Title: Identification of high-risk lower extremity wounds using point-of-care test for bacterial protease activity; a single-centre, single-blinded, prospective study.

Running title: Point-of-care testing for bacterial protease.

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Abstract

Clinician observation is the mainstay to determine if wound infection is present, and focuses on presence of erythema, purulence, and odour. However, non-visible bacterial protease activity can delay wound healing and lead to complications. In this study, a point-of-care test to detect the presence of bacterial protease activity (BPA, tested with Woundchek Bacterial Status test) was appraised. A total of 130 patients with lower extremity wounds were recruited in vascular and podiatry clinics, and across two time-points 182 BPA tests were conducted subsequent to initial (blinded) clinician's wound appraisal. Clinical opinion ('no infection', 'possible' or 'definite' infection) and BPA result (negative or positive test) had a moderate Kendall's tau-c rank correlation coefficient of 0.32 (P <0.001). Binary logistic regression analysis and principal component analysis showed that infection determined by clinical opinion was significantly associated with abovementioned clinical signs and a positive BPA test. However, a positive BPA result was also significantly linked with wound severity, such as number of lesions, chronicity and size. Throughout a 12-week follow-up

period, median ulcer size was larger for wounds positive for BPA test at baseline (*P* 0.001) and week-12 (*P* 0.036; both Mann-Whitney U-test) respectively. As a pilot initiative, clinical staff were allowed to act on the BPA result if they wished; in 11 out of 71 test-positive cases (15%) this happened and antimicrobial dressing was applied instead of planned standard dressing. These results show that protease-releasing bacteria may be active in ulcers that do not (yet) exhibit hallmark signs of infection, and are associated with delayed healing. Targeted point-of-care testing for bacterial protease activity may have the potential to identify and enable pro-active (antimicrobial) management of these high-risk wounds.

Keywords: wound, ulcer, infection, bacterial protease, point-of-care test, non-healing.

Chronic ulcers are associated with considerable expense, morbidity and impaired quality of life.[1,2] The natural history and pathophysiology of lower extremity (foot and leg) wounds – particularly in those patients who have venous insufficiency and or other chronic disease affecting the vasculature such as diabetes and peripheral arterial disease - is a continuous cycle of healing and breakdown over years and sometimes decades.[3] Bacterial infection of wounds carries the risk of further degenerative complications including cellulitis, necrotising fasciitis, and sepsis.[4] It has been long recognised that an additional undesirable effect of wound infection is that it delays, or even stops altogether, the wound healing process.[3,5]

Clinical guidelines in the United Kingdom stipulate that laboratory-based microbiological testing, should only be used to identify the pathogen strain in clinically confirmed infection. Therefore, clinical opinion is the mainstay of predicting and diagnosing infection.[6,7] Yet, microbiological counts and bacterial species identification do not necessarily reflect

infection as defined by other assessments, as demonstrated by different researchers.[8,9,10] Bacteria are capable of secreting protease enzymes which can break down restorative structures in wounds and induce a host response, and this can be a precursor to more invasive infection.[11,12,13] Diagnostic tools, including point-of-care tests (POCT) for rapid availability of tests results, have been developed that determine the presence of bacterial factors, such as elevated bacterial protease activity (BPA), that interfere with wound healing. For one of these POCT, Woundchek™ Bacterial Status, a clear link between elevated bacterial protease activity and host inflammation was observed (using markers IL-1β and TNF-α).[9] The focus on bacterial (protease) activity is step-change away from the established practice focus on purely quantifying the number of bacteria.[14,15]

A systematic review has indicated that further evidence is needed before it can be concluded with certainty that bacterial protease activity is linked to impaired wound healing in lower limbs.[16] This present study investigates the feasibility of introducing the Woundchek™ Bacterial Status (to measure BPA) in a standard National Health Service (NHS) clinic setting. The degree of agreement between BPA test result and clinical opinion is evaluated; both wound and patient characteristics will be considered as part of this evaluation, to determine what factors and variables may be associated with positive clinical opinion and positive BPA results respectively. Furthermore, the degree of impact the BPA test result may have on subsequent wound healing trajectory and clinical management of said lower limb ulcers are explored.

Methods

Study design and patients

Between March 2023 and March 2024, bacterial protease activity (BPA, tested with Woundchek Bacterial Status point-of-care test) was compared to clinical opinion in this controlled, non-randomised, single-blinded, prospective study. Participants were seen at study visits at week 0 (baseline), week 6 and week 12, to coincide with standard clinic visits. Inclusion criteria were patients aged 18 years or older, having mental capacity to provide informed consent, with a clinical diagnosis of a foot or leg ulcer (any underlying pathology) that was present for at least 30 days. Treatment with antibiotics for the index ulcer within the last three weeks was an exclusion criterion. Research governance clearance was obtained from UK ethics (ref 23/NW/0044) and health research authority (reference 314595), plus the sponsor NHS Trust. Written informed consent was obtained from all participating patients, in accordance with the Declaration of Helsinki. Podiatrists and vascular nurses - all with at least two years' experience - managed the patients.

Study dataset

At each clinic visit, first the treating clinical staff recorded whether they felt the wound was not infected, possibly infected, or infected. A clear definition of these three options was not provided to staff, since we wanted clinical staff to apply their usual methodology for this as per standard clinical practice. Then the ulcer was cleaned using a small amount of saline and a swab taken for the Woundcheck Bacterial Status test. Thereafter, the ulcer was managed as per standard clinical practice and this could involve debridement and application of fresh dressing and possibly further bandaging or compression wear. Before the end of the consultation, the Woundchek result was available for the clinician to act on if they so wished to. The following ulcer characteristics were recorded by the clinical staff: erythema around ulcer (absence, mild-moderate presence, and moderate-severe presence), purulence

(grading same as for erythema), odour (none, low, moderate, high levels), patient perception of ulcer-related pain (linear visual display score between 0 and 10); this methodology has been described previously.[10,17] Patient characteristics such as age, sex, diabetes status and patient mobility status (unable to walk, walks with assistance (stick/frame), walks without assistance) were recorded. In addition, wound data such as wound type (diabetic foot ulcer, venous leg ulcer, other), wound chronicity (<3 months or ≥3 months), number of lesions (1 or ≥2), and history of previous ulcer were also noted. The ulcer size was determined using the PUSH score.[18]

Bacterial protease activity testing

At week 0 (baseline) and week 6 patients' wounds were tested for presence of BPA using the Woundchek Bacterial Status point of care test (Woundchek Laboratories, UK). This is a lateral flow test, where a wound swab is taken and introduced to the device. Woundchek Bacterial Status contains an alpha-1-antitrypsin (A1AT) peptide substrate. This is targeted by proteases released by bacteria to inhibit a host immune response to the presence of the bacteria. To avoid measuring host-derived protease activity in the wound site, human matrix metalloproteinases (MMPs) are mostly left undetected and human neutrophil elastase in particular (a natural host-to-host target for A1AT) is inhibited in the test to avoid false-positive results.[19] The swabbing technique has been described previously and involves moistening the wound and rotating the swab across it.[20] For those patients who had multiple lesions, the largest wound was considered the index wound and only this lesion was swabbed. Any impact that the introduction of the Woundchek test had on management of the index ulcer and patient was evaluated as a pilot experiment by

recording any test-derived deviations in management. Clinical staff did not have to act on the BPA test outcome, and it was not dependent on concordance or non-concordance between BPA result and clinical opinion.

Statistical analyses

Data was collated using Microsoft Excel and analysed using IBM's SPSS version 24; a $P < 0.05$ was considered significant. Inferential analyses were applied as indicated in the Results section ($P<0.05$ was deemed statistically significant). Only outcomes for patients who had complete datasets for week 0 and week 6 study visits were included in analyses involving BPA test results, whereas only patients who had complete datasets for week 0, week 6, and week 12 were included in analyses involving wound healing; this could include healed wounds, whose PUSH score in such an instance was set at '0'. To enable binary regression analysis using either BPA test or clinical opinion as dependent, the results for the latter were re-arranged binary as done previously with 'possible infection' and 'infection' merged.[10,17] Principal component analysis (PCA) was conducted to take into account any multicollinearity (presence of high intercorrelations among two or more independent variables in a multiple regression model); the regression coefficient cut-off value was 0.40. A p-value, *P*, of <0.05 was considered statistically significant. A post-hoc power analysis based on the Kendall's tau-b correlation coefficient observed in this study between clinical opinion and BPA test result showed that the sample number was sufficient for an alpha of 0.01 and a power (1-beta) of 90%.[21]

Results

Figure 1 gives an overview of the number of patients who were enrolled into the study and the subsequent patient numbers at each clinic visit for obtaining study outcome measures.

Table 1 outlines the characteristics of the patients and the ulcers they presented with. As mentioned, a total of 130 patients were consented and BPA was measured 182 times. The degree of concordance between clinical opinion and BPA result was significant but only moderate at 0.32 (P <0.001; Kendall's tau-c) as summarised in Table 2. A total of 71 out of 182 (39%) BPA tests proved positive, whereas in 16 out of 182 test cases infection was deemed to be present by clinical opinion. More often was the BPA result positive when the clinical opinion concluded that there was no infection. There was a degree of dynamism; for the 58 patients that week 0 and week 6 test results were available, the BPA test was negative both times for 23 patients, first negative and then positive in 12 cases, first positive and then negative in 13 cases, and positive both times for 10 patients.

Any significant relationship(s) between patient and wound variables and either a positive BPA or positive (ie possible or definite infection) clinical opinion were appraised. Table 3 shows the results of multivariable binary logistic regression analysis whereas Table 4 shows the results of Principal Component Analysis (PCA). As part of the PCA process, suitability of the dataset was confirmed; the Kaiser-Meyer-Olkin measure of sampling adequacy of 0.73 indicated a strong level of relationships among variables, and Bartlett's test of sphericity was significant at *P* <0.001). Patient characteristics were taken from baseline (week 0) measurements, whereas wound characteristics were taken at the time of clinical opinion and BPA assessment (week 0 or week 6). The binary regression model and PCA show similar significant trends. As to be expected, a clinical opinion of infection being present is strongly associated with wound symptoms, stronger odour and increased purulence in particular. For the PCA results in Table 4 this is Component 1. A positive BPA result is also significantly linked to clinical symptoms, such as increased odour, but is also significantly associated with more general wound characteristics that strictly speaking are not appraised by staff when

specifically checking for wound infection. Wounds of patients that are more chronic, larger, and involve multiple lesions are more likely to be BPA positive; this forms Component 3 for the PCA results in Table 4. Diabetic foot ulcers were linked to a positive BPA test in the binary regression model but not PCA, whereas increased odour was the only 'hallmark' clinical sign to be associated with both analysis methods. Components 2 and 4 of the PCA results in Table 4 are not specifically related to either clinical opinion or BPA result.

When patients are stratified by their BPA result at baseline (week 0), a divergent picture emerges in terms of wound healing. Due to the high degree of lost to follow-up in this study, complete data across the study period was available for only 45 patients with a negative BPA test result, and 17 with a positive BPA test result. Nevertheless, Figure 2 shows that, in line with regression and PCA evaluations, wounds with a positive BPA test result are significantly larger at week 0. The median PUSH score was 7.0 (inter-quartile range 5.5) for negative BPA cases and 11.0 (IQR 5.0) for positive BPA cases (*P* 0.001, Mann-Whitney U-test). They continue to be larger on average too. For week 6 the figures were: BPA negative, median of 5.0 (IQR 9.5); and BPA positive, median of 10.0 (IQR 11.5), *P* 0.030. Finally, for week 12 they were: BPA negative, median of 4.0 (IQR 7.5); and BPA positive, median of 8.0 (IQR 14.0), *P* 0.036. There was also a difference in 'wound healed' status but this was not statistically significant. At week 12 of follow-up, healing had been achieved in 47% (n=21) of BPA-negative cases, whereas the figure was 29% (n=5) for BPA-positive cases (*P* 0.26, Fisher exact test).

As a pilot initiative, clinical staff were allowed to act on the BPA result if they wished. Since beforehand it was not known how many cases would result in a BPA positive test, yet negative clinical opinion, and patients were followed up for a period limited to 12 weeks, this was mainly done to measure the degree of openness by clinical staff to act on the BPA

test. In 11 out of 71 test-positive cases (15%), clinical staff changed their management of the wound: an antimicrobial dressing was applied instead of initially planned standard dressing. For only four of the treatment deviation cases both week 0 and week 6 data was available; for all four, a positive BPA test at week 0 had changed to a negative BPA test at week 6. Non-prophylactic oral antibiotics were rarely prescribed to study participants. The total number of prescriptions was five. In three out of five cases that antibiotics were prescribed, the BPA test was positive.

Discussion

Non-healing of chronic wounds continues to be a major issue for patients and healthcare professionals due to the risk of infection and further wound deterioration. There are BPA tests commercially available to determine if bacteria in wound ulcers are releasing (or stimulating host-derived increase in production of) protease enzymes that may pathologically impede the wound healing process.[14,22,23] Here, the following was assessed: the concordance between clinical opinion of wound infection, degree of association between a positive BPA test with patient and wound characteristics, and the openness of clinicians to change wound management in response to the BPA test result.

The BPA test utilised in this study, Woundchek Bacterial Status, showed a moderate – but significant - correlation with clinical opinion. In addition, the BPA test significantly correlates with wound characteristics that can be observed and measured but are typically not taken into account to determine that a wound is infected. The accompanying wound healing data indicate that wound chronicity, number, and type all contribute to delays in healing. The results of this study supports findings by others that Woundchek Bacterial Status detects

bacterial release of proteases, and that in a considerable number of cases this is not accompanied (yet) by clinical signs of infection.[9] These are very similar findings to those obtained previously with a different brand BPA test.[10,17] Since the exact substrate protease in each different brand test kit may differ, each BPA test kit needs to be appraised separately. Previous studies have already reported that not bioburden itself is necessarily a driving force for infection to occur; other patient-specific factors such as poor vascular supply and abnormal inflammatory response may facilitate bacteria to persist and release destructive proteases, or promote release of proteases by the host.[24,25] Although our results suggest that clinical opinion of infection mainly corresponds with hallmark signs of infection (erythema, purulence, odour), there have been others who have also linked it with extended wound features; for example, measured over longer observation periods, multiple lesions and wound chronicity have previously also been found to be associated with eventual wound infection of the lower limb.[25,26]

another outcomes study based on the Woundchek Bacterial Status BPA test has been reported on.[23,28] Baines and colleagues focused on selecting BPA positive wounds and then randomising them to either standard care or the application of antimicrobial dressing.[28] Despite applying similar inclusion and exclusion criteria to this present study, their BPA positive test rate was 100 out of 143 (70%) which is higher than the 39% positive test rate seen here. Furthermore, they reported higher rates of non-prophylactic oral antibiotics use, which was even higher in BPA test positive cases, than observed in our sample of patients. This may possibly reflect a long-standing recognition that there is a degree of variation in wound management despite the availability of national clinical guidelines.[29] Baines and colleagues found that wound healing did not improve with antimicrobial dressing treatment

compared to standard care over a 12-week period, though nursing time and antibiotics use were at least significantly lower.[28] Our study data on wound status and healing associated with a positive Woundchek Bacterial Status BPA test verifies earlier results obtained by Serena and colleagues.[23] They also observed that more chronic and larger ulcers were associated with a positive BPA test. A trend towards multiple lesions and a positive BPA test was also observed, but this was not significant (the vast majority of cases in that study were single lesions, reducing the statistical power of the analysis). A significant difference in wound healing, with BPA positive cases healing slower, was also noted. In this instance, the BPA test positive rate was similar at 38%.[23] By also comparing clinical opinion with BPA testing and by identifying risk factors for a positive BPA test, plus comparing wound healing rates between wounds negative and positive for a BPA test respectively, our study adds to the evidence base for the Woundchek Bacterial Status kit. The results of this study support the findings to date that ulcers with elevated bacterial protease activity are challenging to manage. Applying the test may provide quantitative evidence to clinical staff and test results may support them in discussions with patients.

There are a number of strengths and limitations to note, both in terms of the test kit used and the overall study design. In terms of the Woundchek Bacterial Status BPA test kit, this has the benefit that it incorporates a positive control to show the test process has been done correctly. Due to the COVID-19 pandemic, the lateral flow design of the kit is now common knowledge amongst both healthcare professionals and patients. Informal feedback from staff was that the test procedure did not cause delays in consultation times. The Woundchek Bacterial Status test appears reliable. The few failed tests experienced in this study – i.e. no positive control result showing - all occurred in the same clinic location

operated by the same staff; since batches of kits were distributed evenly amongst different locations, the issue may have been staff (incorrect) use of the kit, despite initial training on its use, rather than a faulty test kit batch. Due to the test's incubation time, in a busy NHS clinic a test cannot be readily redone. As with other test kits, staff do need to take care not to swab volumes of blood since this can affect test results. This study's prospective and single-blind design involving a large sample is a strength, though we did include a mixture of ulcers with different aetiologies. It reflects the caseloads seen in clinic and meant sufficient patients could be recruited in a reasonable timeframe. Best practice would have been to maintain a sufficiently detailed record for the number of patients considered and approached for the study. A comparison with microbiological testing of the wounds was not conducted, since this has been done previously for Woundchek Bacterial Status and it is now established that bacterial load does not correlate strongly with either clinical opinion or BPA test result status.[9,10] The application of Woundchek Bacterial Status testing in clinical practice is effectively an approach advocated by others previously, who recognise that infection can express itself through bacterial virulence (such as release of proteases) rather than purely the bioburden (e.g. biofilm) present in the wound.[11,30]

Conclusions

This study confirms that a considerable proportion of lower limb ulcers have an elevated protease status and may therefore be high-risk wounds for delayed healing and becoming clinically infected. A BPA test is often positive when clinical observation (through focus on any presence of erythema, purulence and odour) suggests a wound is not infected.

By corroborating evidence from another study[23] that the wider wound status (lesion number, chronicity, size) is a significant risk factor for a positive BPA test, clinicians can potentially use this as an initial stratification tool for the application of a BPA test. Since BPA positive ulcers have a significantly slower healing trajectory than BPA negative ulcers, testing can aid clinicians in deciding which patients may benefit from closer monitoring and proactive anti-microbial management to minimise the chance of complications. Pilot data from this study indicates an openness by clinical staff to adopt BPA testing. However, further – larger scale and longer term - evaluations are indicated to determine if (e.g. aggressive antimicrobial) wound management guided by BPA testing will translate in improved clinical and cost-benefit outcomes.

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Table 1, Overview of participants (total n = 124)

Patient variable	
Age, mean years (95% confidence interval)	74 (72 to 76)
Sex	Male: 74 (60%) Female: 50(40%)
Mobility level	Unable to walk: 16 (13%) Walk with assistance: 65 (52%) Walk unaided: 43 (35%)
Type 2 diabetes mellitus diagnosis	No: 67 (54%) Yes: 57 (46%)
Previous history of ulcer	No: 63 (51%) Yes: 61 (49%)
Wound variable	
Number of lesions	One lesion: 81 (65%) Two or more lesions: 43 (35%)
Wound type	Diabetic foot ulcer: 33 (27%) Venous leg ulcer: 49 (40%) Other aetiology: 42 (33%)
Ulcer chronicity	Less than 3 months: 73 (59%) Between 3 and 6 months: 14 (11%) More than 6 months: 37 (30%)

Table 2. Clinical opinion and Woundchek Bacterial Status test outcomes; outcome comparison (total n = 182)

Clinical Opinion (three outcomes)	**Woundchek Bacterial Status test result**	
	Negative	Positive
No infection	87 (48%)	33 (18%)
Possible infection	20 (11%)	26 (14%)
Infection	4 (2%)	12 (7%)
	Kendall's tau-c rank correlation coefficient = 0.32 (*P* <0.001)	

Table 3, Binary logistic regression analysis to assess relationship patient and wound characteristics with positive BPA test result or Clinical Opinion of infection.

Variables[a]	Dependent: Positive BPA test result			Dependent: Clinical opinion of infection[b]		
	Odd Ratio	95% CI[c]	*P* value[d]	Odd Ratio	95% CI[c]	*P* value[d]
Number of lesions	2.51	1.21 to 5.23	0.014			ns[e]
Wound type (DFU)	Reference for variable		0.018			ns[e]
Wound type (VLU)	0.43	0.16 to 1.14	0.091			ns[e]
Wound type (other)	0.31	0.14 to 0.71	0.006			ns[e]
Wound size (PUSH score)	1.19	1.07 to 1.32	0.002			ns[e]
Odour	2.53	1.44 to 4.44	0.001	3.82	1.95 to 7.49	<0.000
Purulence			ns[e]	10.98	4.00 to 30.19	<0.001
Patient age			ns[e]	0.96	0.93 to 0.99	0.035
Wound-related pain score			ns[e]	1.18	1.04 to 1.35	0.011
	Nagelkerke R^2 coefficient for model = 0.34			Nagelkerke R^2 coefficient for model = 0.47		

a: Backward elimination performed. Other variables in initial model: patient age, patient sex, wound chronicity, history of ulcer, patient mobility level, type 2 diabetes mellitus diagnosis, erythema.

b: Binary outcome for clinical opinion, no infection versus possible infection/infection

c: 95% CI = 95% confidence interval

d: Significant at $P < 0.05$

e: ns = not significant, eliminated at earlier stage of regression model.

Table 4, Principal Component Analysis to assess relationship patient and wound characteristics with positive BPA test result or Clinical Opinion of infection.

	Component # (% variance of model explained)			
	1 (23%)	2 (12%)	3 (9%)	4 (8%)
Purulence level	0.80			
Clinical Opinion of infection	0.78			
Erythema level	0.66			
Odour level	0.66			
Sex (reference: male)		0.68		
Patient age		0.61		
Patient mobility		-0.58		
Wound-related pain score		0.57		
Number of lesions			0.81	
Wound chronicity			0.63	
BPA test positive	0.42		0.43	
History of ulcer				0.70
Patient diabetic				0.66
Wound size (PUSH score)	0.49			-0.55

Figure 1, Flowchart overview of the number of patients and BPA testing regime

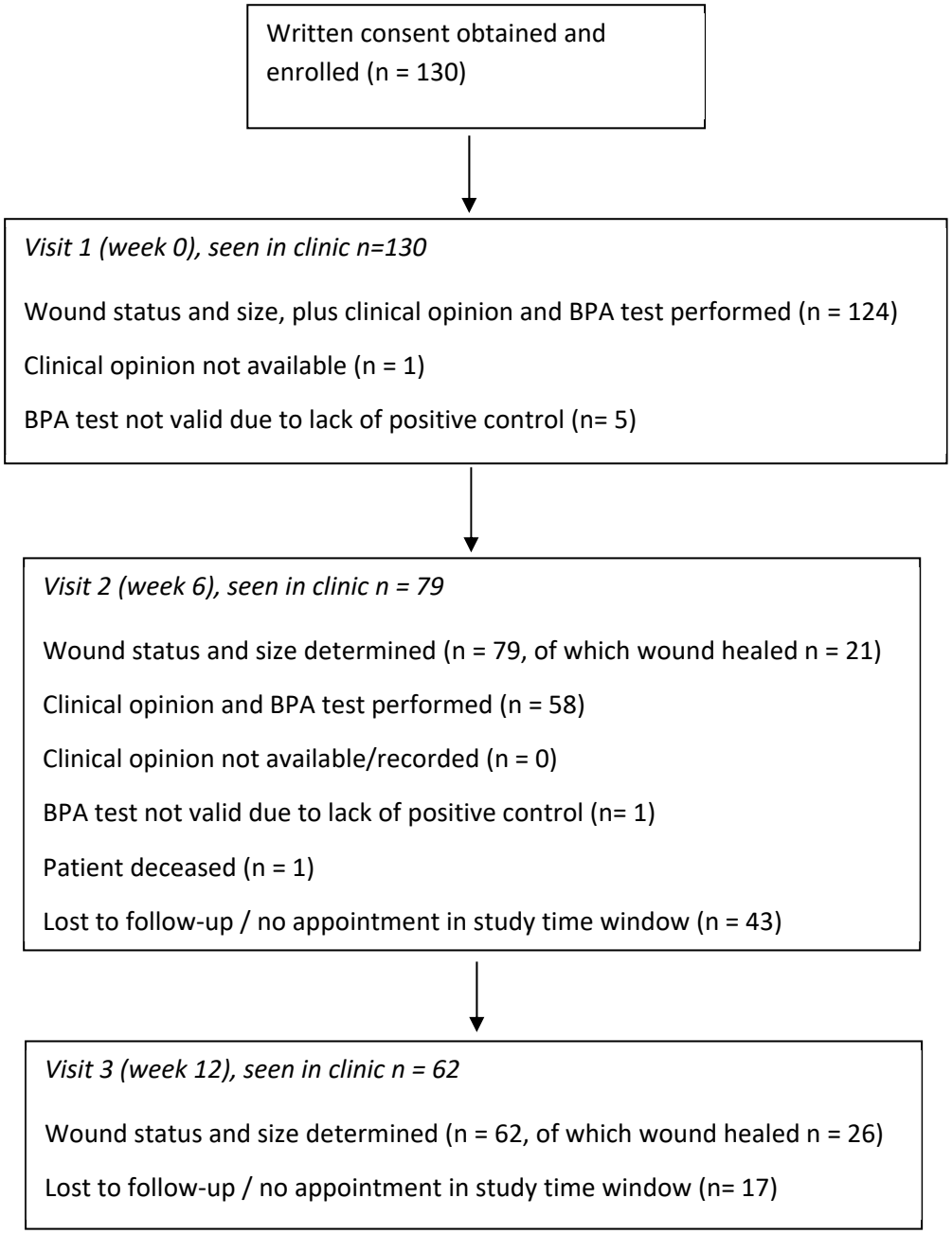

Figure 2, Box plot graph of wound size over time, stratified by BPA (Woundchek Bacterial Status) test result at week 0.