Research: Complications

Levels of wound calprotectin and other inflammatory biomarkers aid in deciding which patients with a diabetic foot ulcer need antibiotic therapy (INDUCE study)

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Abstract

Aims   Deciding if a diabetic foot ulcer is infected in a community setting is challenging without validated point-of-care tests. Four inflammatory biomarkers were investigated to develop a composite algorithm for mildly infected diabetic foot ulcers: venous white cell count, C-reactive protein (CRP) and procalcitonin, and a novel wound exudate calprotectin assay. Calprotectin is a marker of neutrophilic inflammation.

Methods   In a prospective study, people with uninfected or mildly infected diabetic foot ulcers who had not received oral antibiotics in the preceding 2 weeks were recruited from community podiatry clinics for measurement of inflammatory biomarkers. Antibiotic prescribing decisions were based on clinicians' baseline assessments and participants were reviewed 1 week later; ulcer infection was defined by clinicians' overall impression from their two assessments.

Results   Some 363 potential participants were screened, of whom 67 were recruited, 29 with mildly infected diabetic foot ulcers and 38 with no infection. One participant withdrew early in each group. Ulcer area was 1.32 cm^2 [interquartile range (IQR) 0.32–3.61 cm^2] in infected ulcers and 0.22 cm^2 (IQR 0.09–1.46 cm^2) in uninfected ulcers. Baseline CRP for mild infection was 9.00 mg/ml and 6.00 mg/ml for uninfected ulcers; most procalcitonin levels were undetectable. Median calprotectin level in infected diabetic foot ulcers was 1437 ng/ml and 879 ng/ml in uninfected diabetic foot ulcers. Area under the receiver operating characteristic curve for a composite algorithm incorporating calprotectin, CRP, white cell count and ulcer area was 0.68 (95% confidence intervals 0.52–0.82), sensitivity 0.64, specificity 0.81.

Conclusions   A composite algorithm including CRP, calprotectin, white cell count and ulcer area may help to distinguish uninfected from mildly infected diabetic foot ulcers. Venous procalcitonin is unhelpful for mild diabetic foot ulcer infection.

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Introduction

Diagnosis of diabetic foot ulcer infection continues to rely on symptoms, principally pain, and signs, including erythema, warmth, oedema and discharge. However, pain may be absent due to concomitant neuropathy and signs may be attenuated by vasculopathy [1]. Failure to treat mild infection with antibiotics risks progression to severe infection and amputation. Conversely, unnecessary over-prescription of

antibiotics exposes the person to the risk of adverse effects, increases the risk of subsequent infection with resistant organisms, and contributes to increasing antimicrobial resistance in society, one of the highest current public health priorities [2]. The financial burden of diabetic foot ulcers and associated amputations is large, approximately £650 million in England in 2011, exceeding 0.6% of the total health budget [3]. There are still no objective biomarkers of diabetic foot ulcer infection available at the point of care, which is particularly relevant because most diabetic foot ulcer care occurs in the community [3].

A pilot study of 45 individuals with diabetic foot ulcers reported that venous C-reactive protein (CRP) levels combined with venous procalcitonin, another infection

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What's new?

- Distinguishing between mild infection and no infection in diabetic foot ulcers, a frequent position of equipoise in antibiotic prescribing, is challenging in the absence of objective evidence available at point of care.

- We developed a novel wound exudate calprotectin assay which, when combined with venous C-reactive protein, white cell count and ulcer area, provided a diagnostic algorithm for diabetic foot ulcer infection.

- The combined algorithm has a specificity of 0.81 in distinguishing mild infection from no infection in a diabetic foot ulcer.

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biomarker, may help in distinguishing between infected and non-infected foot ulcers [4]. For a CRP cut-off value of 17 mg/L, negative predictive value was 0.91 and positive predictive value was 0.83. Validated point-of-care tests for CRP and other inflammatory biomarkers are now rapidly entering the market, providing the potential for testing in the community [5,6]. Calprotectin is a marker of neutrophilic inflammation and therefore may be useful as a marker of infection. It is currently largely used as a marker of inflammatory bowel disease through testing faecal samples, is available as a dipstick test, and is resistant to protease degradation [7]. This makes it a candidate to directly assess wound exudate, whose relatively high protease levels prevent the use of several other candidate inflammatory biomarkers.

Diagnostic infection assays, including non-specific testing of inflammatory markers, are likely to be most effective when combined with optimal clinical assessment and patient communication. For example, the use of a point-of-care test for CRP combined with an educational intervention more than halved antibiotic prescribing in primary care for lower respiratory tract infections without compromising patient safety [8].

The aim of our study was to determine the diagnostic accuracy of a combined inflammatory biomarker algorithm including venous white cell count, CRP and procalcitonin, and wound exudate calprotectin levels in predicting diabetic foot ulcer mild infection.

Patients and methods

Study design

The Updated List of Essential Items for Reporting Diagnostic Accuracy Studies (STARD) extension of the EQUATOR network has been followed to guide study reporting (Appendix S1) [9]. A sequential recruitment observational design was selected to provide proof of principle and feasibility data, the intention being to use data from this study to inform subsequent evaluations of the potential use

of point-of-care tests in future. Participants were recruited from six community podiatry clinics in South Wales, UK and the study received ethics approval from Wales Research Ethics Committee 6 (ref 14/WA/0085). Inclusion criteria were the presence of a full-thickness skin defect located distal to the ankle that was assessed clinically as either uninfected or mildly infected according to the Infectious Disease Society of America International Working Group on the Diabetic Foot (IDSA-IWGDF) classification system [10], in adults aged at least 18 years who met World Health Organization (WHO) diagnostic criteria for diabetes [11]. Exclusion criteria were immunosuppression by medication or other medical conditions, recent antibiotic treatment, pregnancy or lactation, or IDSA-IWGDF moderate or severe diabetic foot ulcer infection. During the first few weeks of recruitment it was noted that receipt of antibiotics within the preceding 6 weeks excluded a high proportion of potential participants and this criterion was reduced to 2 weeks, based on the dynamics of inflammatory biomarker production in response to infection [12].

It was anticipated that individuals with uninfected diabetic foot ulcers would be more prevalent and easier to recruit, and so it was prospectively decided to allow re-recruitment of participants initially recruited with an uninfected diabetic foot ulcer, if they subsequently presented with a mildly infected diabetic foot ulcer. However, only data from their infected diabetic foot ulcer presentation would be used in the primary analysis of biomarker diagnostic accuracy, to avoid the same participant being counted twice in analyses.

Infection definition

Our infection definition was based on the clinician's assessment 1 week after initial recruitment incorporating any change from baseline, factoring in whether antibiotic therapy had been received in the subsequent week. The clinician obtained all the clinical information, including measurement of ulcer area, vasculopathy and neuropathy, while remaining blinded to assay results, including point-of-care test results.

Data collection and study procedures

At baseline, experienced podiatrists provided a clinical assessment of participants, including an overall assessment of appearing well or unwell, checking for peri-ulcer erythema, tenderness, warmth, lymphangitis and osteomyelitis. Ankle brachial pressure indices (ABPIs) were recorded as a measure of vasculopathy, with a level less than 0.9 considered abnormal, and duplex scanning was undertaken in those with calcified vessels. Neuropathy was assessed by 10 g monofilament in the relevant foot. If the diabetic foot ulcer was judged to be infected at baseline on clinical grounds, oral antibiotics and antimicrobial dressings were prescribed based on local guidelines; oral antibiotics and antimicrobial

dressings were not permitted in those whose diabetic foot ulcer was judged to be uninfected.

Wound swabs, without debridement, were collected for calprotectin measurement, as well as microscopy, culture and antibiotic sensitivity analysis. Venous blood was sampled for automated measurement of white cell count and CRP; serum was stored at –80 °C for analysis of procalcitonin levels. Quantification of procalcitonin was performed in duplicate by ELISA (Abcam, UK, ab100630). CRP was also measured from a pin prick of blood using a point-of-care instrument (QuikRead go CRP, Orion Diagnostica, Finland); levels were measured by a researcher and the result was not communicated to the clinician until after they had provided their overall assessment of ulcer infection status at the week 1 visit.

Participants were asked to return after 1 week for a repeat clinical examination and to repeat sampling of venous blood, wound exudate and point-of-care CRP testing. They also kept a diary during the initial first week and three subsequent weeks, recording ulcer symptoms, instigation of any antibiotics, adherence to treatment, other medications and dressings required, quality of life [13], functional impairment [14] and any ulcer complications, including hospitalization.

Calprotectin assay

Ulcer swabs were pre-processed by vortexing the head of the swab in 3 ml of sterile saline for 5 s followed by sonication for 5 min; the head of the swab was then centrifuged at 100 g for 1 min. Duplicate supernatant samples were aliqoted and frozen at –80 °C for batched enzyme-linked immunosorbent assay (ELISA) analysis within 1 year. A two-site sandwich Calprotectin ELISA (MRP 8/14, S100A8/A9, DRG Diagnostics) at an initial dilution of 1 : 2 was used, absorbance being measured at 450 nm with a FLUOstar Optima microplate reader (BMG LABTECH Ltd, UK). Samples outside the upper limit of measurement were re-run at dilutions of 1 : 20 or 1 : 200.

Sample size and statistical analysis

The largest previous study in the field recruited 23 participants with uninfected diabetic foot ulcers and 23 with infected diabetic foot ulcers, with a designed clinical prevalence for infection of 50% [4]. Our intention was to exceed this study size, ideally recruiting 50 participants with uninfected diabetic foot ulcers and 50 with mildly infected diabetic foot ulcers, providing 95% confidence intervals (95% CI) of 0.77 to 0.96 around a positive predictive value (PPV) of 0.90 for the diagnostic algorithm. Inflammatory biomarker levels along with the gold standard dichotomous classification of diabetic foot ulcer infection formed the primary dataset. Cut-off points that optimized sensitivity, specificity and the area under the receiver operating characteristic curve (AUROCC) were calculated using Youden's index and the Euclidean distance from the upper left hand corner to the co-ordinates of the curve [15]. Analyses to obtain AUROCC utilized bootstrapping in Stata/IC 13.1 with 5000 repetitions and bias-corrected estimates are reported.

Results

During a 12-month recruitment period, from October 2014 to September 2015, 363 potential participants were screened, of whom 67 were recruited. A breakdown of reasons for study non-recruitment is given in Table 1, with recent antibiotic treatment being the most common cause. Some screened participants were excluded because their ulcer was not full thickness or was located proximal to the ankle, in the context that the clinics assessed a spectrum of patients with skin integrity problems. The primary dataset included 34 participants with uninfected diabetic foot ulcers and 27 with mildly infected diabetic foot ulcers (Fig. 1). Table 2 details the baseline characteristics of participants included in the primary dataset. Median ulcer area for the uninfected group was 0.22 cm^2 [interquartile range (IQR) 0.09–1.46 cm^2] compared with 1.32 cm^2 for mildly infected diabetic foot ulcers (IQR 0.32–3.61 cm^2).

Results for the inflammatory biomarker tests at baseline are in Table 3, subdivided by their infection status as determined by the study infection definition. The median wound exudate calprotectin level in samples from mildly infected diabetic foot ulcers was 1437 ng/ml (IQR 664–6420 ng/ml) compared with 879 ng/ml (IQR 586–2674 ng/ml) in uninfected ulcers. Venous procalcitonin results were limited by 41 of the 59 baseline samples having a level below the lower limit of assay detection, including 21 samples from those with infected ulcers.

The inflammatory biomarker values were then combined in logistic regression models as predictors of diabetic foot ulcer infection, in each case creating a 'likelihood score for

Table 1 Breakdown of reasons for study ineligibility in the 294 potential participants who were not recruited into the study. In some individuals more than one reason was identified

Exclusion criterion	No. of participants	Percentage of ineligible individuals
Antibiotic treatment in last 2 weeks (post-Nov 2014)	82	28
Ulcer not full thickness/below ankle	74	25
Moderate /severe infection	48	16
Not able to attend week 1 follow-up	37	13
Antibiotic treatment in last 6 weeks (pre-November 2014)	27	9
Not interested in participating	26	9
Immunosuppression	20	7
Unable to provide informed consent	15	5
People without diabetes	11	4
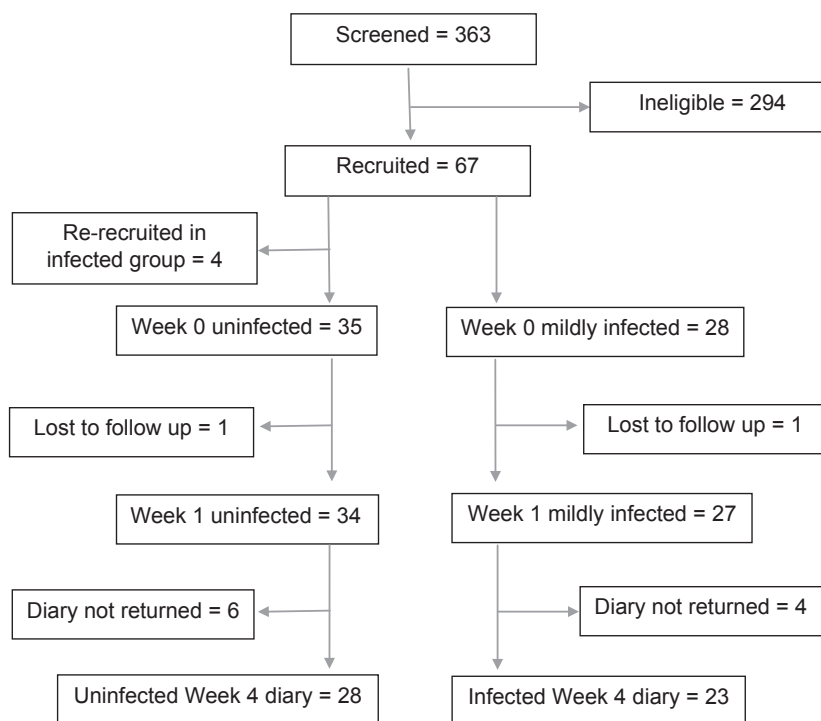
FIGURE 1 Participant flow diagram. A flow diagram summarizing participant recruitment into the INDUCE study. Uninfected or mildly infected status is defined by the clinician's overall clinical impression of the diabetic foot ulcer at week 1, incorporating response to antibiotics, if prescribed at baseline, while blinded to all test results. Four participants who were recruited with uninfected ulcers subsequently re-presented with a mildly infected ulcer and were recruited again, but their data is only counted once, for the episode of infection.

infection' (LSI) from which receiver operating characteristic curves could be plotted. Our original intention was to combine point-of-care test CRP, white cell count, procalcitonin and calprotectin values, however, the lack of measurable procalcitonin levels led to exclusion of this variable from the model. Participants lacking data for any of the required variables were not included, and so the LSI for the remaining three variables was based on 31 uninfected and 25 mildly infected diabetic foot ulcer participants. The AUROCC was 0.63 (95% CI 0.47–0.78), indicating no benefit for prediction of diabetic foot ulcer infection.

Having excluded procalcitonin from the combined algorithm, we decided to substitute a clinical parameter as the fourth variable. Ulcer area was chosen because results were available for all participants, there was a six-fold difference in the mean value between infected and uninfected ulcers, and it is a straightforward measurement for clinicians to obtain. Combination of the three remaining biomarkers, point-of-care test CRP, white cell count and calprotectin with baseline ulcer area produced a composite algorithm demonstrating significant improvement in prediction of diabetic foot ulcer infection, with an AUROCC of 0.68 (95% CI 0.52–0.82). Using Youden's index and the Euclidean distance from the upper left hand corner provided a consistent cut-off point for LSI of 1.77, giving a sensitivity of 0.64 and a specificity of 0.81, with a corresponding

positive predictive value of 0.73 and negative predictive value of 0.75 (Appendix S2).

In terms of study safety, there were no serious adverse events during the 4 weeks covered by trial diaries. Five of the 28 participants (18%) with uninfected diabetic foot ulcers who returned their diary cards required oral antibiotics for diabetic foot ulcer infection in the subsequent 3 weeks after the week 1 assessment, compared with 15 of 23 with infected diabetic foot ulcers (65%) who required additional antibiotics during this period.

Discussion

Our results show that an algorithm including white cell count, CRP, wound exudate calprotectin and ulcer area has an AUROCC which is insufficient to recommend its use without further refinement. A sensitivity of 0.64 means that if 100 people with a mildly infected diabetic foot ulcer were tested, 64 would be correctly identified as having infection, and there would be 36 false negatives. A specificity of 0.81 means that if 100 people with an uninfected diabetic foot ulcer were tested, 81 would be correctly identified as having no infection and there would be 19 false positives indicating mild infection. Levels of the novel wound calprotectin biomarker were nearly doubled in mild diabetic foot ulcer infection, however, this result did not reach statistical

Table 2 Baseline characteristics of participants, subdivided by ulcer infection status

	Uninfected* ($n = 34$)	Infected* ($n = 27$)
Age at recruitment (years)	64.9 (11.0)	66.4 (14.4)
Male[†]	27 (79)	19 (70)
Ethnicity White British/Welsh[†]	32 (94)	26 (96)
BMI (kg/m^2)	33.6 (5.5)	28.3 (6.3)
Current smoker[†]	2 (6)	6 (22)
Ex-smoker[†]	18 (53)	13 (48)
Years since diabetes diagnosis	20.4 (9.9)	16.6 (11.3)
HbA$_{1c}$ (mmol/mol)[‡]	64 (54–77)	68 (54–110)
HbA1c (%)[‡]	8.0 (7.1–9.2)	8.4 (7.1–12.2)
At least 1 previous ulcer in past year	16 (47)	11 (43)
Current ulcer area (cm^2)[‡]	0.215 (0.090–1.463)	1.320 (0.320–3.610)
Current ulcer duration (weeks)[‡]	16.0 (8.4–73.5)	10.0 (1.1–66.0)
Previous antibiotic treatment of current ulcer	23 (68)	13 (48)
Ankle brachial pressure index abnormal in ipsilateral lower limb	8 of 23 (35)	8 of 15 (53)
Peripheral neuropathy in ulcerated foot	33 (97.1)	21 (77.8)

*Mean (sd) unless stated otherwise; [†]number (%) and [‡]median (25th to 75th percentile).

consideration when most diabetic foot ulcer care occurs in the community and antibiotic decisions have to be made swiftly. We chose to restrict study participants to mild or no ulcer infection because this distinction represents the greatest challenge in diabetic foot ulcer antibiotic stewardship, in the context that moderate to severe infection is straightforward to diagnose from clinical parameters.

Venous procalcitonin or a combination of procalcitonin and CRP have been used as biomarkers of diabetic foot ulcer infection in several other studies. Jeandrot et al. calculated an AUROCC of 0.95 (sd 0.029) for procalcitonin combined with CRP [3], Uzun et al. demonstrated an AUROCC of 0.86 for procalcitonin alone [16], and Massara et al. found a 100-fold increase in procalcitonin levels between infected and uninfected diabetic foot ulcers [17]. In comparing our results with these other studies, it is possible that technical issues using different assays might account for some of the discrepancy, however participant selection criteria are the most likely reason. Most of the other studies recruited from hospitalized patients, with more severe diabetic foot ulcer infections. Massara et al. did not restrict recruitment to mild diabetic foot ulcer infection and 11 of the 15 patients in their infected group were pyrexial, with a temperature > 38.5 °C, graded as severe infection by the IDSA-IWGDF classification system [17]. The increased severity of infection is reflected by a mean CRP level of 121 mg/L, more than 10 times the level in our patients judged to have infected diabetic foot ulcers. Uzun et al. recruited participants who required admission to hospital; 28 of the 49 were receiving antibiotics at the time of admission and 7 of the 27 patients in the infected group had MRI evidence of osteomyelitis, indicating at least moderate infection [16]. Jeandrot et al. did use the IDSA-IWGDF classification system and compared people with mildly infected diabetic foot ulcers with those with uninfected diabetic foot ulcers [4]. However, their study population was again recruited in a hospital setting, suggesting that more severe infection was likely, and people with other inflammatory conditions were excluded, reducing external validity.

We chose to screen and recruit sequential participants in a community setting because this is where most people with

significance. By contrast, venous procalcitonin levels were of no value in detecting mild diabetic foot ulcer infection. The parameters included in our algorithm are, or soon will be, available at point of care, which is an important

Table 3 Results of inflammatory biomarker tests, subdivided by ulcer infection status

Test	Uninfected*	Infected*	AUROCC (95% CI)
White cell count (10^9 cells/L)[†]	7.43 (1.50) N = 32	8.15 (1.95) N = 27	0.62 (0.47–0.76)
Laboratory venous C-reactive protein (mg/L)	4.5 (2.0–11.0) N = 32	7.0 (3.0–22.0) N = 27	0.62 (0.48–0.77)
Point-of-care venous C-reactive protein (mg/L)	6.0 (5.0–15.5) N = 34	9.0 (5.0–17.0) N = 27	0.54 (0.39–0.69)
Venous procalcitonin (pg/ml)	21.8 (13.2–108) N = 13 (20 below limit)	4.8 (1.96–14.1) N = 4 (21 below limit)	Unable to calculate
Wound exudate calprotectin (ng/ml)	879 (586–2674) N = 33	1437 (664–6420) N = 25	0.56 (0.41–0.71)

*Values are median (25th to 75th centiles), except [†]mean (sd).

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diabetic foot ulcers are treated, and where clinical equipoise occurs most frequently in considering possible systemic or topical antibiotic therapy for people with diabetic foot ulcers. Biomarkers available at point of care in a community setting have the greatest potential to avoid early infection being missed and to support clinicians in avoiding inappropriate empirical antibiotic therapy in the absence of objective evidence. In most people with moderate or severe infection requiring hospitalization, clinical parameters such as overt wound inflammation are sufficient to diagnose infection with confidence and biomarkers are relatively redundant. Inflammatory biomarkers, in particular erythrocyte sedimentation rate, may have a specific role to assist in the diagnosis of occult osteomyelitis, which can be difficult to diagnose clinically in the absence of immediate MRI resources [18].

In considering other strengths of our study, we selected an infection definition that is more robust than relying on a single clinical impression at one snap-shot in time. Our definition was based on two separate clinical assessments, 1 week apart, allowing the clinician to integrate data regarding the natural history of the ulcer and incorporating response to antibiotic treatment, if given. All the clinicians involved in the study were podiatrists who were experienced in application of the IDSA-IWGDF classification system and had completed an online educational tool to reinforce their clinical skills. Our definition has not, however, been subjected to validation studies to confirm that it performs better than a single assessment. We avoided incorporating microbiology results from ulcer swabs into our definition because all wounds are colonized with organisms and the method of sampling, comparing swabs with tissue samples, is known to produce inconsistent results [19].

In terms of study limitations, our definition for infection diagnosis has not been validated against the most robust gold standard of a peri-ulcer tissue biopsy Gram stain and culture. We chose not to incorporate a tissue biopsy in our study because of the logistical challenge of performing biopsies in the community and ethical considerations in terms of enlarging the size of the diabetic foot ulcer in people with uninfected or only mildly infected ulcers. Difficulty in applying the gold standard for infection diagnosis in the field of diabetic foot ulcers persists, which led to our methodology of applying the IDSA-IWGDF criteria on two separate occasions, one week apart, as a refinement when using clinical parameters.

Regarding other study limitations, it would have been ideal to measure toe pressures rather than ABPIs in our study population, but ABPI measurement remained standard practice when our study was performed. In addition, it should be noted that the dynamics of biomarker responses to infection caused us to exclude people who had received oral antibiotics within the preceding 2 weeks, resulting in 28% of screened individuals being ineligible for the study. It follows that our results are not generalizable to those who have received oral antibiotics in the previous fortnight.

Building on the results from our calprotectin wound swab assay, it may be that the focus of biomarker development for diabetic foot ulcer infection should be directed towards further wound fluid assays. It is perhaps unlikely that circulating venous biomarkers are substantially raised by mild infection in a foot ulcer, particularly when the vascular supply to the foot is compromised. In addition, interference from other comorbid inflammatory conditions is less likely to affect the results. Other wound exudate biomarkers under investigation include levels of lactate [20], interleukin-6 [21], human neutrophil elastase and cathepsin G [22]. These could be combined with calprotectin to produce a composite wound exudate assay. Subsequent clinician education is important to encourage behaviour change and to prevent over-reliance on test results. Ultimately, an RCT is needed utilizing a composite point-of-care test and accompanied by an educational resource, to determine whether antibiotic prescribing can be safely targeted for people with infected diabetic foot ulcers, avoiding inappropriate antibiotic use in people without diabetic foot ulcer infection.

In summary, our study demonstrated that a novel wound exudate calprotectin biomarker shows promise in assisting with the diagnosis of mild diabetic foot ulcer infection, a scenario in which clinical equipoise is frequently encountered regarding whether to offer antibiotic therapy. Combination of calprotectin with venous white cell count and CRP, and ulcer area improved diagnostic accuracy but the AUROCC of 0.68 remains insufficient to recommend the composite algorithm without further refinement in a primary care setting. Nevertheless, our algorithm's specificity of 0.81 provides some much-needed objective evidence to help clinicians avoid mass antibiotic prescribing in non-infected diabetic foot ulcers, contributing to prevention of antimicrobial resistance.

Competing interests

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Updated List of Essential Items for Reporting Diagnostic Accuracy Studies (STARD).

Appendix S2. Receiver operating characteristic curve for 'likelihood score for infection' and infection status.