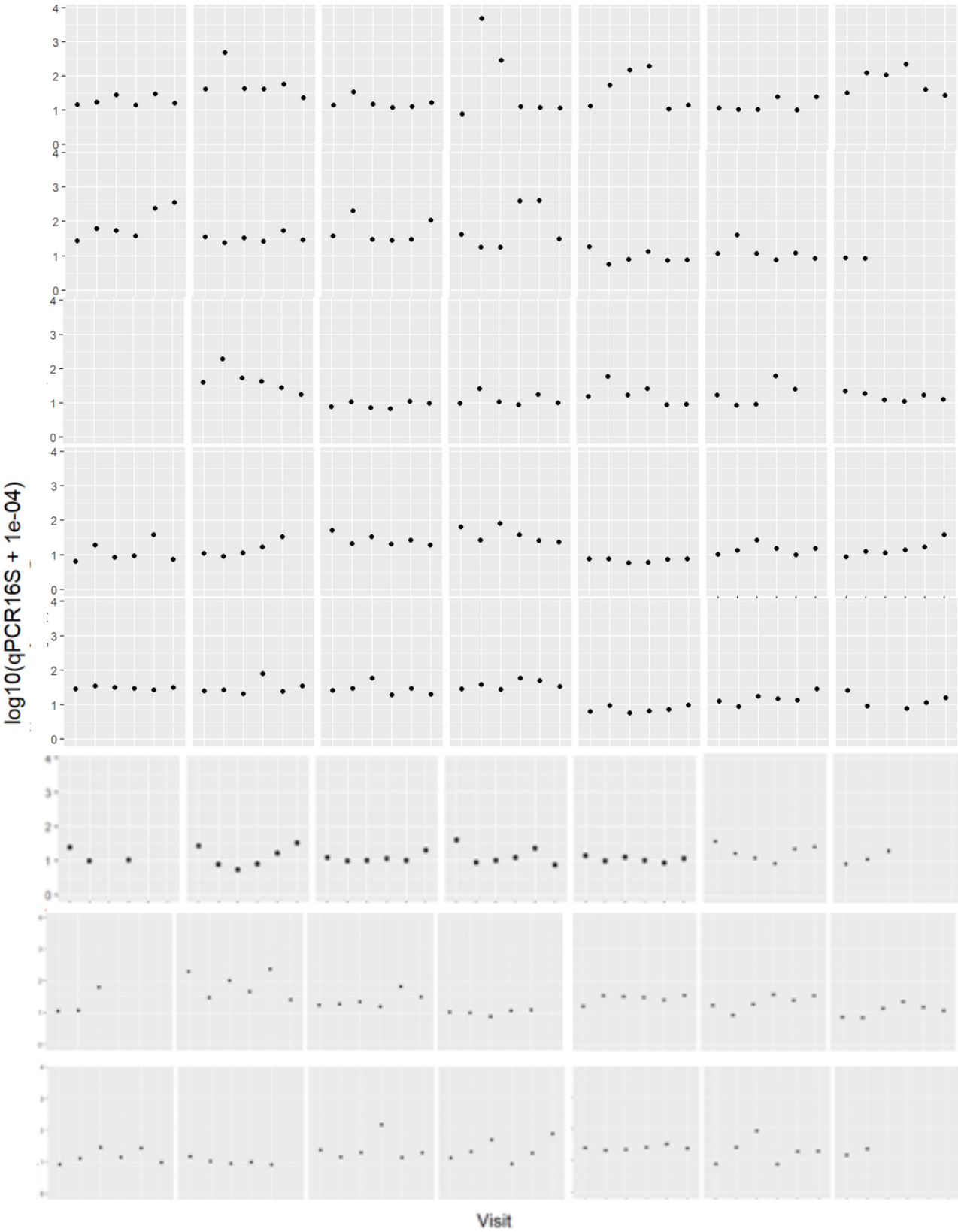


Appendix 3. Description of subgroups

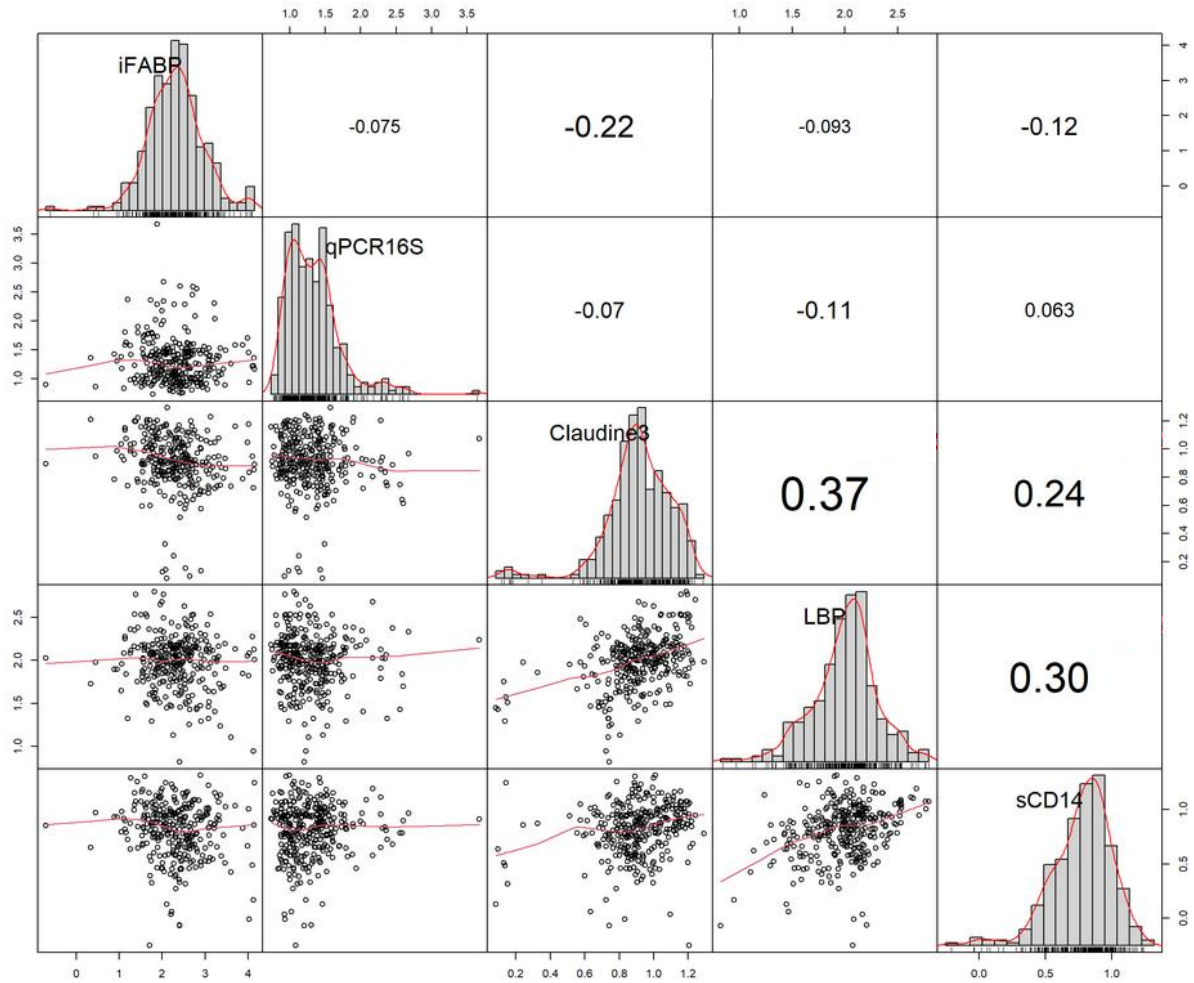
Severity				
SOFA score at D0 (SOFA D0)	Low (≤ 7) n = 20	Moderate (8-13) n= 34	High (≥ 14) n = 6	
SOFA cluster	Cluster 1 n = 42	Cluster 2 n=9	Cluster 3 n=9	
IGS II	Low (< 50) n =7	Moderate (50-75) n=19	High (> 75) n=34	
Characteristics of the infection				
Infected site	Lung n=21	Urinary tract n=16	Digestive tract n=8	Other n=15
Bacteriema at initial infection	Yes n=26	No n=34		
Digestive conditions				
Digestive symptoms at D0	Yes N=40		No N= 19	
Digestive symptoms at D3	Yes N= 29		No N=19	
IAP D0	Normal (≤ 12 mmHg) N=29		High (> 12 mmHg) N=23	
IAP D3	Normal (≤ 12 mmHg) N=29		High (> 12 mmHg) N=17	
Local AGI score at D0	Stage 0 n= 14	Stage 1 n= 14	Stage 2 n= 31	
Local AGI score at D3	Stage 0 n=21	Stage 1 n= 10	Stage 2 n=25	
Local AGI score cluster	Cluster 1 n= 7	Cluster 2 n= 36	Cluster 3 n= 17	
Nutritional status at D3	Yes (enteral or oral) n=48		No (parenteral or death) n=12	

Cluster (SOFA and AGI local score) reflects the evolution profile during the stay

Appendix 4. Evolution of qPCR 16S rDNA for each patient during the 7 first days of septic shock



Appendix 5. Study of the correlation between the different biomarkers by calculating the Spearman coefficient.



*The Spearman coefficient (**rs**) that links 2 biomarkers is the value written at the intersection of the right side of the boxes corresponding to these biomarkers. The values of the different biomarkers are placed on a logarithmic scale to the left of the boxes corresponding to the biomarkers.*

16S rDNA was not associated with iFABP (**rs** = -0.075), claudin-3 (**rs** = -0.07), LBP (**rs** = -0.11) or sCD14 (**rs** = 0.063). I-FABP was not associated with 16S rDNA, claudin-3 (**rs** = -0.22), LBP (**rs** = -0.09) or sCD14 (**rs** = -0.12). Claudin-3 was not associated with 16S rDNA, iFABP, LBP (**rs** = 0.37) or sCD14 (**rs** = 0.24). LBP was not associated with 16S rDNA, iFABP, claudin-3 or sCD14 (**rs** = 0.30).

SERMENT

- *En présence des Maîtres de cette école, de mes chers condisciples et devant l'effigie d'Hippocrate, je promets et je jure, au nom de l'Être suprême, d'être fidèle aux lois de l'honneur et de la probité dans l'exercice de la médecine.*
- *Je donnerai mes soins gratuits à l'indigent et n'exigerai jamais un salaire au-dessus de mon travail.*
- *Admis dans l'intérieur des maisons, mes yeux ne verront pas ce qui s'y passe, ma langue taira les secrets qui me seront confiés, et mon état ne servira pas à corrompre les mœurs, ni à favoriser le crime.*
- *Respectueux et reconnaissant envers mes Maîtres, je rendrai à leurs enfants l'instruction que j'ai reçue de leurs pères.*
- *Que les hommes m'accordent leur estime si je suis fidèle à mes promesses. Que je sois couvert d'opprobre et méprisé de mes confrères si j'y manque.*

Résumé

Introduction : Plusieurs théories présentent la dysfonction intestinale comme moteur de la défaillance d'organe dans le choc septique. Notre hypothèse est que le choc septique entraîne une insuffisance intestinale aiguë entraînant des altérations de la fonction barrière de l'intestin et du microbiote intestinal favorisant la translocation bactérienne.

Matériel et méthodes : Il s'agit d'une étude observationnelle prospective, monocentrique, chez 60 patients durant les 7 premiers jours du choc septique.

L'évaluation clinique de la fonction digestive reposait sur les critères de l'ESICM.

La translocation bactérienne était évaluée par la qPCR ADNr 16S, le LBP et le sCD14.

L'altération de la paroi intestinale était évaluée par l'I-FABP et la Claudine-3.

Le microbiote fécal était étudié à J0 et J7.

Résultats : 90% des patients en choc septique ont une PCR ADNr 16S positive (>12.25 copies/ μL) au moins une fois durant les 7 premiers jours du choc septique et plusieurs patients ont eu ce critère à plusieurs reprises.

La cinétique des différents biomarqueurs n'était pas associée aux caractéristiques du choc septique (foyer infectieux, bactériémie...), la symptomatologie digestive ou le support nutritionnel à J3. Aucune corrélation forte entre ces biomarqueurs n'a été montrée.

Le taux d'I-FABP était associé à la positivité de la PCR ADNr 16S ($> 12,25$ copies/ μL) et à la mortalité à J7.

La diversité du microbiote intestinal diminuait au cours des 7 premiers jours du choc septique.

Conclusion : La translocation bactérienne semble fréquente au cours du choc septique. La PCR ADNr 16S est un marqueur prometteur pour lequel un seuil ayant un intérêt clinique doit être défini.

Mots-clés : translocation bactérienne, perméabilité intestinale, microbiote intestinal, réanimation, choc septique, PCR ADNr 16S, I-FABP, Claudine-3