Diabetes does not influence activation of coagulation, fibrinolysis or anticoagulant pathways in Gram-negative sepsis (melioidosis)

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Introduction

Activation of coagulation is almost universal in sepsis, regardless of aetiology (1, 2), and is an important contributor to morbidity and mortality (2). Diabetes mellitus is a major risk factor for the development of infection (3, 4), and roughly 20% of all sepsis patients have diabetes as a risk factor (3, 4), regardless of aetiology; yet the influence of diabetes on sepsis and sepsis-related organ dysfunction remains unclear. It is known that diabetes per se is pro-thrombotic and pro-inflammatory, probably as a direct effect of hyperglycaemia (5, 6). Yet, once sepsis is established, the evidence for an influence of diabetes on pathology and prognosis is contradictory, with some studies finding no effect on mortality (7–9), some finding a higher mortality rate (10–12), and some a lower mortality rate (3, 9, 13).

Melioidosis (Burkholderia pseudomallei infection) is a common form of community-acquired sepsis in Southeast Asia and northern Australia (14–16). Mortality from melioidosis is 14–50% despite appropriate antimicrobial therapy (17–19). Since diabetes is a risk factor in around half of all patients with melioidosis and is the most commonly identified risk factor in all published melioidosis cohorts (20–22), melioidosis is an ideal clinical model for studying the interaction of diabetes and sepsis. We showed in a previous study of 34 patients that melioidosis is characterised by activation of coagulation, activation and inhibition of fibrinolysis and inhibition of anti-coagulant pathways. Of note, the extent of coagulation activation correlated with mortality (23). Furthermore, despite being a strong risk factor for melioidosis, a diagnosis of diabetes in patients with melioidosis is associated with remarkable lower mortality rate (24). We hypothesised that diabetes induces
dysregulation of procoagulant, anticoagulant or fibrinolytic pathways that could partly account for this observation. The aim of the current study, performed in a new cohort of melioidosis patients, was to investigate the contribution of pre-existing diabetes on the coagulation and fibrinolytic systems during sepsis caused by *B. pseudomallei*.

### Methods and materials

#### Patients

The patients were recruited at Sappasithiprasong Hospital (Ubon Ratchathani, Thailand) from 31 January to 31 October, 2008. Eligible patients were aged 18–75, had culture-proven melioidosis, had received active antimicrobial chemotherapy for less than 48 hours (cefazidime, amoxicillin-clavulanate, meropenem or imipenem) and had two out of four criteria for systemic inflammatory response syndrome (SIRS) (25). None of these patients were part of our previous study on the impact of melioidosis on coagulation (23). Controls were seen once and not followed-up; patients were seen daily until death or discharge and then seen at the first follow-up outpatient clinic (total follow-up time 696 days, mean 16 days per patient).

Access to healthcare is limited in our setting: diabetes may be diagnosed late, and melioidosis is often the first presentation of diabetes (20). We identified patients with diabetes not just from their history but also by admission glycosylated haemoglobin (HbA$_1c$). Melioidosis patients were classed as diabetic if they had a diagnosis of diabetes prior to the onset of illness, or an admission HbA$_1c$ ≥7.8% (26) (Bio-Rad D-10, Bio-Rad Laboratories, Hercules, CA, USA). The HbA$_1c$ measurement allowed us to identify patients with previously unrecognised diabetes who would otherwise die before a diagnosis can be made by WHO criteria (27). Details of admission random glucose concentration, diabetes medication (insulin, metformin or sulphonylurea treatment) were also recorded. We recruited as controls, healthy blood donors and otherwise healthy diabetics attending a routine out-patient clinic. We excluded pregnant women and patients on anticoagulants or immunosuppressive therapy. The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2008–001–01) and the Oxford Tropical Research Ethics Committee (OXTREC 018–07). Written consent was obtained from all subjects or next-of-kin by a native Thai speaker.

#### Coagulation assays

Blood samples were collected once only from controls, and up to three times from patients (at recruitment, seven days later and at the first follow-up clinical ≥28 days from discharge). No samples were collected at any other time points, because previous studies have showed no advantage of doing so (4).

Citrated plasma (Vacutainer® 369714, Becton Dickinson, Franklin Lakes, NJ, USA) was stored at −70°C pending analysis. Prothrombin time (PT), activated partial thromboplastin (PTT), antithrombin (AT) and D-dimer were assayed on an automated blood coagulation analyzer (BCS® XP, Siemens Healthcare Diagnostics, Marburg, Germany). Fibrinogen levels were derived from the change in optical signal in the PT. Prothrombin fragment F$_{1+2}$ ($F_{1+2}$) (Enzygnost®, Siemens Healthcare Diagnostics), thrombin-antithrombin complexes (TAT) (Enzygnost®) and plasmin-antiplasmin complexes (PAP) (DRG Diagnostics, Marburg, Germany) were determined by enzyme-linked immunosorbent assays (ELISA). Total protein S levels were determined with an in-house ELISA (using antibodies from Dako, Glostrup, Denmark). Free protein S was measured by precipitating the C4b-binding protein-bound fraction with polyethylene glycol 8000, then measuring the concentration of free protein S in the supernatant. Protein C (PC) was assayed by kinetic assay (Coamatic®, Chromogenix™, Mölndal, Sweden). Endogenous thrombin potential and associated measures (lag time, peak, time-to-peak, area-under-the-curve and velocity index) were assayed on the Calibrated Automated Thrombogram® (Fluoroscan Ascent, ThermoLab systems, Helsinki, Finland) and Thrombinoscope® software (Thrombinoscope BV, Maastricht, The Netherlands) as previously described (28). Resistance to activated protein C was determined by testing the effect of activated protein C on the ETP with the CAT assay. The sensitivity to APC (Enzyme Research Laboratories, South Bend, IN, USA) of each plasma sample was determined in both the presence and absence of 4 nM APC. The APC concentrations used were adjusted to maintain a residual thrombin generation activity of approximately 10% in normal pooled plasma. The normalised ratio (APCsr) was determined by dividing the APCsr of an individual by the APCsr of the pooled plasma (a ratio >1.6 reflects an APC resistant phenotype). The diffuse intravascular coagulation (DIC) score was calculated for patients using the International Society on Thrombosis and Haemostasis (ISTH) standardised method (29).

### Statistical analysis

Analyses were performed on GraphPad Prism 5.0b for Macintosh (GraphPad Software, San Diego, CA, USA). We calculated that 40 melioidosis patients would give us >80% power to detect a ≥1.0 standard deviation difference in means by Student t-test with a 5% level of significance if half the patients were diabetic (sampsi command, Stata 9.0, StataCorp, College Station, TX, USA). After study completion, we found that a much higher proportion had diabetes than originally expected, but a retrospective power calculation showed our study retained >80% power to find a difference of ≥1.1 standard deviations in the parameters we studied. Quantile-quantile plots were used to check normality and select transformations (gladder command, Stata 9.0). The distributions of age, PT, AT, protein C, total protein S, ETP velocity index and area under the curve were Gaussian. Glucose, HbA$_1c$, D-dimer, free protein S and PAP were log-normal; a square-root transform was applied to APC
sensitivity ratio; an inverse square-root transform was applied to fibrinogen, F1+2, TAT and TAT/PAP ratio; for ETP lag time and time-to-peak (ttPeak), an inverse transform; for PTT, an inverse square transform. The Student t-test was used for pairwise comparisons of continuous variables with Welch’s correction applied where appropriate; the Mann-Whitney U test was applied for DIC scores as this is on an ordinal scale. Categorical variables were compared by Fisher exact test. Correlations were assessed by Pearson’s test on transformed values. Two-tailed p-values were reported to two decimal places if ≥0.01 or three decimal places if <0.01 and ≥0.001; p-values less than 0.001 were reported as such.

Results

Clinical characteristics

We recruited 30 healthy blood donors, 52 otherwise healthy diabetics and 44 melioidosis patients. Of the melioidosis patients, the proportion with diabetes was greater than expected (34 patients, or 74%), and of these, 10 were new diagnoses made on the basis of admission HbA1c concentration. Mean glucose was 214 mg L–1 in diabetic melioidosis patients versus 124 mg L–1 in those without diabetes. Clinical features are summarised in Table 1.

Influence of diabetes on coagulation during melioidosis

After adjusting for the effect of diabetes, melioidosis was associated with activation of coagulation as reflected by elevated plasma concentrations of TAT, F1+2, and fibrinogen as compared to non-diabetic controls (Fig. 1, Table 2). In the controls, F1+2 and fibrinogen levels were also higher in diabetics than in non-diabetic blood donors (Fig. 1, Table 2). In patients with established melioidosis, however, pre-existing diabetes was not associated with differences in the extent of coagulation activation: no differences between TAT, F1+2 and fibrinogen levels were observed between septic patients with or without diabetes (Fig. 1, Table 2). Moreover, in patients with sepsis caused by B. pseudomallei, PT and PTT were both prolonged irrespective of the presence of diabetes (Fig. 1, Table 2).

Influence of diabetes on anticoagulant pathways during sepsis

The activation of coagulation seen in septic melioidosis was accompanied by depression of anticoagulation: antithrombin (AT), protein C, total and free protein S levels were all depressed in non-diabetic patients with melioidosis (Fig. 2, Table 2). In otherwise healthy subjects, diabetes per se did not alter levels of AT or protein C, but total protein S concentrations were higher in diabetic controls compared to blood donors (Fig. 2, Table 2). In the context of septic melioidosis, diabetes did not influence the circulating levels of these anticoagulant proteins (Fig. 2, Table 2). We were motivated by the results of a previous observational study that APC resistance was increased in endotoxemia (30) to look for differences in the APC sensitivity ratio, but found none (Fig. 4, Table 2).

Influence of diabetes on fibrinolysis during melioidosis

D-dimer and PAP were strongly elevated in melioidosis (Fig. 3, Table 2). D-dimer and PAP levels were also higher in diabetic controls compared to blood donors (Fig. 3, Table 2), consistent with previous research (31). Within the context of melioidosis, there was no additional effect of diabetes on D-dimer or PAP.

Table 1: Characteristics of study subjects.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th></th>
<th>Melioidosis patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood donors</td>
<td>Diabetes patients</td>
<td>No diabetes</td>
</tr>
<tr>
<td></td>
<td>n=30</td>
<td>n=52</td>
<td>n=10</td>
</tr>
<tr>
<td>Mean age, years</td>
<td>41.5 (37.5–45.4)</td>
<td>57.5 (54.1–60.9)</td>
<td>51.6 (40.9–62.3)</td>
</tr>
<tr>
<td>Male sex</td>
<td>80.0% (24 of 30)</td>
<td>34.6% (18 of 52)</td>
<td>90.0% (9 of 10)</td>
</tr>
<tr>
<td>Glucose, mg L–1</td>
<td>101 (87–117)</td>
<td>126 (117–136)</td>
<td>124 (97–159)</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.8 (5.4–6.3)</td>
<td>8.2 (7.8–8.5)</td>
<td>6.0 (5.5–6.5)</td>
</tr>
<tr>
<td>Sulfonylurea treatment</td>
<td>—</td>
<td>55.8% (29 of 52)</td>
<td>—</td>
</tr>
<tr>
<td>Metformin treatment</td>
<td>—</td>
<td>57.7% (30 of 52)</td>
<td>—</td>
</tr>
<tr>
<td>Insulin treatment</td>
<td>—</td>
<td>32.7% (17 of 52)</td>
<td>—</td>
</tr>
<tr>
<td>Mortality</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Age, glucose and HbA1c are reported as mean (95% confidence interval). HbA1c, haemoglobin A1c. *This total includes 10 patients given a new diagnosis of diabetes by admission HbA1c concentration.
lioidosis is characterised by elevated DIC scores, but there was no difference in DIC scores between melioidosis patients with and without diabetes (Table 2).

Influence of diabetes on endogenous thrombin potential (ETP) during melioidosis

Haemostasis is activated through low concentrations of tissue factor (TF), which is regarded as the primary initiator of the inflammation-induced coagulation cascade and is known to be upregulated during melioidosis (23). Furthermore, the rate of clot formation, and in particular the rate of thrombin generation, is crucial for the formation of a stable fibrin clot (23). We made use of a thrombin generation assay (ETP) in order to investigate the influence of diabetes on clot formation in the event of sepsis. Lag times, peak and time-to-peak were all prolonged in melioidosis (Fig. 4, Table 2). In controls, we found that diabetes was significantly associated with abnormalities of the ETP: diabetes was associated with higher peak thrombin concentrations, shorter time-to-peak, increased velocity index and a smaller area under the curve (Fig. 4, Table 2). Diabetes per se had no influence on any of the measured parameters of thrombin generation in the context of melioidosis (Fig. 4, Table 2).

Effect of HbA1c, admission glucose levels and pre-admission diabetes medication on coagulation, anti-coagulation and fibrinolysis during melioidosis

We postulated that sepsis-induced hyperglycaemia, and not just pre-existing diabetes, might influence the coagulation cascade in the event of sepsis. We therefore looked for associations between enrolment HbA1c and glucose levels for each marker of coagulation, anti-coagulation and fibrinolysis in all patients. There were no correlations between HbA1c or glucose levels and any of the parameters measured (data not shown). In addition, type of diabetic treatment (metformin, sulphonylurea or insulin) did not influence

Figure 1: Markers of coagulation activation compared by group. Plasma concentrations of TAT, F$_{1+2}$ and fibrinogen were elevated, and PT and PTT were prolonged in patients with sepsis caused by *B. pseudomallei* when compared to controls, even after adjusting for the effect of diabetes. These results are presented numerically in Table 2. No difference was found between melioidosis patients with or without diabetes. TAT, thrombin-antithrombin complexes; F$_{1+2}$, prothrombin fragment F$_{1+2}$; PT, prothrombin time; PTT, partial thromboplastin time; DM, diabetes mellitus.
any of the parameters examined (data not shown). Lastly, admission blood glucose levels and enrolment HbA1c did not vary with metformin, sulphonylurea or insulin treatment in either diabetic controls or diabetic melioidosis patients.

**Effect of diabetes on association between markers of coagulation and clinical outcome during melioidosis**

Having characterised the influence of diabetes on the coagulation, anti-coagulation and fibrinolysis pathways in patients with septic melioidosis, we next sought to examine differences in the coagulation profile on clinical outcome in the context of diabetes. Overall mortality in this cohort of patients with septic melioidosis was 27%. We observed no deaths in melioidosis patients without diabetes (Table 1). Admission glucose levels were not different between survivors (mean 199 mg L\(^{-1}\)) and non-survivors (228 mg L\(^{-1}\); \(p = 0.33\)), and HbA1c concentrations did not correlate with mortality (8.8% in survivors vs. 10.2% in non-survivors, \(p = 0.23\)). Deficiencies in anti-coagulation and increased fibrinolysis correlated with mortality: patients who died had lower Protein C and antithrombin concentrations and increased D-dimer concentrations, which corresponds with the findings of our previous cohort study (data not shown) (23). All measured markers of coagulation, anticoagulation and fibrinolysis normalised in survivors from whom follow-up samples were drawn, especially those markers most strongly associated with mortality, i.e. Protein C, antithrombin and D-dimer (Fig. 5). Diabetes did not influence the recovery of these parameters after successful treatment (Fig. 5).

**Discussion**

In this study, we examined a cohort of diabetic patients with gram-negative sepsis caused by *B. pseudomallei* and compared these
Diabetes does not influence coagulation in melioidosis

Figure 2: Markers of anticoagulation compared by group. Plasma levels of antithrombin, total and free protein S and protein C are depressed in melioidosis. Diabetes was associated only with a depression in protein S levels, but this did not translate into a difference in patients with melioidosis. These results are presented numerically in Table 2. DM, diabetes mellitus.

Figure 3: Markers of fibrinolysis compared by group. D-dimer and PAP levels are both elevated in melioidosis and this difference persisted even after adjusting for the effect of diabetes. D-dimer and PAP levels were higher also in diabetic controls, but this did not translate into a difference between melioidosis patients with or without diabetes. These results are presented numerically in Table 2. PAP, plasmin-antiplasmin complexes; DM, diabetes mellitus.
against otherwise healthy controls. Both diabetes and melioidosis are associated with multiple abnormalities of coagulation, anti-coagulation and fibrinolysis, yet we found no influence of diabetes on the coagulation system when superimposed on the more severe changes due to melioidosis.

The activation of coagulation in diabetics who are otherwise healthy is well-described, but this was done mainly in Western populations, so this study both confirms and extends those findings (32). That we were unable to find any differences between the melioidosis patients with and without diabetes, despite differences in coagulation parameters between blood donors and otherwise healthy diabetes patients, is consistent with the findings of our previous study of 830 sepsis patients, of whom 22% had a pre-admission diagnosis of diabetes (4). We found no differences in markers of inflammation or coagulation between patients with or without a diagnosis of diabetes prior to presentation (4). However, one important limitation of that study was that no admission HbA1c measurements were available, so patients with undiagnosed diabetes are likely to have been included in the control group, potentially confounding the results. We have shown in another cohort of patients that melioidosis is characterised by activation of coagulation, activation and inhibition of fibrinolysis and inhibition of anticoagulant pathways. Of note, the extent of coagulation activation correlated with mortality (23), but limitation of that study was that healthy blood donors were used as controls while around two-thirds of patients with melioidosis have diabetes which is itself a pro-thrombotic condition (33), again complicating the interpretation of the potential effect of diabetes on the coagulation cascade.

Since sepsis and diabetes both stimulate coagulation and innate immunity, the lack of a strong influence of diabetes on the coagulation pathways during sepsis is remarkable (4). Preclinical studies in young healthy volunteers have shown that acute hyperglycaemia and/or insulin resistance can directly influence inflammation and coagulation (5, 34). The effects of hyperglycaemia and hyperinsulinaemia on coagulation, and fibrinolytic responses during
systemic inflammation were investigated in 24 healthy humans during clamp experiments in which either plasma glucose, insulin, or both, was increased after Escherichia coli endotoxin was injected intravenously to induce a systemic inflammatory and procoagulant response (6). That study showed that during experimental endotoxaemia, hyperglycaemia reduced neutrophil degranulation and increased coagulation as reflected in elevated concentrations of TAT complexes and soluble TF (6). Our current study in patients with sepsis does not, however, show any correlation between glucose levels or HbA1c and any of the parameters measured. A small proportion of melioidosis patients with localised disease do not present with sepsis. It remains possible that examination of these non-septic melioidosis patients might reveal differences in coagulation responses undetected by our study.

The role of protein C in melioidosis is an area of active research, since the protein C system has been shown to be deficient in melioidosis (23, 35), and because recombinant activated protein C supplementation has been shown to reduce mortality in sepsis (36). Activated protein C inactivates factor Va and VIIIa, and is thus anti-coagulant. The activated protein C (APC) sensitivity ratio was first described in 1993 (37): addition of APC to plasma in vitro normally results in prolongation of the PTT, and the ratio of clotting times before and after the addition of a fixed amount of APC is the APC sensitivity ratio (APCsr). Failure of APC to prolong the PTT (APC resistance) may be seen in patients with factor V Leiden mutation (38), but is also seen in abnormalities of factors II, V, VIII and protein S (39, 40). De Pont et al. found increased APC resistance in an endotoxaemia human model of sepsis (30), but we found no difference in APCsr in melioidosis compared to controls.

The question of how to identify previously undiagnosed diabetes in the setting of acute sepsis is a vexed one. Neither fasting glucose nor a glucose tolerance test can be relied on in acute sepsis, and the only remaining option is glycated haemoglobin concentrations (HbA1c), which is the method we elected to use in this study. HbA1c is a measure of the average glucose level over the life-span of the circulating erythrocyte and is highly consistent over time within individuals; furthermore, it seems reasonable to as-

What is known about this topic?
- Sepsis is associated with abnormalities of coagulation, fibrinolysis and anti-coagulation when compared to healthy blood donors. Melioidosis (Burkholderia pseudomallei infection) is an important cause of community-acquired sepsis in northeast Thailand.
- Diabetes is a major risk factor for melioidosis.
- Diabetes is itself associated with abnormalities of coagulation, fibrinolysis and anti-coagulation.

What does this paper add?
- This study includes a control group of patients with diabetes, not just healthy blood donors.
- We confirm that patients with diabetes have multiple abnormalities of coagulation, fibrinolysis and anti-coagulation, but that the changes due to diabetes are not detectable on the background of larger abnormalities due to melioidosis. 

Figure 5: The normalisation of three parameters (protein C, antithrombin and D-dimer) over time in melioidosis patients. The three time points are enrollment, 7 days following enrollment and the first follow-up clinic (≥28 days after discharge).
assume that HbA1c concentrations are largely unaffected by the rapid onset of hyperglycaemia in sepsis. The results of diabetes diagnosed by HbA1c do not correlate completely with fasting glucose or glucose tolerance tests and false positives do occur (41). Despite these limitations, there are no alternative assays for diagnosing diabetes in the context of sepsis.

The mortality data from this study appear inconsistent with previous studies from Thailand that seem to show patients with diabetes have better survival in melioidosis (19, 24). The main difference between this study and previous ones is that this study uses HbA1c measurements to identify patients with undiagnosed diabetes, whereas previous studies relied solely on history obtained from the patient or family. Although HbA1c has been held up as a gold standard for diagnosing diabetes in sepsis studies, there are in fact no studies validating this. It should also be noted that this study was not designed to look for differences in mortality and the number of patients who turned out not to have diabetes by study criteria was very small (only 10), so caution should be taken not to over-interpret the mortality data presented here.

In conclusion, diabetes is associated with abnormalities of coagulation, anticoagulation and fibrinolysis, however these changes are not significant on the background of the larger abnormalities attributable to sepsis caused by B. pseudomallei.

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Conflict of interest

None declared.

References