

SYSTEMATIC REVIEW

Protease activity as a prognostic factor for wound healing in complex wounds

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Abstract

Healing mechanisms are disrupted in complex wounds. Proteases may persist longer in nonhealing wounds. We sought to investigate whether protease activity, protease inhibitor activity, or their combinations are independent prognostic factors for healing of complex wounds. We searched MEDLINE, EMBASE, CINAHL, and *The Cochrane Library* to March 2019. Study selection comprised longitudinal studies assessing the independent effect of proteases, their inhibitors or ratios of the two, on healing of complex wounds, while controlling for confounding factors. Two reviewers independently extracted data and assessed risk of bias. We conducted meta-analyses separately for proteases, inhibitors, and ratios. We graded the evidence certainty (quality). We identified eight eligible studies in 10 cohorts involving 343 participants. Risk of bias was moderate or high. Elevated protease activity may be associated with less wound healing (standardized mean difference [SMD]: -0.41, 95% CI -0.72 to -0.11; nine cohorts); and elevated protease inhibitor activity with more healing (SMD: 0.37, 95% CI 0.06-0.68; five cohorts), this is low certainty evidence. Increased protease: inhibitor ratios may be associated with less healing (SMD -0.47, 95% CI -0.94 to -0.01; four cohorts), but this evidence is of very low certainty. Heterogeneity in protease activity was unexplained by prespecified subgroup analyses for wound type or protease activity status, but partially explained by protease class. Posthoc analysis suggested elevated levels of a particular protease, MMP-1, may be associated with more healing and other proteases with less healing. This is low/very low certainty evidence. Limitations were small included studies at moderate or high risk of bias, and the use of posthoc analyses. Elevated protease activity and protease: inhibitor ratios may be associated with less healing, and elevated inhibitor levels with more healing. There may be important differences between MMP-1 and other proteases. High quality research is needed to explore these new findings further.

Abbreviations: HNE, human neutrophil elastase; MMP, matrix metalloproteinase; SMD, standardized mean difference; TIMP, tissue inhibitor of MMPs.

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1 | INTRODUCTION

Complex wounds are open wounds that heal by the formation of new tissue, which is often described as “healing from the bottom up.” In this work, we focus on four types of complex wound: venous leg ulcers (VLU), pressure ulcers (PU), diabetic foot ulcers (DFU), and surgical wounds healing by secondary intention (SWHSI). People with complex wounds report that complete wound healing is the most important outcome to them.

Wound healing is a complex process involving coordinated interactions between diverse biological systems.¹ Proteases are active in all phases of wound healing, which is achieved via a cascade of precisely regulated steps and events that align with the appearance of various cell types in the wound bed during four distinct phases: haemostasis/coagulation, inflammation, proliferation/repair, and epithelialization/remodelling.²⁻⁴ The process of healing may be similar, regardless of the aetiology, or site of the wound.

Evidence from both human and animal studies suggests individual proteases may have varying roles at different times in the healing process.^{3,5,6} The principal proteases involved are the matrix metalloproteinases (MMPs) and the serine proteases, which in concert have the ability to break down all components of the extracellular matrix (ECM) such as collagens and elastin.^{2,4}

MMPs are divided into seven broad subtypes on the basis of their substrates and their chemical structure:³ collagenases (MMP-1, MMP-8, and MMP-13); gelatinases (MMP-2 and MMP-9); stromelysins (MMP-3, MMP-10, and MMP-11); metalloelastase (MMP-12); matrilysins (MMP-7 and MMP-26); membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25); and other MMPs which fail to sit clearly in any of the described categories. In healthy tissue, MMP expression is limited but is rapidly induced by an array of molecules, including cytokines and growth factors released on, for example, wounding.⁷ MMPs are released into the ECM as latent pro-enzymes, requiring activation, which is achieved via multiple mechanisms, including self-catalysis and digestion by other members of the same enzyme family.⁷ Control of MMP activation is complicated by the role of other molecules that inhibit the action of MMPs, most notably the tissue inhibitors of MMPs (TIMPs). These are small molecules, which are promiscuous in their stoichiometric binding of multiple MMPs to regulate the activity of the degrading enzymes, hence, the balance of proteases and their inhibitors may be important in wound healing.⁴

The main serine proteases are mast cell tryptase and chymase, plasmin, human neutrophil elastase (HNE), cathepsin G, urokinase-type plasminogen activators (uPA), and tissue-type plasminogen activators (t-PA).⁸ Other protease biomarkers include modular proteins that combine proteases with other biological species, which also break down collagen: for example, the families of A disintegrin and metalloproteinase (ADAM),⁹ and ADAM with thrombospondin motifs (ADAMTS).¹⁰ Complex wounds also contain proteases associated with several types of bacteria and there may be synergy between proteases from host and the skin-resident microbiome.^{4,11-14}

In complex wounds in humans, where healing mechanisms are disrupted, some proteases are reported to persist for longer than in normally healing wounds.^{4,15,16} It is possible that any association of protease level with delayed wound healing may be a general wound phenomenon, although differences between wound types have been reported.^{4,17}

This systematic prognostic factor review expands on previous work,¹⁸ and aims to investigate whether protease activity, protease inhibitor activity or combinations of the two are independent prognostic factors for wound healing in people with complex wounds. Studying prognostic factors for wound healing has a threefold purpose,¹⁹ helping us to: understand mechanisms related to healing; identify wounds at increased risk of nonhealing (which may permit selective treatment of these wounds if the prognostic factor is modifiable); and monitor response to therapy.

2 | METHODS

2.1 | Criteria for considering studies for this review

We included reports of prospective and retrospective longitudinal studies in humans that measured, or gave sufficient data, to assess the independent effect of individual proteases on the healing outcome while controlling for key confounding factors. We determined these following both consideration of pathophysiological processes and possible pathways to nonhealing at the cellular level, and also from a review of the literature (see methods in Westby et al¹⁸). Key confounders were considered to be age, sex, BMI, infection, and smoking. Controlling for confounders meant either taking account of these factors in the analysis or in the design of the study. For example, the study design could involve matching or stratification or limited study eligibility (such as having a narrow age range). Alternatively, the analysis could involve multivariable regression with adjustment for the key confounding factors or their omission from the analysis because they are not significantly associated with the outcome in univariate analyses. We included studies reporting multivariable analyses regardless of the adjusting factors involved. In the absence of formal matching or regression analysis, we also included studies with data on univariate associations of protease activity with wound healing, provided the study also reported data for healing and nonhealing groups for at least some of the key confounding factors. We did not include studies that solely reported univariate associations between the prognostic factor and the outcome, with no consideration of key confounding factors.

We included studies with any follow-up period. The study design could be cohort studies or randomized controlled trials (RCTs) analyzed as cohort studies. We excluded cross-sectional studies because the prognostic association under study is inherently longitudinal and there is likely to be a substantial risk of bias associated with reverse causation. We did not include case-control studies



(unless there were no cohort studies) because of the high risks of recall bias. We excluded studies with a sample size of fewer than 10 people, taking into consideration experience from our previous review¹⁸ and rules of thumb for estimating regression coefficients.²⁰

We included studies of people with the following types of complex wounds, accepting authors' definitions of these wounds: VLUs, DFUs (Wagner grade I or above), PUs that are at least stage II, and SWHSI (whether by design or following dehiscence).

We included studies that assessed as prognostic factors any type of protease or combination of proteases, any type of specific protease inhibitor (eg, TIMP-1 to TIMP-4) and combinations of proteases and their inhibitors, either from the host or from bacteria. The prognostic factor could be examined as a continuous or categorical variable with any cut-off point. A prognostic factor can be defined as any measure that, among people with a given health condition (ie, a startpoint), is associated with a subsequent clinical outcome (an endpoint),¹⁹ therefore we only included studies if the prognostic factor was measured at an earlier time than the outcome, preferably at study baseline. Prognostic factors could be measured at baseline only or change from baseline to a second time point, with wound healing measured at a third (later) time point.

Protease activity is measured in both wound fluid and wound tissue using various biochemical tests, including gelatin zymography, quenched fluorescence substrate hydrolysis, and enzyme-linked immunosorbent assays (ELISAs). A range of methods of obtaining wound fluid has been used and these methods vary with the type of protease measured. We permitted any approach to obtaining samples from wounds, including wound fluid and tissue samples. We did not include measures of proteases in the blood, urine, saliva, or other systemic fluids. We excluded studies solely measuring pro-proteases or other bound forms (because the active form is required for enzymatic action).^{7,11}

Our primary outcomes were: (a) time to healing (analyzed by time-to-event analysis) and (b) the proportion of people with wounds completely healed, at any follow-up duration. If there were insufficient complete wound healing data reported for a particular association, we included data on the change (and percentage change) in wound size, either reported as continuous outcomes or dichotomised with any cut-off point. We termed changes in wound size or area as "partial healing."

2.2 | Search strategy

We used a search strategy developed for a scoping review of all wound biomarkers, based only on population terms and biomarker terms. One review author conducted a preliminary assessment of the titles and abstracts of retrieved records and then two authors assessed the filtered abstracts and titles for protease biomarkers. We searched MEDLINE, EMBASE, CINAHL, and the Cochrane Central Register of Controlled Trials (CENTRAL) up to March 18, 2019; the MEDLINE search strategy is shown in Appendix 4.

2.3 | Data extraction and assessment of risk of bias

Data were extracted by one reviewer into a piloted data extraction spreadsheet, and checked by a second. Data extracted included the following: study design, participant details (especially those of the key confounding factors); ulcer type, size and duration; details of each prognostic factor (measurement methods, whether the active form was measured, type of data (eg, continuous/any cut-off points); details of each outcome; type of analysis and how the study accounted for the key confounding factors; duration of follow-up and loss to follow-up; association statistics or raw data where no regression analysis was conducted.

Two reviewers independently appraised the included studies using an approach based on the Quality In Prognosis Studies (QUIPS) tool, which is appropriate for prognostic factor review questions.^{21,22} We assessed risk of bias per study for each protease-outcome combination, considering six domains: study participation, study attrition, prognostic factor measurement, outcome measurement, study confounding, and statistical analysis and reporting. Each domain was rated as having high, moderate, or low risk of bias. For the study participation domain, we assessed representativeness of the population, also assigning studies with fewer than 8 weeks' follow-up to be at high risk of bias, a duration set a priori based on clinical experience of healing times for complex wounds, alongside evidence from time to healing plots (eg, Ashby et al.²³ 2014): this duration is expected to be insufficient to capture healing, such that any population that did heal within that time would not be representative. For the outcome measurement domain, we considered the validity of partial healing measures (such as 50% reduction in size), but were careful to avoid double counting with study participation (because these measures typically had short follow-up times). For the prognostic factor measurement domain, we considered whether the study measured solely the active form of protease, or reported 'total' protease levels (the combination of both active and bound forms): activation of proteases is needed for enzymatic action.^{7,11} We assigned low risk of bias only when the active form of protease was measured and more than one measurement method was used. If total protease levels were reported we assigned moderate risk of bias. For the study confounding domain, we considered how studies had taken account of the following key confounding factors (identified on the basis of literature risk factors and/or clinical or basic science experience): age, sex, BMI, smoking, and infection. We assessed risk of bias in this domain in terms of the proportion of key confounding factors accounted for, taking into consideration how accounting had been done (which could include regression analysis; reporting a lack of difference between unhealed and healed groups; stratification; and a narrow inclusion criterion). For multivariable analyses, we reported all adjusting factors included in the regression analysis. To assess the "statistical analysis and reporting" domain, we considered whether the analyses took into account treatment type (protease modulating vs not protease modulating). We also assigned moderate risk of bias when the study only reported the median and IQR for the *t*-test analysis (see Section 2.4).

We defined an all-domain risk of bias per study for each prognostic factor, taking into account all the above domains. We assigned all-domain low risk of bias if all domains had low risk of bias; moderate if there was high risk of bias for one domain or moderate risk of bias for at least two domains (with the rest as low risk of bias); and high risk of bias if there was high risk of bias for at least two key domains. This assessment of overall risk of bias is based on guidance in the literature.^{22,24,25}

2.4 | Data synthesis

At the outset, we stratified the analyses into three protease groups, recognizing that (a) proteases, (b) their inhibitors, and (c) ratios of proteases to inhibitors were very different potential biomarkers. We also initially separated complete and partial healing (dichotomous) outcomes, but investigated the appropriateness of this separation in subgroup analyses.

Studies reported their available data in different ways: outcomes and protease activity could each be presented as dichotomous or continuous data, and combinations of these required different analytical approaches, detailed in Table 1. We would have preferred to synthesize the data by meta-analyzing the results from multivariable logistic regression or Cox regression analyses, in which the independent variable is continuous protease activity and the dependent variable is the dichotomous healing outcome, and account is taken of confounders. However, this analysis was rarely reported by the studies and we did not have individual participant data to allow us to perform regression analyses.¹⁹ Consequently, we took an alternative analytical approach: all included studies summarized protease activity as the mean and SD for each of the healing and nonhealing outcome 'groups'. Therefore, we conducted a *t*-test analysis for each study, with the continuous prognostic factor (protease activity) as the dependent variable and the

dichotomous healing outcome as the independent variable, and then we meta-analyzed the results as appropriate. The *t*-test determines whether the mean protease activity differs between people with healing and nonhealing wounds. Statistically, when considering univariate associations, the *t*-test and logistic regression models can be shown to be compatible. We also note the use by other studies of the *t*-test in comparing baseline characteristics by outcome category for continuous predictors, and in identifying candidate prognostic factors for multivariable logistic regression analysis (eg, Antwi et al.²⁶ 2017). Our application of the statistical *t*-test enabled data synthesis, but the results require careful interpretation: a negative mean difference in the protease levels indicates that lower baseline levels of protease are associated with higher proportions of healing wounds at follow-up (compared with nonhealing).

We conducted a random effects meta-analysis for the *t*-test analysis. When protease levels were reported differently in studies (eg, as concentration per quantity of total protein or as activity per milliliter), we analyzed the *t*-test results as standardized mean differences (SMD), and reported the meta-analysis findings as the summary SMD with its 95% confidence interval. Where studies reported results for more than one protease, we included all results, recognizing the limitation that there may have been multiple counting. Where protease level data were reported as median and IQR, we converted the data to mean and SD using the method of Wan et al.²⁷ We planned to apply the Cohen interpretation of the size of the association according to the SMD numerical value, such that 0.2 represents a small association, 0.5 a moderate association, and 0.8 a large association,²⁸ but in this exploratory study, we were more concerned with investigating the direction and heterogeneity of association.

We presented individual study findings on forest plots, stratifying by protease type and outcome definition; we grouped study data across all wound types, protease activity measurements, individual proteases and types of sample. The decision on whether to pool data was based on clinical and methodological heterogeneity in these variables.

We assessed heterogeneity by visually inspecting the forest plots, examining the overlap of confidence intervals and the variation of point estimates, as well as statistical measures (Chi-squared test and the I^2 measure), taking into consideration the fact that some studies reported results for more than one protease. We considered whether the definition of healing (complete or partial) gave important differences and combined these data where results were similar, reassessing the overall heterogeneity.

2.5 | Subgroup analyses and sensitivity analyses

First, we carried out sensitivity analyses to explore the impact of all-domain risk of bias, restricting the analyses to studies rated as having moderate or low risk of bias. If this did not explain any observed heterogeneity, we conducted a number of a priori subgroup analyses by: (a) type of wound (DFU, VLU, PU, and SWHSI); (b) type of protease - MMP collagenases (MMP 1 and MMP-8), MMP gelatinases (MMP-2

TABLE 1 Summary of analyses undertaken based on available data

Healing measure	Protease measure	
	Dichotomous	Continuous
Dichotomous (including categorical which had been dichotomized ^a)	Logistic or Cox regression Chi-squared test using raw data (calculated from sensitivity and specificity ^b)	Logistic or Cox regression <i>t</i> -test using raw data (mean and standard deviation for healed and unhealed groups)
Continuous	Linear regression	Linear regression; Correlation

^aWhere healing data were reported as categorical data, we dichotomized them, selecting the highest cut-off point (in order to approximate more closely complete healing).

^bWhere studies reported sensitivity and specificity data for a protease cut-off level, we transformed these data into 2 × 2 tables allowing us to calculate the risk ratio and its 95% confidence interval.

and MMP-9), other MMPs, and serine proteases; (c) protease activity (active only, and mixed active and latent); (d) presence or absence of infection at baseline; (e) type of sample (wound fluid or wound tissue); and (f) type of treatment (protease modulating or other). We explored all of these subgroup analyses, provided there was at least one study per subgroup, applying the test for subgroup differences to determine whether splitting explained any observed heterogeneity (including the absence of residual heterogeneity in subgroups).²⁹

One of the heterogeneity assessments suggested possible differences in the direction of association for one individual protease and we conducted an exploratory posthoc subgroup analysis by individual proteases to investigate this.

2.6 | Summary of findings table and GRADE assessment

We used an approach modified from the GRADE framework to assess the summarized evidence for overall quality (certainty) for each protease-outcome combination.^{22,24,25} GRADE defines the certainty of a body of evidence as the extent to which one can be confident that an association is close to the true quantity of specific interest. We rated the overall strength of evidence as high, moderate, low or very low, considering within-study risk of bias, indirectness of the evidence, inconsistency, imprecision of association estimates and risk of publication bias.^{24,29,30} We also considered two “upgrading” factors, large association and “dose effect”, although we note that high risk of bias may artificially lead to larger recorded associations. For assessing overall risk of bias in a meta-analysis, we considered the weight of each study and its risk of bias. For imprecision, we considered the total number of events (dichotomous outcomes) or participants (continuous) across all relevant studies, and also noted whether the summary CI included no effect and clinically meaningful benefits or harms. For assessing inconsistency, we considered the variation in the direction and magnitude of the point estimates, alongside heterogeneity statistics of I^2 and the P -value.

We present the main results of the review in ‘Summary of findings’ tables, which give key information concerning the certainty of the evidence, the magnitude of the associations examined and the sum of the available data, and include an overall grading of the evidence.^{29,30} For completeness, we included different types of analyses as separate rows in the Summary of findings tables, even though they are reporting different approaches to the same association.

3 | RESULTS

The search generated 14 737 records (for all biomarkers): we obtained 93 full papers; 10 studies had two reports, leaving 83 possible studies (see Figure 1). Eight studies (described in 11 reports) were deemed to meet the study inclusion criteria (Appendix 1). We excluded, and listed separately a further eight studies which reported only univariate results and took no account of key confounding factors and so were not considered further (Table A2 in Appendix 1). We excluded

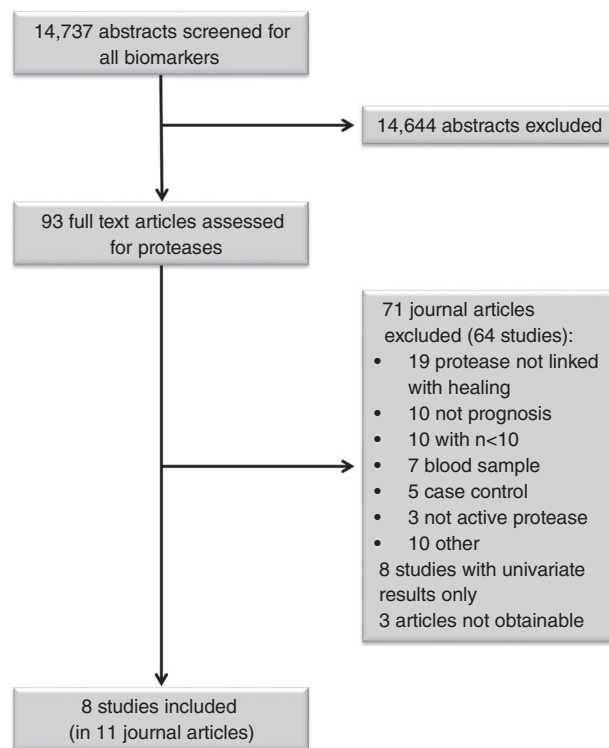


FIGURE 1 Flow of studies

64 studies (see Figure 1 for reasons). Nine of the 11 studies in our earlier review focusing on protease activity in VLU were excluded, either because of more stringent inclusion criteria in the current review (five studies) or because they did not take account of confounders (four studies).¹⁸

3.1 | Included studies

Eight studies, with 343 participants, met the inclusion criteria for the review.^{31–38} One study split the participants into two cohorts;³² (a) a “discovery” set formed from people with “rapidly healing” wounds (healed after 6 months) and an age and sex-matched set of people with non-healing (deteriorating) wounds, and (b) a “validation” set formed from the remaining participants. One RCT combined the results for two randomized arms (protease modulating matrix and placebo)³⁵ and another reported results separately for each randomized group (doxycycline and placebo)³⁷, giving a total of 10 cohorts. The size of the cohorts was small, with a median (overall range) of 31.5 (16–62) participants.

Six cohorts (five studies) took account of at least half the prespecified confounding factors either in the study design or analysis^{31–34,36} and four cohorts (three studies) considered less than half of the key confounding factors.^{35,37,38} Three studies conducted a multi-variable analysis.^{31,32,34}

Seven cohorts (six studies) involved people with DFUs and three cohorts (two studies) involved people with VLUs.^{37,38} No studies were identified in people with PUs or SWHSI. See Table A1 (Appendix 1) for more detail.

Seven studies (eight cohorts) measured wound protease levels; some examining more than one protease: MMP-1;^{31,33,35} MMP-2;^{31,34} MMP-8;³¹ MMP-9;^{31,33-35,37,38} the serine protease, HNE;^{35,36} and a combination of proteases, MMP-9 plus HNE.³⁵ Five cohorts (four studies) investigated protease inhibitors: TIMP-1^{31,33,34} and the serine protease inhibitor, Serpin B3.³² Four cohorts (four studies) investigated ratios of proteases with protease inhibitors: MMP-1:TIMP-1,^{31,33} MMP-2:TIMP-1,³⁴ MMP-9:TIMP-1,³³⁻³⁵ giving a total of 27 cohort-protease combinations.

Four cohorts^{31,33-35} reported results separately for the active form of at least some proteases (MMP-9,^{31,33} HNE,³⁵ MMP-2, and MMP-9³⁴) and the remainder reported total levels of proteases or used ELISA analysis, which we assumed measured the total level unless stated otherwise. See Table A1 for more detail.

Three cohorts in 112 people reported complete healing as a dichotomous measure;³²⁻³⁴ and three cohorts reported partial healing as a dichotomous outcome.^{31,32,35} Three further included cohorts (two studies) compared two different degrees of healing,^{36,37} and one reported categorical healing (high, low, and no healing),³⁸ which we dichotomised into high healing vs low and no healing combined. See Table A1 for more detail. Three studies additionally reported the outcome as a continuous measure, the percentage change in size from baseline.^{31,33,34}

3.2 | Risk of bias

Figure 2 shows risk of bias judgements for each cohort-protease combination, also indicating differences according to analysis approach; full details are given in Table A3, Appendix 1.

For four cohorts (three studies) we assigned high all-domain risk of bias for each protease^{35,37,38} and two cohorts had high all-domain risk of bias for one protease.^{31,34} Otherwise, the all-domain risk of bias was moderate; no cohorts had low all-domain risk of bias.

3.3 | Summary of data and investigation of heterogeneity

All cohorts reported continuous protease data and accompanying dichotomous healing data, and this formed our main analysis (using the *t*-test approach). Results are shown in Figure 3, stratified into proteases, protease inhibitors and ratios of proteases: inhibitors, and, in terms of outcomes, initially separated into complete healing vs non-healing and partial healing vs nonhealing. Three cohorts (three studies) had results for more than one protease^{31,33,35} and one cohort (one study) investigated more than one ratio of protease to inhibitor.³³

3.3.1 | Protease activity as a prognostic factor for healing

t-test analysis

For the *t*-test analysis, eight cohorts (266 participants) investigated whether there was any difference in protease activity (continuous)

	Selection bias	Attrition bias	Prognostic factor measurement	Outcome measurement	Adjustment factors	Analysis and reporting	ALL DOMAIN RISK OF BIAS
KEY ● High risk of bias ■ Moderate ◇ Low							
HNE_Fadini 2016_DFU	■	◇	■	■	■	■	■
HNE_Gottrup 2013_DFU	●	◇	■	■	■	■	■
MMP1_Gottrup 2013_DFU	●	◇	■	■	■	■	■
MMP1_Luanraksa 2018_DFU	■	◇	■	■	■	■	■
MMP1_Muller 2008_DFU	●	◇	■	■	■	◇	■
MMP2_Liu 2009_DFU	■	◇	■	■	■	■	■
MMP2_Muller 2008_DFU	●	◇	■	■	■	■	■
MMP8_Muller 2008_DFU	●	◇	■	■	■	■	■
MMP9_Gottrup 2013_DFU	●	◇	■	■	■	■	■
MMP9_Liu 2009_DFU	■	◇	■	■	■	■	■
MMP9_Luanraksa 2018_DFU	■	◇	■	■	■	■	■
MMP9_Muller 2008_DFU	●	◇	■	■	■	■	■
MMP9_Serra 2013_VLU	■	◇	■	■	■	■	■
MMP9_Serra 2015_dox_VLU	■	◇	■	■	■	■	■
MMP9_Serra 2015_plac_VLU	■	◇	■	■	■	■	■
MV_Fadini 2014_Serpin_DFU	●	◇	■	■	◇	■	■
MV_Liu_MMP9_TIMP1_DFU	●	◇	■	■	■	■	■
MV_Muller_MMP1 to T1_DFU	●	◇	■	■	■	■	■
Ratio_MMP1:T1_Luanraksa_DFU	■	◇	■	■	■	■	■
Ratio_MMP1:T1_Muller_DFU	●	◇	■	■	■	■	■
Ratio_MMP2:T1_Liu_DFU	■	◇	■	■	■	■	■
Ratio_MMP9:T1_Gottrup_DFU	●	◇	■	■	■	■	■
Ratio_MMP9:T1_Liu_DFU	■	◇	■	■	■	■	■
Ratio_MMP9:T1_Luanraksa_DFU	■	◇	■	■	■	■	■
Serpin_Fadini 2014(1)_DFU	●	◇	■	■	■	■	■
Serpin_Fadini 2014(2)_DFU	●	◇	■	■	◇	■	■
Sum_HNE_MMP9_Gottrup_DFU	●	◇	■	■	■	■	■
TIMP1_Liu2009_DFU	■	◇	■	■	■	◇	■
TIMP1_Luanraksa2018_DFU	■	◇	■	■	■	■	■
TIMP1_Muller2008_DFU	●	◇	■	■	■	■	■

FIGURE 2 Risk of bias per study and protease

for each healing status group (dichotomous; Figure 3). For each of the complete healing and partial healing outcomes, the pooled results suggested, on average, that higher levels of protease at study baseline may be linked with less healing, but estimates were imprecise.

The statistical test for subgroup differences suggested there is no significant difference between complete and partial healing outcomes ($I^2 = 0\%$, $P = 0.94$), so these studies were pooled for subsequent sensitivity analyses and prespecified subgroup analyses.

The random effects summary statistics for the meta-analysis with pooled complete and partial healing were, SMD: -0.41 (95% CI -0.72 to -0.11), with I^2 inconsistency of 48% ($P = .02$) (Figure A1 in Appendix 2 and Table 2). The negative SMD indicates that lower baseline levels of protease may be associated with higher proportions of healing wounds at follow-up (compared with nonhealing). The evidence certainty is low, downgraded once for risk of bias and once for inconsistency and some imprecision. Sensitivity analysis by risk of bias (restricting to low and moderate all-domain risk of bias) did not explain the heterogeneity amongst the protease associations: the summary estimate decreased, SMD (random effects) was -0.29 (95% CI -0.79 to 0.20) and I^2 increased to 64% ($P = .007$; Table 2).

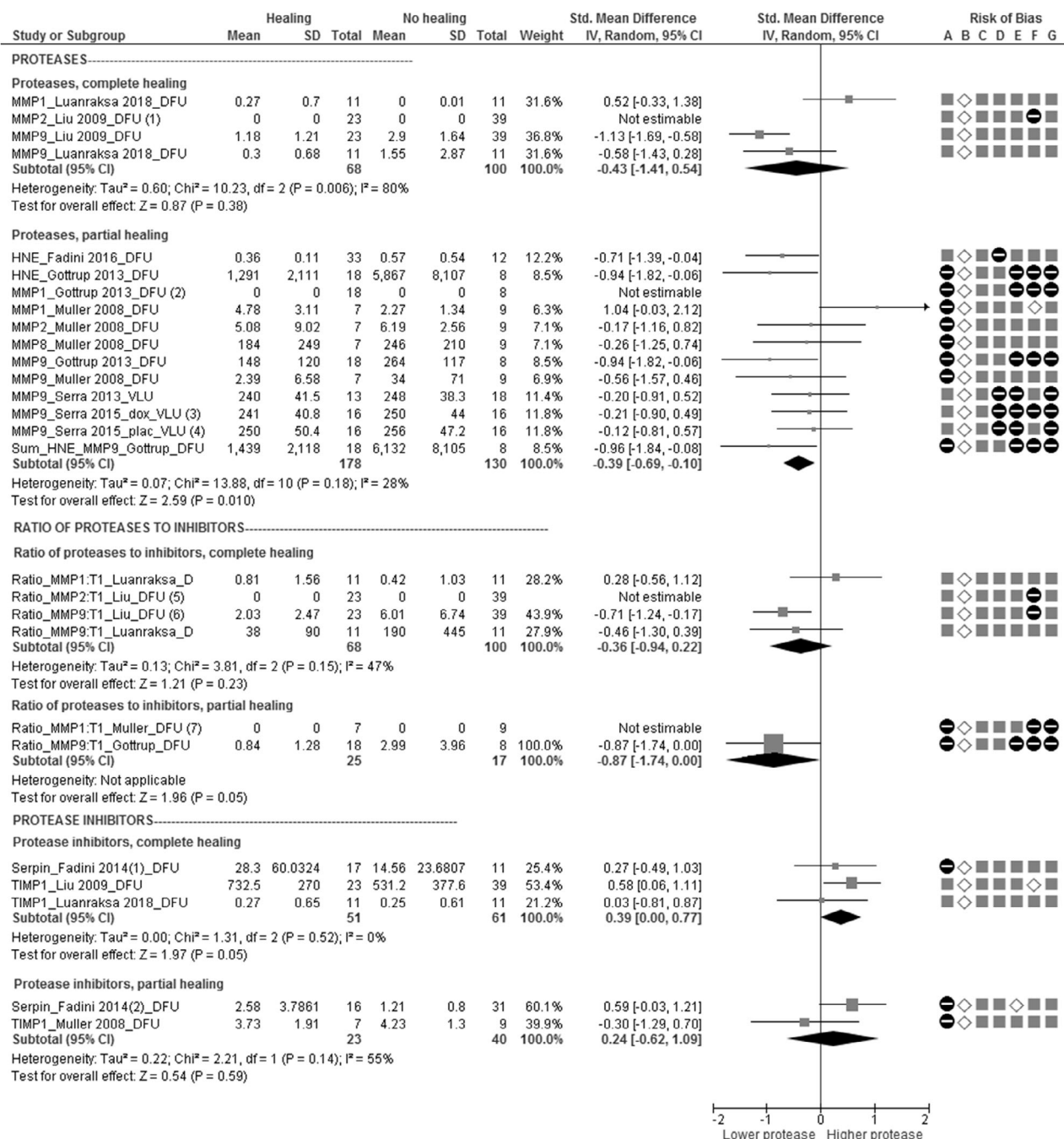


FIGURE 3 All results for dichotomous healing and continuous protease

Key (risk of bias): A - Selection bias; B - Attrition bias; C - Prognostic factor measurement bias; D - Outcome measurement bias. E - Adjustment/confounding bias; F - Analysis and reporting bias; G - All-domain risk of bias. Symbols: white diamond = low risk of bias; grey square = moderate risk of bias; black circle with white line = high risk of bias.

Footnotes: (1) "not significant" and 1.5 times higher for unhealed; (2) "no significant difference" between responders and non-responders; (3) and (4) standard error provided, number healed assumed; (5) "attained significance" and 3-fold increase for unhealed versus healed; (6) we assumed the reported 'SD' was the 'SE' (7) "not significantly different in healers/non healers"; $P = 0.064$

Prespecified subgroup analyses undertaken for associations of proteases with healing are described in Table 3. None of the subgroup analyses completely explained the observed heterogeneity in direction of association: the results for DFU and VLU wound types, and the results for active and total protease activity subgroups were qualitatively in the same direction; there was also residual

heterogeneity in one of the subgroups for each subgroup analysis. The subgroup analysis by protease class partly explained the observed heterogeneity, with increased collagenase activity mainly favoring healing in these data and the opposite direction of association for the other protease classes. Some residual heterogeneity in the direction of association remained within the collagenase class ($I^2 = 36\%$), which may be

TABLE 2 Summary of findings table for all proteases, all protease inhibitors, and all ratios of proteases-to-inhibitors

Elevated activity of proteases, protease inhibitors and ratios of protease: inhibitor, and their associations with healing.				
Population: people with complex wounds.		Setting: any.	Prognostic factor: Protease activity.	Outcome: healing.
Outcomes/analysis	Associations (95% CI): random effects	Participants (studies)	Evidence quality (GRADE)	Comments
Proteases				
Design part-adjusted for confounders; <i>t</i> -test investigation of protease level and "healing" (dichotomous) association. Outcomes: (a) high healing vs immediate + no healing at 8 weeks (b) at least % reduction in size of 50% at 4 weeks (c) complete healing at 12 weeks ³³⁻³⁶	MMP-1, MMP-2, MMP-8, MMP-9, HNE, sum of HNE, and MMP-9 <ul style="list-style-type: none"> SMD for all proteases: −0.41 (−0.72 to −0.11) One cohort had results from four proteases³¹, one from three proteases³⁵, and one from two proteases³³ Sensitivity analysis (restricted to moderate all-domain risk of bias), SMD: −0.29 (95% CI −0.79 to 0.20) Separating the combination of proteases: <ul style="list-style-type: none"> for sum of HNE and MMP-9 (one study): SMD: −0.96 (−1.84 to −0.08)³⁵ for the remainder (MMP-1, MMP-2, MMP-8, HNE): SMD: −0.37 (−0.69 to −0.06) 	266 (seven studies, eight cohorts) HNE + MMP-9: 26 (1 study)	⊕⊕⊖⊖ LOW ^a	Most studies suggested higher levels of proteases at baseline may be associated with less healing, but there is some heterogeneity in the direction of association. Overall, $I^2 = 48\%$, $P = .02$ For the sensitivity analysis by risk of bias, $I^2 = 64\%$, $P = .007$ Prespecified subgroup analyses only partly explained the inconsistency Figure A1
Design part-adjusted for confounders; association of healing (dichotomous) with elevated protease level (dichotomous): Outcome: complete healing at 12 weeks ³³	MMP-9 activity; threshold above 0.38 pg/μg protein <ul style="list-style-type: none"> RR 0.40 (0.16-0.96) Probability of healing below the threshold: 778 per 1000 wounds Probability of healing above the threshold: 311 per 1000 (124-746) 	22 (1) 11 healed	⊕⊖⊖⊖ VERY LOW ^b	High vs low levels of proteases at baseline may be associated with healing in 467 fewer wounds per 1000 (from 654 to 32 fewer) Figure A2
Design part-adjusted for confounders; Correlation coefficients: Outcome: % reduction in area at 4 weeks ³³	MMP-9 total activity <ul style="list-style-type: none"> Correlation coefficient: −0.53 (−0.90 to −0.15) 	22 (1)	⊕⊖⊖⊖ VERY LOW ^b	Higher levels of proteases may be associated with less healing (% reduction in area at 4 weeks) Figure A3
MULTIPLE linear regression: Outcome: % change in area per day at 4 weeks ³⁴	No results, but model that included active MMP-9, pro-MMP-9, and TIMP1 accounted for 32% of the variance	62 (1)	⊕⊖⊖⊖ VERY LOW ^c	Figure A3
Protease inhibitors				
Design part-adjusted for confounders; <i>t</i> -test association of protease inhibitor level and "healing" (dichotomous): Outcomes: (a) complete healing at 12 weeks or 6 months (b) % reduction in size of at least 82% at 4 weeks ³¹⁻³⁴	TIMP-1 and Serpin B3: <ul style="list-style-type: none"> SMD: 0.37 (0.06-0.68) One result per cohort 	175 (four studies, five cohorts) 74 healed	⊕⊕⊖⊖ LOW ^d	Most studies suggested higher levels of protease inhibitors at baseline may be associated with more healing, but there is some heterogeneity in the direction of association. Overall, $I^2 = 0\%$, $P = .47$ Figure A1
MULTIVARIABLE logistic regression: Outcome: "healing" (improved versus not improved at 6 months; reference 32, cohort 2 ³²)	Serpin B3 Odds ratio per unit increase in Serpin B3/total protein (ng ml ^{−1} μg ^{−1}):3.5 (1.1-11.9)	47 (1) 16 healed	⊕⊖⊖⊖ VERY LOW ^e	Higher levels of Serpin B3 may be associated with more healing. Adjusted for coronary artery disease (only significant factor)
Design part-adjusted for confounders; association of healing (dichotomous) with elevated protease inhibitor level (dichotomous): Healing (improved versus not improved at 6 months); reference 32, cohort 2 ³²	Serpin B3; threshold above 1.13 ng ml ^{−1} μg ^{−1} <ul style="list-style-type: none"> RR 2.9 (1.1-7.6) Probability of healing below the threshold: 174 per 1000 wounds 	47 (1) 16 healed	⊕⊖⊖⊖ VERY LOW ^e	High vs low levels of Serpin B3 at baseline may be associated with healing in 327 more wounds per 1000 (from 15 to 826 more) Figure A2

TABLE 2 (Continued)

Elevated activity of proteases, protease inhibitors and ratios of protease: inhibitor, and their associations with healing.				
Population: people with complex wounds.		Setting: any.	Prognostic factor: Protease activity.	Outcome: healing.
Outcomes/analysis	Associations (95% CI): random effects	Participants (studies)	Evidence quality (GRADE)	Comments
	<ul style="list-style-type: none"> Probability of healing above the threshold: 501 per 1000 (189-1000) 			
<i>Ratio of proteases to inhibitors</i>				
<i>Design part-adjusted for confounders; t-test association of protease/inhibitor ratio and "healing" (dichotomous):</i> Outcomes: <ul style="list-style-type: none"> Complete healing at 12 weeks At least 50% reduction in size at 4 weeks^{31,33-35} 	MMP-1/TIMP-1, MMP-2/TIMP-1 and MMP-9/TIMP-1: <ul style="list-style-type: none"> SMD: -0.47 (-0.94 to -0.01) One study contributed results for two protease-to-inhibitor ratios³³ Additional studies without numerical data: <ul style="list-style-type: none"> Threefold higher MMP-2/TIMP-1 ratio for unhealed (compared with healed) No significant difference for MMP-1/TIMP-1 	126 (four studies with six results for different ratios, two without numerical data) 52 healed	⊕⊕⊕⊕ VERY LOW ^f	Most studies suggested higher levels of protease/inhibitor ratios may be associated with less healing, but there may be heterogeneity in the direction of association, represented by one study. Overall $I^2 = 35\%$, $P = .20$ Figure A1
<i>MULTIVARIABLE logistic regression:</i> Outcome: "healing" (at least 82% reduction in size at 4 weeks) ³¹	MMP-1/TIMP-1 ratio: <ul style="list-style-type: none"> No numerical data Study stated that "ratio is a predictive factor of healing independent of wound area and depth" 	16 (1) 7 healed	⊕⊕⊕⊕ VERY LOW ^b	Insufficient information
<i>Design part-adjusted for confounders; association of healing (dichotomous) with elevated protease/inhibitor ratio (dichotomous):</i> Outcomes: <ul style="list-style-type: none"> Complete healing at 12 weeks³³ At least 85% reduction in size at 4 weeks³¹ 	MMP-1/TIMP-1; threshold above 0.056 ³³ and above 0.39. ³¹ MMP-9/TIMP-1; threshold above 9.06 ³³ Wide range of findings: <ul style="list-style-type: none"> MMP-9/TIMP-1: <ul style="list-style-type: none"> RR 0.33 (0.14-0.78) Probability of healing below the threshold: 875 per 1000 wounds Probability above the threshold: 289 per 1000 (123-683) MMP-1/TIMP-1: <ul style="list-style-type: none"> RR 2.1 (0.9-4.7)³³ and RR 4.2 (1.2-15.1)³¹ Probability of healing below the threshold 357 per 1000³³ and 200 per 1000³¹ Probability above the threshold 750 per 1000 (336-1000)³³ and 834 per 1000 (230-1000)³¹ 	38 (two studies, one with two proteases) 18 healed	⊕⊕⊕⊕ VERY LOW ^g	Very serious inconsistency precludes summarizing the findings. There may be a large difference between MMP-1/TIMP-1 ratio and MMP-9/TIMP-1 ratio findings Figure A2
<i>Design part-adjusted for confounders; Correlation coefficients:</i> Outcome: % reduction in area at 4 weeks ^{31,33}	MMP-1/TIMP-1 and MMP-9/TIMP-1 Correlation coefficients: <ul style="list-style-type: none"> MMP-1/TIMP-1: 0.33 (0.01 to 0.66) and 0.65 (0.17 to 1.13) MMP-9/TIMP-9: -0.33 (-0.66 to -0.01) 	38 (two studies, one with two proteases)	⊕⊕⊕⊕ VERY LOW ^g	Very serious inconsistency precludes summarizing the findings. There may be a large difference between MMP-1/TIMP-1 ratio and MMP-9/TIMP-1 ratio findings Figure A3

Abbreviations: HNE, human neutrophil elastase; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP; SMD, standardized mean difference.

^aSerious risk of bias, some inconsistency and some imprecision.

^bSerious risk of bias, very serious imprecision.

^cVery serious risk of bias, very serious imprecision.

^dSerious risk of bias, serious imprecision.

^eSerious risk of bias, very serious imprecision.

^fSerious risk of bias, serious imprecision, serious inconsistency.

^gSerious risk of bias, very serious imprecision, very serious inconsistency.

TABLE 3 Subgroup analyses for protease associations with healing

Subgroup analysis	Results (random effects) SMD (95% CI)	Heterogeneity and test for subgroup differences
Protease class <ul style="list-style-type: none"> Collagenases (MMP-1 and MMP-8) Gelatinases (MMP-2 and MMP-9) Elastases Sum MMP-9 and HNE 	<ul style="list-style-type: none"> Subgroup analysis may account for the overall heterogeneity Collagenases showed a trend for increased protease activity being associated with <u>more</u> healing, SMD: 0.42 (−0.28 to 1.12) Gelatinases, serine protease HNE, and sum of MMP-9 and HNE showed a trend for increased protease activity being associated with <u>less</u> healing. <ul style="list-style-type: none"> Gelatinases, SMD: −0.52 (−0.82 to −0.21) HNE, SMD: −0.80 (−1.33 to −0.26) Sum of MMP-9 and HNE, SMD: −0.96 (−1.84 to −0.08) 	Test for subgroup differences: $I^2 = 66\%$, $P = .03$ Heterogeneity within subgroups: <ul style="list-style-type: none"> Collagenase variability in point estimates: $I^2 = 36\%$ ($P = 0.21$; $N = 4$, 1 with no data) Gelatinase fairly consistent association: $I^2 = 22\%$ ($P = .26$; $N = 9$, 1 with no data) HNE very consistent association $I^2 = 0\%$ ($P = .69$; $N = 2$)
Wound type <ul style="list-style-type: none"> DFU VLU 	<ul style="list-style-type: none"> Results for both wound types are qualitatively in the same direction (increased protease associated with less healing) <ul style="list-style-type: none"> DFU, SMD: −0.48 (−0.87 to −0.10) VLU, SMD: −0.17 (−0.58 to 0.23) The magnitude of association was smaller for VLU, which had wide CIs crossing zero VLU studies all at high risk of bias 	Test for subgroup differences: $I^2 = 15\%$, $P = .28$ Heterogeneity within subgroups: <ul style="list-style-type: none"> DFU: variability in direction of point estimates; $I^2 = 55\%$ ($P = .01$; $N = 13$, 2 with no data) VLU highly consistent $I^2 = 0\%$ ($P = .98$; $N = 3$)
Active/total protease	<ul style="list-style-type: none"> Results for both active and total protease level subgroups are qualitatively in the same direction (increased protease associated with less healing) <ul style="list-style-type: none"> Active, SMD: −0.85 (−1.25 to −0.44) Total or unclear, SMD: −0.27 (−0.62 to 0.07) 	Test for subgroup differences: $I^2 = 77\%$, $P = .04$ Heterogeneity within subgroups: <ul style="list-style-type: none"> Active: slight variability in point estimates; $I^2 = 6\%$ ($P = .36$; $N = 5$, 1 with no data) Total/unclear: variability in the point estimates; $I^2 = 44\%$ ($P = .07$; $N = 11$, 1 with no data)
Type of sample Infection Treatment	Insufficient evidence for these subgroup analyses	

Abbreviations: HNE, human neutrophil elastase; MMP, matrix metalloproteinase; SMD, standardized mean difference.

attributable to the type of collagenase: two small studies reported results for MMP-1 (two studies) and MMP-8 (one study). In the study reporting both collagenases, the direction of association was different for the two proteases ($I^2 = 67\%$).³¹

Other analyses: chi-squared test and correlation

The evidence for other types of analysis (chi-squared test and correlation) for protease activity involves one study in 22 participants;³³ the evidence is of very low certainty (downgraded for risk of bias and twice for imprecision). One study in 62 participants³⁴ conducted multiple linear regression for the percentage change in wound area per day at 4 weeks: no results were reported, but the model (which included active MMP-9, pro-MMP-9, and TIMP-1) accounted for 32% of the variance. The evidence is of very low quality (downgraded twice each for risk of bias and imprecision). Full details are given in Table 2 and Appendix 2.

3.3.2 | Protease inhibitor activity as a prognostic factor for healing

t-test analysis

For the t-test analysis, five cohorts (four studies; 175 participants) investigated possible associations between continuous protease inhibitor activity and dichotomous healing data (Figure 3 and Table 2). The random effects summary statistics for the meta-analysis across complete and partial healing were, SMD: 0.37 (95% CI 0.06 to 0.68), with I^2

inconsistency of 0% ($P = .47$; Figure A1 in Appendix 2). The positive SMD result indicates that, on average, higher baseline levels of protease may be associated with higher proportions of healing wounds at follow-up (compared with nonhealing). The evidence certainty is low, downgraded once for risk of bias and once for imprecision.

Other analyses: logistic regression and chi-squared test

One study (47 participants³²) conducted a multivariable logistic regression analysis to investigate the serine protease inhibitor Serpin B3 as a prognostic factor for healing (improved vs not improved) at 6 months. While the study findings suggest higher levels of Serpin B3 may be associated with more healing after adjustment for coronary artery disease (odds ratio per unit increase in Serpin B3/total protein [$\text{ng mL}^{-1} \mu\text{g}^{-1}$]: 3.5, 95% CI 1.1–11.9), this is very low certainty evidence (downgraded once for risk of bias and twice for imprecision), meaning we are highly uncertain about any potential relationship. The evidence for the chi-squared test is also of very low certainty for the same reasons (see Table 2).

3.3.3 | Ratio of protease to inhibitor as a prognostic factor for healing

t-test analysis

For the t-test analysis, there is very low certainty evidence on associations between continuous protease to inhibitor ratios and dichotomous



healing from four cohorts (126 participants). Random effects meta-analysis summary statistics across complete and partial healing were: SMD -0.47 (95% CI -0.94 to -0.01 ; Figure A1), meaning that, on average, lower baseline levels of protease to inhibitor ratios may be associated with higher proportions of healing wounds at follow-up (compared with nonhealing). The evidence certainty is very low (downgraded once for risk of bias, once for inconsistency and once for imprecision). We note that one study contributes two results to the meta-analysis of the protease to inhibitor ratio (MMP-1:TIMP-1 and MMP-9:TIMP-1) and there is inconsistency in the direction of the results within this study ($I^2 = 32\%$).³³

Other analyses: logistic regression, chi-squared test, and correlation

One study (16 participants³¹) conducted a multivariable logistic regression analysis to investigate the ratio MMP-1:TIMP-1 as a prognostic factor for healing (at least 82% reduction in size) at 4 weeks, but gave no numerical data, stating that the “ratio is a predictive factor of healing independent of wound area and depth”. This is very low certainty evidence (downgraded once for risk of bias and twice for imprecision).

Two studies in 38 participants (one with data for two ratios) reported results for the chi-squared test and correlation.^{31,33} In each analysis, very serious inconsistency in the direction of association (between the results for MMP-1:TIMP-1 and MMP-9:TIMP-1) precluded meta-analysis. The evidence is of very low certainty (downgraded once for risk of bias, twice for inconsistency and twice for imprecision). Further details are in Table 2 and Figures A2 and A3.

3.4 | Post-hoc subgroup analyses by individual proteases

3.4.1 | Protease activity

Our qualitative assessment of the *t*-test analysis of protease activity suggested differences between MMP-1 (a particular collagenase) and other proteases (including another collagenase, MMP-8) in terms of direction of association with healing, so we conducted two related posthoc subgroup analyses (see Appendix 3). We present the final analyses of MMP-1 vs all other proteases and across healing outcomes (Figure 4 and Table A4). For the ‘other protease’ subgroup, some studies reported data for more than one protease, so we selected results for MMP-9 to minimize multiple counting. We also conducted a risk of bias sensitivity analysis (Table A4).

Two cohorts (38 participants, plus one cohort without results in 26 participants³⁵) explored whether MMP-1 was prognostic for healing (over 12 weeks³³ or 4 weeks³¹). Random effects meta-analysis summary statistics were: SMD 0.73 (95% CI 0.06 – 1.39), meaning that, on average, higher baseline levels of protease may be associated with higher proportions of healing. The evidence is rated as very low certainty for the MMP-1 subgroup (downgraded once for risk of bias and twice for imprecision). In contrast, for the eight cohorts (266 participants) that investigated whether proteases other than MMP-1 were prognostic for healing, the random effects meta-analysis results were: SMD -0.57 (95% CI 0.86 to -0.29), indicating that, on average, lower baseline levels of protease may be associated

with higher proportions of healing. The evidence is rated as low/very low certainty for the other proteases (downgraded once each for risk of bias and imprecision, together with some uncertainty about the posthoc subgroup analysis). The test for subgroup differences gave $I^2 = 92\%$ and $P = .0005$; there was an even greater distinction in the sensitivity analysis: $I^2 = 94\%$, $P < .0001$, with no heterogeneity in either subgroup.

3.4.2 | Ratio of proteases to protease inhibitors

We observed similar differences between ratios involving MMP-1 and those involving MMP-9 for the *t*-test, chi-squared test, and correlation analyses, although sample sizes were small and the evidence is of very low certainty for the MMP-9:TIMP analyses (downgraded for risk of bias and very serious imprecision): results for MMP-1:TIMP-1 may be in a different direction to those for ratios involving MMP-9 (Figures A1–A3).

4 | DISCUSSION

We sought to investigate whether protease activity was an independent prognostic factor for wound healing in people with complex wounds. We had broad inclusion criteria, permitting studies that considered at least one key confounding factor in the analysis or study design, but we also took account of this potential confounding in risk of bias assessment, assigning high risk of bias to those studies accounting for less than half the key confounding factors. The inclusion criteria were met by 10 cohorts (eight studies) in 343 participants; however, all cohorts were small (median 32 participants, overall range 16–62) and there was clinical heterogeneity around the type of protease, measurement of protease activity and cut-off points, definition of healing and the analysis type. Most studies did not conduct regression analyses, but all reported continuous protease data linked to dichotomous healing data, and we were able to conduct a meta-analysis using standardized mean differences and a *t*-test approach.

All evidence is of low or very low certainty. A GRADE rating of low certainty means that our confidence in the association is limited: the true association may be substantially different. The evidence suggests that elevated protease levels may be associated with less wound healing; elevated protease inhibitor levels with more healing; and increases in the ratio of protease to inhibitor may also be associated with less healing. However, there was also statistical heterogeneity that was not fully explained by our prespecified subgroup analyses of wound type, protease class or protease activity status.

In an exploratory posthoc subgroup analysis, the evidence suggests different directions of association with healing for MMP-1 or ratios including MMP-1 (increased protease activity associated with more healing) compared with other proteases (association with less healing). This posthoc evidence is of low or very low certainty. The exploratory nature of the analysis together with the clinical and

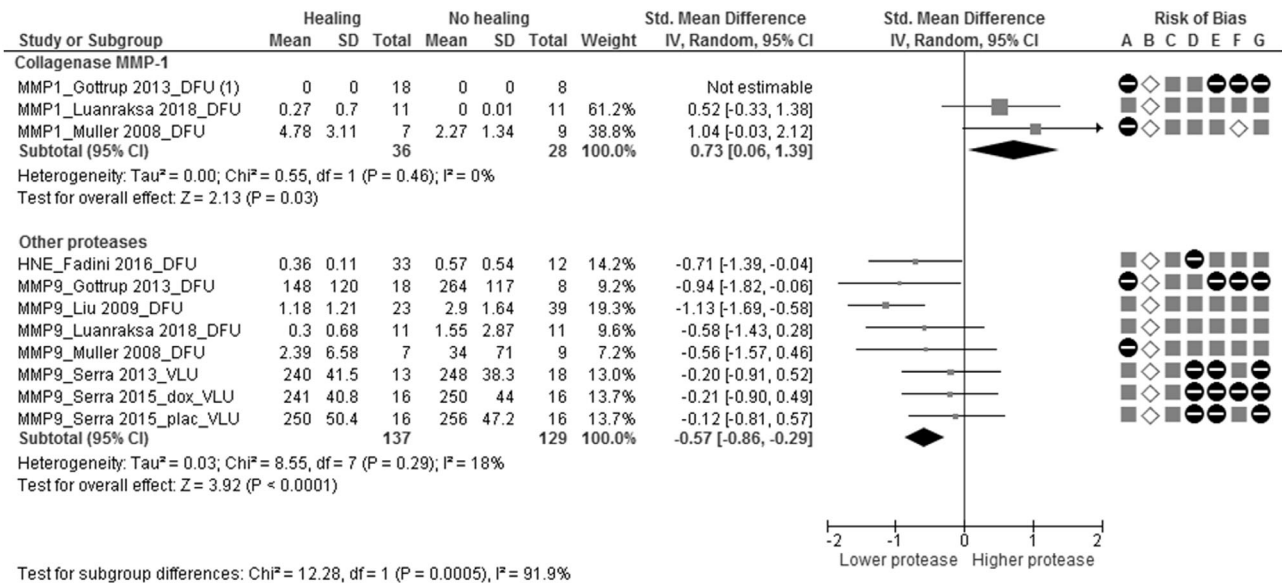


FIGURE 4 Posthoc subgroup analysis MMP-1 vs other proteases (one protease per study)

Key (risk of bias): A - Selection bias; B - Attrition bias; C - Prognostic factor measurement bias; D - Outcome measurement bias; E - Adjustment/confounding bias; F - Analysis and reporting bias; G - All-domain risk of bias. Symbols: white diamond = low risk of bias; grey square = moderate risk of bias; black circle with white line = high risk of bias.

(1) "No significant difference" between responders and non-responders

methodological heterogeneity and small studies means that any conclusions are tentative and should only be used to inform further research. However, further research would only be appropriate if there is a biological hypothesis regarding the different types of proteases.

The Muller et al 2008 study suggests a biological rationale for different protease findings. MMP-1 is the major collagenase implicated in wound healing: its specific role involves initial proteolysis of type I collagen, which enables keratinocyte migration leading to re-epithelialization.³¹ The authors hypothesize that the higher level of MMP-1 in healing wounds permits the proliferative phase to be completed and allows progression of the healing process. Additionally, the association of healing with TIMP-1 suggests regulation of MMP-1 activity. On the other hand, MMP-9 and MMP-8 are synthesized by inflammatory cells, which if present in excess may impact the healing process.³¹

There are a number of limitations. The included studies were small and had moderate or high risk of bias. Few studies reported the primary outcome of complete healing and we consequently also included data on partial healing. Although the results were not different for partial and complete healing, there is an implicit assumption that partial healing is an adequate surrogate, which it may not be.

The preferred data synthesis would have been to meta-analyze results from multivariable regression analyses that took account of key confounding factors, or to conduct individual patient data meta-analyses. However, there were no large cohort studies reporting multivariable regression analyses, and studies that did report multivariable analyses were too small to give reliable results. We therefore synthesized the data in a different way using an application of the *t*-test; we note that the *t*-test assumption of approximately normal distributions

may not have been met for some studies. Most of the evidence is from studies that took account of confounding factors only in the study design and in an unplanned way, which decreases their reliability. The main analysis (*t*-test) involved multiple counting when individual studies reported results for more than one protease; this occurred for three studies. Only three studies clearly reported the active form of protease.

The observed heterogeneity may have been explained by the posthoc subgroup analysis comparing MMP-1 and other proteases, but this proposed explanation should be treated with caution. Apart from being posthoc, all subgroup analyses are nonrandomized comparisons and may be confounded by other factors that we have and have not examined. In addition, the subgroup analysis relies on two very small studies that did not take full account of confounding factors, for one of which we transformed the median (IQR) data.

5 | CONCLUSIONS

This is the first systematic review of protease activity as a prognostic factor for healing across all wound types. We restricted the review to the better-conducted studies and applied rigorous systematic review methods. However, the evidence base is poor, our certainty about the findings is low or very low, and our conclusions are necessarily tentative. Higher levels of proteases may be associated with less healing, and higher levels of protease inhibitors may be associated with more healing. There may be a place for the ratio of protease to inhibitor as a predictor of healing. There may be important differences between MMP-1 and other proteases, a finding that our review has investigated explicitly, following earlier indications in two included studies.^{31,33}

This is an important review because there is much interest, both commercially and in health services, in identifying wounds at greatest risk of nonhealing and in developing targeted treatments. There are already point-of-care tests for protease activity commercially available³⁹ and a number of protease modulating matrix dressings have been tested in trials and are marketed widely, but the evidence for their effectiveness is uncertain.⁴⁰ If the various proteases indeed have qualitatively different predictive abilities for healing, there will be an even greater need for highly targeted predictive tests and treatments; it is unclear whether current protease modulating dressings can distinguish between proteases.

The tentative evidence from this work should be confirmed (or otherwise) by a large cohort study that measures protease activity appropriately, explores the role of different proteases, considers different wound types and chronicity, and measures the time to healing (survival analysis). Only then can we develop effective treatments to improve the quality of life for the many people with complex wounds.

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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