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Full Length Research Paper

Metabolic markers as possible diagnostic tools to distinguish between Gram positive and Gram negative septicaemia in baboons

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Septicemia is a disease with high mortality and morbidity. Most patients die within 48 h after infection because directed treatment can only start after the bacterium is identified as gram positive or gram negative. This may take up to 72 h. Early identification of the causative pathogen can therefore decrease the high mortality rate following infection. The aim was to identify possible metabolic markers of gram positive and gram negative septicemia in appropriately infected baboons. Ten baboons, anaesthetised with ketamine hydrochloride and pentobarbitone for 24 h, were used in this pilot study. Blood and urine samples were collected at various intervals during the 24 h. Four baboons were inoculated with *S. pyogenes* H305 and four with *E. coli* O111:B4. Two baboons served as controls. Acyl carnitine, amino acids, organic acids, very long chain fatty acids, glucose, pyruvate and lactate were measured in blood plasma and in urine using standardised methods. No metabolic markers could distinguish between gram positive and gram negative septicemia. α-Amino-adipic acid, citramalic acid and xanthurenic acid, produced only by bacteria, show promise. Alanine and glycine increased significantly over 24 h and can be used as diagnostic markers and perhaps as markers of disease progression. In conclusion, (in PDF file it is conclusively) metabolites can be used to diagnose septicemia and possibly its progression, but not to distinguish between gram positive and gram negative septicemia.

Key words: Septicaemia, baboon, Gram negative, Gram positive, metabolic marker.

INTRODUCTION

Septicaemia is caused by either Gram positive or Gram negative bacteria in the circulation (Martin et al., 2003) where they actively multiply and overwhelm the immune system. This can then rapidly develop into septic shock and death (Van Amersfoort et al., 2003). Over the last 22 years, the incidence of septicaemia increased at a rate of approximately 8.7% per year (Martin et al., 2003) in spite of vast technical development in intensive care units and advanced support treatments (Bone, 1993; Riedemann et al., 2003). Mortality depends on the type, site and extent of the organism involved, how early the patient is hospitalized, diagnosed and how quickly the appropriate treatment is started (Martin et al., 2003). Organ failure also determines the prognoses; the risk of death increases with increasing numbers of failing organs (Das, 2000; Levy et al., 2003; Watson et al., 2003). It is important to note that more than 50% of patients with sepsis die during the first 48 h after infection (Bone, 1993).

The problem to adequately manage patients with septicaemia and the reason for the relatively high mortality is to a large extent the delay to correctly treat septicaemia.

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Abbreviations: CRP, C-reactive protein, **PCT,** procalcitonin, **PCR,** polymerase chain reaction.

Current standard microbiological techniques identify infecting organisms after culture of a blood sample/isolate in conditions suitable for replication of the infectious bacterium. This may be difficult if patients already receive antibiotics (Gao et al., 2008). Preliminary classification is usually done within 24 h, with full species identification and antimicrobial sensitivity data becoming available only 48 to 72 h after blood sampling. The slowness of the investigation usually mandates the use of broad-spectrum antibiotics while awaiting results (Jansen et al., 2000; Mishra et al., 2006; Gao et al., 2008). Only after it is known if the causing bacterium is Gram positive or Gram negative can the specific antibiotic treatment start. In addition, drug selection not only depends on the causing bacterium, but also on the site or presumed site of infection, the place from where the infection was acquired, the underlying disease status of the patient and possible drug toxicities and drug resistance all of which complicates the matter further (Bone, 1993; Watson et al., 2003).

Biomarkers provide a fresh approach to diagnose infection, to assess its severity and to design a specific treatment response (Mueller et al., 2008). In septicaemia, products of activation of the immune response lead to leukocyte recruitment at the site of infections, where they start an inflammatory response, leading to an increase in biomarkers of the immune system. Established biomarkers, especially procalcitonin (PCT) and to a lesser extent, C-reactive protein (CRP), can improve the diagnostic and prognostic assessment of bloodstream infections. It is, however, important to note that these biomarkers can currently only be used as complementary tools to reinforce a clinical diagnostic workup (Mueller et al., 2008). PCT is currently preferred as a biomarker for sepsis because of several advantages that it has over other inflammatory markers. These include an earlier increase in response to an infection that is maintained even with immunosuppressive medication, a better negative predictive value and a better correlation with outcome (mortality). CRP on the other hand, increases relatively late in the infectious process. It is, however, attenuated by the concomitant use of steroids and may be elevated as a direct consequence of a malignancy rather than from an infection (Dahaba et al., 2006; Müller et al., 2007; Monneret et al., 2008). In addition to clinical and microbiological parameters, PCT may further help differentiate blood contamination from bloodstream infection (Müller et al., 2007; Mueller et al., 2008). PCT levels also increase far less than the CRP levels while the period of unspecific induction is much shorter. PCT is therefore the better choice to diagnose sepsis and infection early after surgery. One must bear in mind that, contrary to previous studies, established cut-off values only indicate the risk to develop complications during the further course of the disease, and not the actual sensitivity or specificity to diagnose sepsis (Meisner et al., 2006).

Limitations of every biomarker include false-positive and false-negative results, and the time kinetics of the test. It is possible to obtain false-positive results in patients post-operatively who may present with very high levels of PCT and CRP and no signs of infection. This is also possible during post-traumatic periods and after mechanical trauma. Emphysema is associated with variable increased levels of CRP, but relatively low PCT levels. Caution is also required in settings where infection with coagulase negative *staphylococci* or certain intracellular bacteria are suspected. These are often associated with low PCT concentrations and may be overlooked using standard assays. PCT and CRP are also independently increased following cardiogenic shock (Meisner et al., 2006; Mueller et al., 2008). A major limitation of these biomarkers is that it cannot determine if the infective organism is Gram positive or Gram negative and also the associated patterns of antibiotic susceptibility (Müller et al., 2007; Mueller et al., 2008).

Several other techniques that can speed up the identification of infecting organisms are at different stages of development. Most of these techniques use the polymerase chain reaction (PCR) or fluorescent *in situ* hybridization. Although these methods are fast and accurate, routine bacteriological analysis still rely on classical culturing techniques which is still the mainstay of distinguishing between Gram positive and Gram negative bacteria (Jansen et al., 2000; Llewellyn and Cohen, 2007; Müller et al., 2007). It is theoretically possible to do PCR-based amplification of sufficient magnitude to detect low copy numbers of DNA sequences, thereby eliminating the requirement for an initial period of standard culture. However, the use of these techniques is limited by difficulties in differentiating between contaminants and non-living or degraded bacteria from clinically relevant isolates. Infrared vibration spectroscopy allows the identification of bacterial specific proteins in whole blood. This emerging technique does not require amplification or extraction of the proteins. Although none of these methods have been evaluated extensively in clinical practice, they may offer potential advantages. First, they can enable the earlier use of antibiotics with narrower spectra, but known efficacy, against a particular organism. Second, they promote better understanding of the heterogeneity of infection in sepsis. Third, they may also enable the use of some of the specific anti-mediator therapies that are being investigated (Gao et al., 2008). It must also be noted that not all pathology laboratories have PCR and/or *in situ* hybridization readily available.

It is clear that the methods currently in use to diagnose septicaemia and to differentiate between Gram positive and Gram negative bacteria is either slow, which delays appropriate treatment, or, the new approaches using biomarkers, PCR or infrared vibration spectrometry are still in their development phase. There is thus still scope to investigate other approaches to quickly diagnose septicaemia and to differentiate between disease causing

bacteria. The hypothesis in this pilot study is that infection with bacteria will quickly affect the metabolism in infected individuals and that this will change the metabolic profile. We further hypothesised that Gram positive and Gram negative bacteria may affect the metabolism in the host differently and that this can be measured in the metabolite profile. We tested the hypotheses in baboons (*Papio ursinus*) that were inoculated with either *E. coli* (O111:B4; Gram negative) or *Streptococcus pyogenes* (H305; Gram positive). It was postulated that "different microbes may induce a distinct response in various organs, resulting in a variable repertoire of circulating biomarkers and mediators" (Mueller et al., 2008). Blood and urine were collected at regular intervals during the first 24 h after infection and the metabolite profile measured.

MATERIALS AND METHODS

Study design

Ten healthy baboons (*Papio ursinus*), weighing between 5 and 9 kg, were used in a study approved by the Ethics Committee of the North-West University (project 03D15) in accordance with the National Code for animal use in research, education, diagnosis and testing of drugs and related substances in South Africa (South African Medical Research Council, 2004). Ketamine hydrochloride (8 to 10 mg/kg) was used to enable handling. The baboons were intubated and connected to a ventilator (FiO_2 of 0.25 ± 0.02, Servo ventilator 900C, Siemens-Elma, Sweden). General anaesthesia was maintained for 24 h with pentobarbitone (2 to 5 mg/kg) and halothane. Blood for analysis was collected from the femoral artery through a permanent catheter that was placed to ease blood collection. The baboons were then infused with either saline (sham group; n = 2), serum resistant *E. coli* O111:B4 (1.5×10^7 CFU/kg in saline; n = 4) or *S. pyogenes* H305 (1.5×10^8 CFU/kg in saline; n = 4) over 2 h, followed by a monitoring period of 22 h (total length of experiment 24 h). Blood samples were collected at time 0 and again after 4, 8, 16 and 24 h. Urine samples were collected before inocculation and again after 4, 8, 12, 16, 20 and 24 h using an urinary catheter placed in the bladder. Urine samples were immediately frozen at -80°C. While anaesthetized, the animals received an infusion of at least 5 ml/kg/h saline to keep pulmonary wedge pressure stable in order to avoid hypovoleamia as a complicating factor. The pulmonary wedge pressure was measured continuously using a catheter placed in the pulmonary artery. At the end the treatment, the animal was euthanised by intravenous infusion of 150 mg/kg pentobarbitone.

Analysis

Electron spray tandem mass spectrometry, Micromass Quatro micro™ API (Chemetrix, Midrand, South Africa) was used to quantify acylcarnitines in butylated urine samples as described in Matern (2008). Amino acid quantification was done on serum and urine samples. The amino acids were extracted using the EZ: faast™ kit (Separations KGO-7166, Randburg, South Africa) according to the instructions of the manufacturers. The amino acids were analysed and quantified using a Hewlett-Packard model 6890/5973 GC–MS system (Chemetrix, Midrand, South Africa) equipped with a Zebron ZB-AAA GC amino acid analysis GC column (10 m x 0.25 mm) as described by Duran (2008).

Organic acids in urine were extracted and quantified as described (Rinaldo, 2008). A Hewlett-Packard model 5880/5988A GC–MS system (Chemetrix, Midrand, South Africa) equipped with a MN30962-52 fused silica capillary column (32 m x 0.32 mm x 0.25 µm) (SMM Instruments, Johannesburg, South Africa). Very long chain fatty acids in plasma was extracted and quantified using a Hewlett-Packard model 6890/5973 GC–MS system (Chemetrix, Midrand, South Africa) equipped with a 122-0132DB 1ms capillary column (30 m x 0.25 mm x 0.25 µm) (SMM Instruments, Johannesburg, South Africa) as described (Wanders and Duran, 2008). The glucose, lactate and pyruvate levels in blood were determined using standard techniques that are available in Chemical Pathology laboratories.

Statistical analysis

A total of 40 metabolites in plasma and 186 in the urine were analysed. Most of the metabolites, when statistically analysed, had no pattern in time and group and are therefore not discussed. All the results were analyzed as mixed repeated measures using the Statistical Analysis System (SAS) procedures. This method is described in Littell et al. (1998). If the Analysis of variance (ANOVA) was significant at $P < 0.02$ between group and time X group, the results were further analysed using Tukey's test (Studentized range statistic) to assess if time after inoculation played a role in changes in metabolite concentration and if there were differences between specific groups. Again, only those metabolites that differed significantly at $P < 0.02$ are discussed. Since the data were not normally distributed, the statistical analyses were done on the log-values.

This high level of significance ($P < 0.02$) was chosen for two reasons. First, it was to markedly reduce the number of metabolites of interest. Second, the high level of significance will at least in part negate the fact that there were only a few baboons per group.

RESULTS

The values given in all the figures are the original measurements. The box-plots illustrate the 25th and the 75th percentile as a block. The mean value is given and the minimum and maximum values as bars. There were no significant differences between the control, the Gram positive and the Gram negative group at time 0 in all the metabolites that were measured in plasma and urine. It is possible that ventilation with O_2 may have influenced the metabolite profile. We believe that, if it was the case, the differences that we measured are still valid because the control group was also intubated and ventilated similar to the groups that were inoculated with the bacteria.

Metabolites of interest in blood plasma

α-Amino-adipic acid levels differed significantly between groups and in time x group (ANOVA $P < 0.02$ and $P < 0.002$, respectively). The concentration increased significantly at 16 and 24 h in both Gram positive and Gram negative baboons. No α-amino-adipic acid was measured in the control animals. There were no significant differences between the plasma levels in the Gram positive and Gram negative groups (Figure 1).

Both alanine and glycine differed significantly between groups (ANOVA $P < 0.0004$ and $P < 0.0001$, respectively)

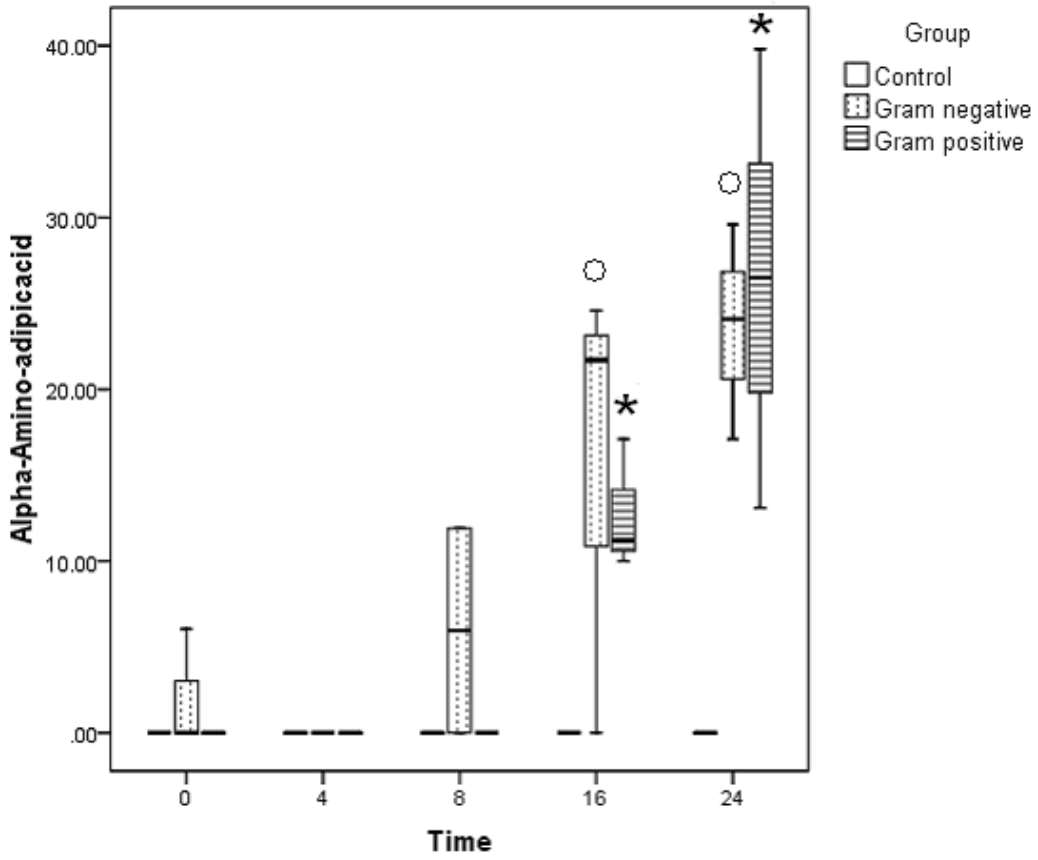

Figure 1. Changes in the levels of plasma α-amino-adipic acid (mmol/mol creatinine) over time (h) in the control, Gram positive and Gram negative groups ($^{O}P < 0.008$ and $P < 0.0001$ at 16 and 24 h respectively, control versus Gram negative and $*P < 0.02$ and $P < 0.0001$ at 16 and 24 h, control versus Gram positive).

and in time x group (ANOVA $P < 0.0001$ and $P < 0.002$, respectively). The control group also differed significantly from the experimental groups (Figures 2 and 3).

Metabolites of interest in urine

Citramalic (2-methyl malic acid), xanthurenic acid, lactic acid and 2-hydroxybutyric acid all differed significantly between groups and in time x group (ANOVA $P < 0.02$). Of these, only the changes in citramalic acid and xanthurenic acid are worth mentioning. Although the changes in the other metabolites differ significantly from control animals and not between Gram positive and Gram negative animals, the changes followed no distinguished pattern and will not be discussed.

Citramalic acid and xanthurenic acid levels in urine in the control group were significantly less than in the experimental groups after 4 and 12h following inoculation with Gram positive and Gram negative bacteria (Figures 4 and 5). There were no significant differences between the urine levels in the Gram positive and Gram negative groups

DISCUSSION

It is important to note that there were no significant differences at time 0 in the levels of metabolites of interest between the three groups. The levels in the control group also remained almost unchanged during the 24 h of the study. The changes that were measured in the metabolites of interest in the experimental groups can therefore not be attributed to the anaesthesia. It is also reasonable to assume that the changes in metabolite levels in the experimental groups can be attributed to inoculation of the baboons with bacteria. Also, conditions such as environment, anaesthesia, fluid replacement and medical care of all the animals were the same. This could therefore not have influenced the results obtained in the experimental groups.

There were a few metabolites in plasma that showed promise as possible metabolic markers of early (<24 h) diagnosis of septicaemia since their levels in plasma

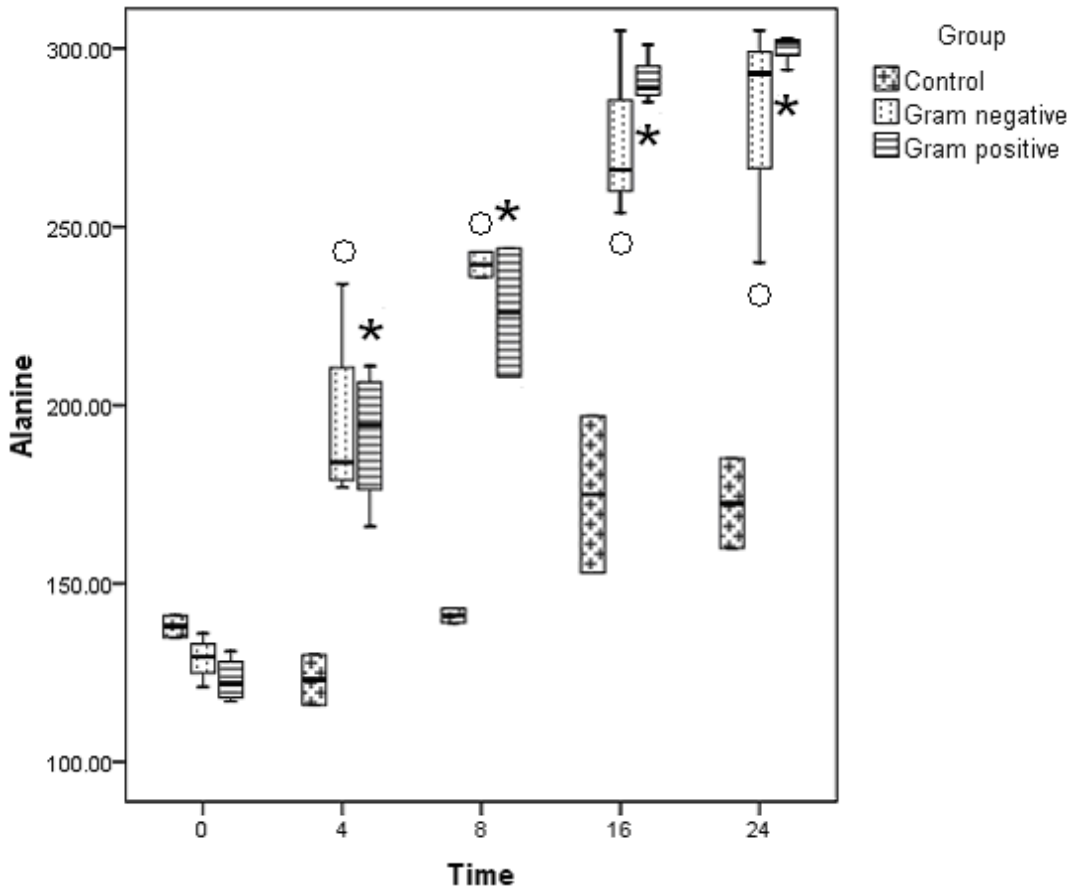

Figure 2. Changes in the levels of plasma alanine (mmol/mol creatinine) over time (hours) in the control, Gram positive and Gram negative groups ($^{O}P < 0.0001$ at 4 to 24 h, control versus Gram negative and *P < 0.0001 at 4 to 24 h, control versus Gram positive).

increased as early as four to eight hours after the baboons were infected with the bacteria. They include α-amino-adipic acid, alanine and glycine (Figures 1 to 3). These findings may be of particular importance since it is generally believed that current methods can only measure detectable levels of molecular markers after 24 hours of infection (Jansen et al., 2000; Müller et al, 2007; Monneret et al., 2008; Gao et al., 2008).

The changes in α-amino-adipic acid are of special interest since it could not be measured in the plasma of the control animals over the 24 h of the study (Figure 1). This is so because α-amino-adipic acid is an intermediate of lysine biosynthesis in bacteria (Elpeleg et al., 1990; Capo-Chichi et al., 2000). It is very rarely measured in the plasma of humans and can therefore be used as a differential diagnosis of septicaemia. One must bear in mind that α-amino-adipic acid can be formed in patients with an α-amino-adipic aciduria defect. This defect is very rare and these patients are usually diagnosed early in life (Fischer et al., 1974; Elpeleg et al., 1990).

α-Amino-adipic acid, alanine and glycine cannot be used as markers to distinguish between Gram positive and Gram negative septicaemia. The changes that were measured over time did not differ significantly between the two groups infected with the bacteria (Figures 1 to 3). The increases over the 24 h study period strongly suggest that alanine and glycine can be used as markers for the progression of septicaemia, at least in the early stages after infection since they increased during the 24 h of the study. In this regard, alanine can be a more important marker since its magnitude of increase was larger than that of glycine (Figures 2 and 3). α-Amino-adipic acid can also be used to monitor progression of septicaemia, especially at later stages (Figure 1).

It is not certain whether the increases in α-amino-adipic, alanine and glycine can be used to monitor the effectiveness of treatment, that is, will the increase over time be less when patients are treated? This can only be investigated in patients with septicaemia. Levels that remain constant following treatment, or even decrease, can indicate effectiveness of treatment.

It is noteworthy that both citramalic acid and xanthurenic acid were not present in the urine of the control animals, but increased markedly, especially citramalic acid, over time in both Gram positive and Gram negative baboons (Figures 4 and 5). However, care must be taken

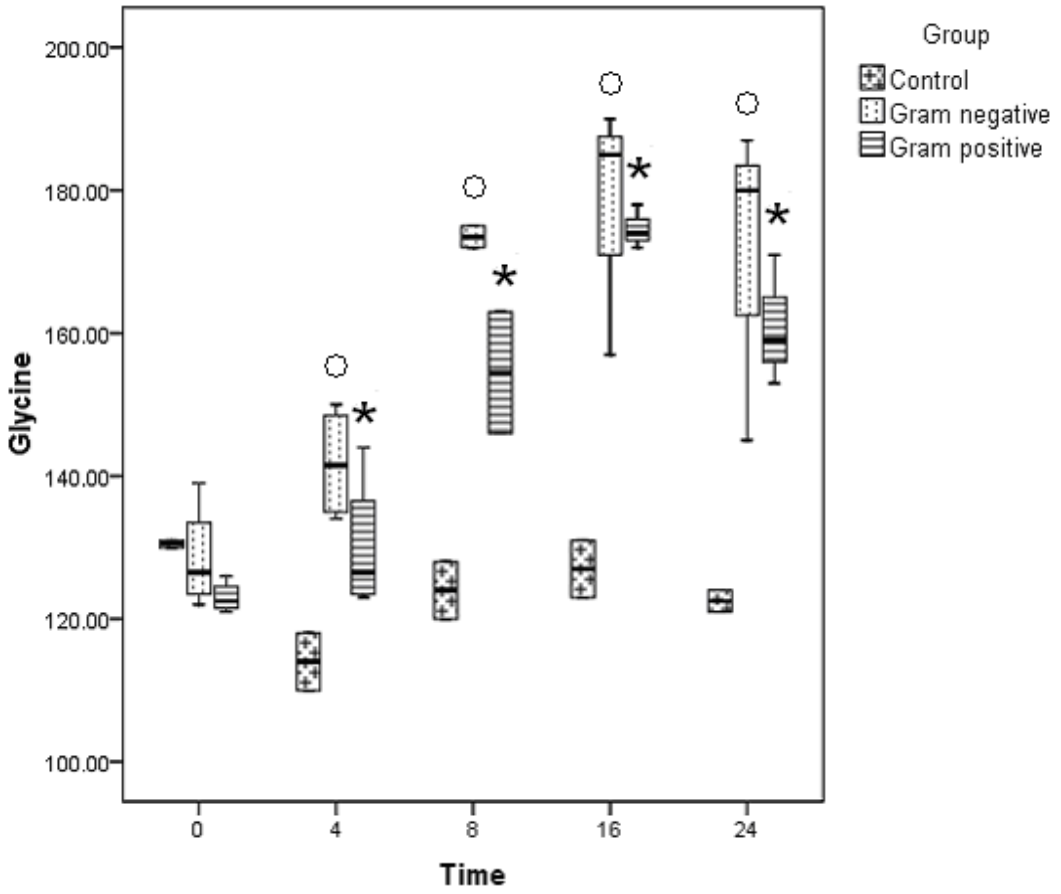

Figure 3. Changes in the levels of plasma glycine (mmol/mol creatinine) over time (hours) in the control, Gram positive and Gram negative groups ($^{O}P < 0.0007$ at 4 h and $P < 0.0001$ at 8 to 24 h, control versus Gram negative and $*P < 0.03$ at 4 h, $P < 0.002$ at 8 h and $P < 0.0001$ at 16 to 24 h, control versus Gram positive).

since the increases were not as extensive as that of, for example, alanine in plasma (Figure 1). It is also evident that citramalic acid and xanthurenic acid cannot be used to distinguish between Gram positive and Gram negative bacteria, similar to those metabolites measured in the plasma. The increase in citramalic acid and xanthurenic acid levels in urine can be used as possible metabolic markers of progression of septicaemia (Figures 4 and 5). Since the differences are perhaps not as highly significant as, for example, alanine in plasma, care must be taken if these metabolites are to be used as such markers. Further investigation into its suitability is called for, simply because collection of urine is non-invasive as opposed to drawing blood.

Citramalic acid is not present in the urine of baboons since no citramalic acid was measured in the urine of the control animals during the 24 h of the study (Figure 4). Citramalic acid is currently used as a marker for autism and was also identified in the cerebrospinal fluid of patients with bacterial meningitis (Perlman and Carr, 1984; Shaw et al., 1995). False diagnosis of sepsis in autism cannot happen because of the typical behaviour of autism patients. Bacterial meningitis is caused by bacteria and this may complicate the diagnosis of sepsis proper if citramalic acid is also excreted in the urine of patients with bacterial meningitis. This underlies the importance that molecular markers must be used in conjunction with clinical symptoms and pathological findings (Gao et al., 2008). Citramalic acid is an intermediate of the anaerobic metabolism of glutamate in bacteria (Howell et al., 1999). In this pathway, glutamate is metabolised to pyruvate and acetate (Barker, 1967). The significance of citramalic acid in the baboons cannot be established from the results of this study, but needs to be investigated in order to assess its use as a molecular marker in the diagnosis of septicaemia.

Xanthurenic acid is rarely excreted by healthy humans. Xanthurenic acid is an intermediary metabolite of L-tryptophan catabolism (Han et al., 2001) and is usually used as a metabolic marker of a Vitamin B_6 deficiency (Liu et al., 1996; Anderson, 2003). The significance of xanthurenic acid in the urine of the infected baboons

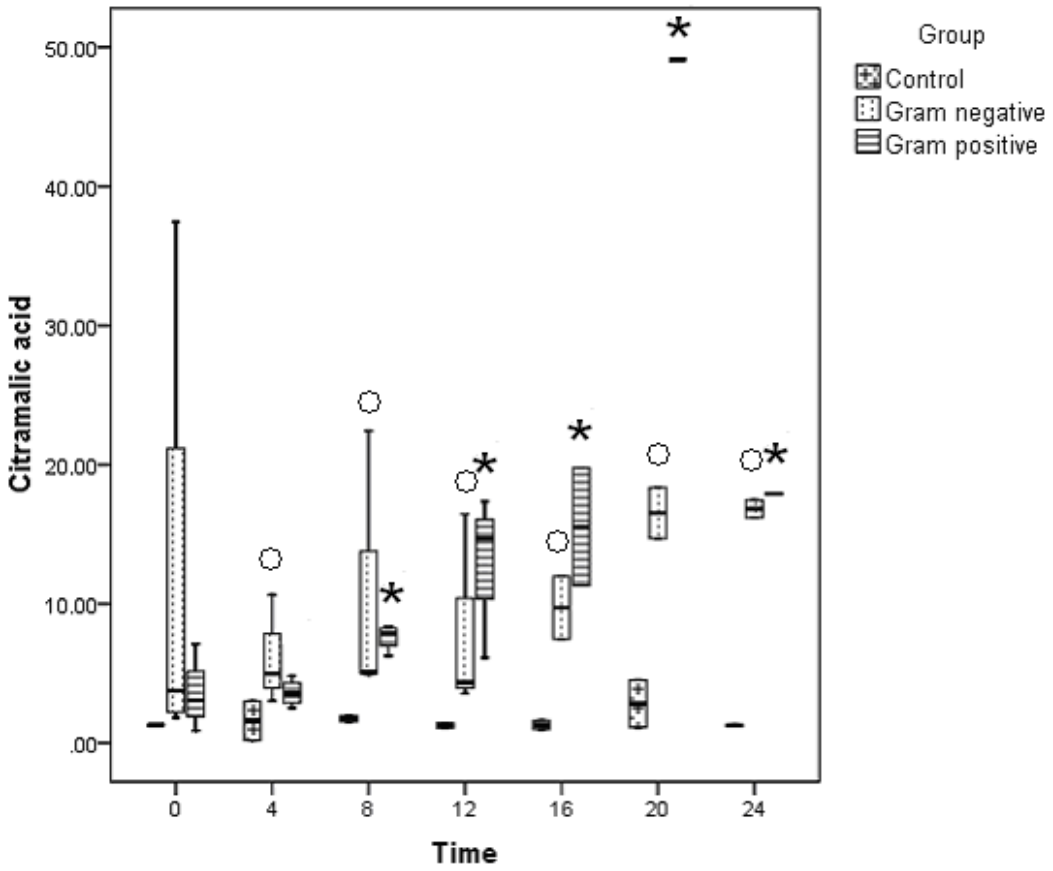

Figure 4. Changes in the urine levels of citramalic acid (mmol/mol creatinine) over time (hours) in the control, Gram positive and Gram negative groups ($^{O}P < 0.04$ at 4 h, $P < 0.02$ at 8 h, $P < 0.03$ at 12 h, $P < 0.01$ at 16 h, $P < 0.007$ at 20 h and $P < 0.001$ at 24 h, control versus Gram negative and $*P < 0.03$ at 8 h, $P < 0.001$ at 12 and 16 h, $P < 0.0005$ at 20 h and $P < 0.005$ at 24 h, control versus Gram positive).

needs to be established before its use as a molecular marker to diagnose septicaemia can be adjudged.

The α-amino-adipic acid that was measured in the plasma, and citramalic acid and xanthurenic acid in the urine are of interest. It is formed only during septicaemia and was not detected in the control animals. This is because they are formed by the bacteria itself. Another important point is that these metabolites were detected as early as four to eight hours after inoculation. It is therefore not farfetched to speculate that it can be used to help to diagnose septicaemia at an early stage, especially if used in conjunction with one another. This should be tested in patients.

It is important to keep in mind that this study must be regarded as a pilot study searching for such markers. Their specificity and sensitivity will have to be determined in patients. Similarly, their possible roles as indicators of progression of infection and as markers of the effectiveness of treatment must be assessed in patients. Since infections are complex, a single marker may not be enough (Mueller et al., 2008) and combinations between molecular markers on the one hand, and/or molecular markers and clinical signs and symptoms and laboratory results needs to be investigated in order to get a definitive diagnosis.

We could not distinguish between baboons infected by Gram positive and Gram negative bacteria by searching for differences in the metabolism of the animals. It may be that the 24 h of study was too short to manifest in a variable metabolic response. This will have to be tested in patients with septicaemia. Perhaps it is not so surprising that we could not distinguish differences in meta-bolism. The main difference between Gram positive and Gram negative bacteria is in their membrane structure (Opal and Cohen, 1999; Levy et al., 2003; Gao et al., 2008). We hoped that the difference in the immune response of the baboons to the infection could affect metabolism differently.

In summary, α-amino-adipic acid, alanine and glycine in plasma and citramalic acid and xanthurenic acid in urine were identified as possible markers to be used for septicaemia. The fact that they also increased during the study strongly suggest that they may be used to assess progression of sepsis. One must bear in mind that these results were obtained in a well controlled experimental setup for only 24 h. Changes after 24 h will have to be

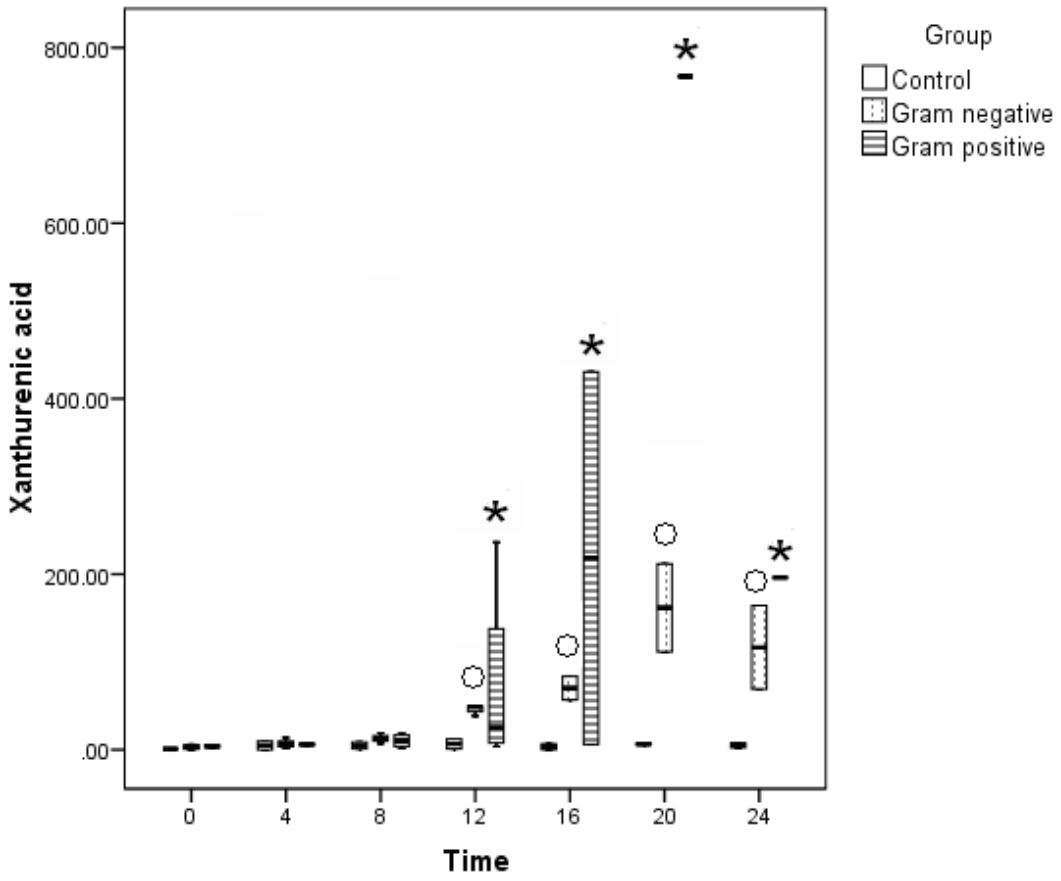

Figure 5. Changes in the urine levels of xanthurenic acid (mmol/mol creatinine) over time (hours) in the control and Gram negative groups ($^{O}P < 0.03$ at 12 h, $P < 0.002$ at 16 h, $P < 0.006$ at 20 and 24 h) and control versus Gram positive groups (*$P < 0.08$ at 12 h, $P < 0.008$ at 16 h, $P < 0.003$ at 20 h and $P < 0.02$ at 24 h).

assessed to give conclusive results, preferably in patients. None of the metabolites that were measured could distinguish between Gram positive and Gram negative septicaemia. This is perhaps not so surprising since the main difference in Gram positive and Gram negative bacteria is in the composition of their outer membrane.

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