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Original Research

Low-molecular-weight heparin adherence and effects on survival within a randomised phase III lung cancer trial (RASTEN)

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Abstract ***Background:*** Coagulation activation is a hallmark of cancer, and anticoagulants have shown tumour-inhibiting properties. However, recent trials have failed to demonstrate improved survival with low-molecular-weight heparin (LMWH) in cancer populations. This has raised the question of suboptimal adherence as a possible explanation for the lack of benefit. Still, there is no standardised method to directly monitor LMWH in patient plasma. Here, we directly determine LMWH levels in patients using the Heparin Red assay to objectively assess adherence and how this associates with the patient outcome in the RASTEN trial.
Methods: RASTEN is a multicentre, randomised phase III trial investigating if the addition of LMWH to standard therapy can improve survival in small-cell lung cancer. LMWH was measured in plasma ($N = 258$) by the Heparin Red assay and compared with the anti–factor Xa (anti-FXa) activity assay.
Results: Both methods could differentiate patients in the LMWH arm from the control arm and patients receiving therapeutic LMWH owing to thrombosis. Receiver Operating Characteristic (ROC) analysis yielded adherence rates of 85% for anti-FXa and 68% for

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Heparin Red. No survival benefits were found in the adherent subgroup compared with the control arm (hazard ratio [HR]: 1.26; 95% confidence interval [CI]: 0.95–1.67; $P = 0.105$ and HR: 1.19; 95% CI: 0.89–1.60; $P = 0.248$ for anti-FXa and Heparin Red, respectively). Heparin Red could define patients with high probability of adherence to LMWH treatment, which warrants prospective studies for further validation. Our finding that the LMWH-adherent subpopulation did not show improved survival excludes that the negative outcome of RASTEN was due to poor adherence.

1. Introduction

Increased coagulation activation and the subsequent risk of venous thromboembolism (VTE) are well-known complications in malignancy. A large body of preclinical evidence suggests that the activated coagulation factors not only involve in the development of VTE but also contribute to malignant processes such as metastasis and angiogenesis [1–3]. Early, pivotal studies suggested that anticoagulants may improve survival when administered prophylactically to patients with cancer, particularly in small-cell lung cancer (SCLC) [4–6], but these positive effects on survival have not been confirmed in more recent trials [7–9].

We conducted an international, randomised phase 3 trial of the low-molecular-weight heparin (LMWH) enoxaparin as an adjunct to standard treatment in patients with SCLC (RASTEN trial), the results of which have been presented elsewhere [7]. The primary aim of the study was to determine whether the addition of LMWH could improve survival in SCLC. We found no differences in overall mortality between the study groups despite a significant reduction of VTE in the LMWH treatment arm. However, this was an open-label study in which adherence was assessed by patient reports. Moreover, another recent randomised trial, FRAGMATIC [8], showed no survival benefit by the addition of LMWH in patients with lung cancer, suggesting suboptimal adherence based on patient information. Thus, strategies to directly measure LMWH levels in patient plasma are warranted to objectively investigate if the conflicting outcomes from clinical studies could be explained by inadequate adherence. However, to date, there is no standardised method that measures the actual levels of LMWH or the anticoagulant effect in patient circulation.

Measurement of anti–factor Xa (anti-FXa) activity is considered the 'gold standard' for monitoring the anticoagulant effect of LMWH in the clinical setting, but the method is based on measurement of only the FXa-inhibiting capacity that may not fully reflect the actual anticoagulant effect of LMWH [10]. The anti-FXa assay provides an indirect measure of LMWH effect by detecting degrees of FXa inhibition without considering other effects exerted by LMWH, such as inhibition of thrombin, release of tissue factor pathway inhibitor or inhibition of heparin-binding proteins in the circulation [11,12]. In addition, anti-FXa has been questioned because the measurement principle differs between assays that in some cases rely on the addition of antithrombin, whereas in others, the antithrombin level of the patient decides the anticoagulant effect of LMWH. This has prompted the development of a fluorescent probe assay (Heparin Red) to directly determine the level of heparin and heparin-derived compounds in plasma [13], independent of their anticoagulant activity. The fluorescent probe forms a supramolecular complex with its target heparin structure, resulting in contact quenching of fluorescence. Importantly, so far, the method has only been examined in heparin-spiked plasma from healthy donors [14] and not in a clinically relevant setting with plasma derived from patients receiving LMWH.

The RASTEN phase 3 lung cancer trial offers a unique opportunity to objectively assess the adherence to prophylactic LMWH and to compare the Heparin Red and anti-FXa assays. Here, our aims were to validate the Heparin Red assay in a clinical setting, to identify a subgroup of 'adherent' *vs* 'non-adherent' patients and to correlate adherence to survival. We also investigated whether the adherence rates varied over time, considering the long enrolment period. To our knowledge, this is the first study where plasma levels of LMWH have been measured quantitatively in the context of the survival outcome in a clinical study.

2. Materials and methods

2.1. RASTEN clinical trial

A complete description of the study design of RASTEN has been previously reported [7]. In brief, 377 patients were randomised 1:1 between a control arm receiving standard therapy and a treatment arm receiving standard therapy with the addition of enoxaparin. Standard treatment involved 4–6 cycles of a platinum compound and a topoisomerase inhibitor, with radiotherapy given according to local guidelines. In the treatment arm, the

LMWH enoxaparin was administered at supra-prophylactic dosages of 1 mg/kg once daily as subcutaneous (s.c.) injections for the duration of the chemotherapy regimen. To assess adherence within the trial, patients were instructed to bring the empty syringe boxes to each study visit, and reasons for treatment interruptions were recorded. The trial was conducted in accordance with the Declaration of Helsinki with approval from the Regional Ethics Committee of Lund University.

2.2. Patient selection and plasma sampling

Blood samples were collected longitudinally during the clinical trial, at baseline, before the third chemotherapy cycle and at 2-month follow-up. The plasma samples in citrated tubes were stored in a −80 °C freezer at the Clinical Research Unit, Lund University Hospital, Sweden. For the present study, an initial biomarker cohort was established on 1st November 2013, consisting of the first 199 patients, for which blood samples collected during treatment, that is, before cycle three, were available. This batch was analysed in September 2017. The cohort was later extended to include the remaining study participants, yielding further 59 patients, analysed in December 2017. Citrated plasma from four healthy donors was collected using the same procedure as for patient samples, pooled and stored at −80 °C.

2.3. Anti-FXa assay

For measurement of anti-FXa activity, 100 µl of plasma was analysed using a commercially available chromogenic FXa substrate assay, run on a BCS-XP (Siemens Healthcare, Marburg, Germany) with Coamatic Heparin (Chromogenix, Instrumentation Laboratories, Bedford, USA) at the Department of Clinical Chemistry, Malmö, Sweden. At therapeutic levels, the reference range for anti-FXa activity is 0.2–1.3 IU/mL [15].

2.4. Heparin Red assay

The Heparin Red® kit is a 'research-use-only' assay and was a kind gift from Redprobes UG, Münster, Germany. The assay was performed in-house using the FluoStar Optima fluorescence reader (BMG Labtechnologies, Ortenberg, Germany) according to the instructions by the provider, with minor adaptations of the protocol. In brief, a mixture of 220 µl Heparin Red and 25 ml enhancer solution was freshly prepared (working solution). On a 96-well microplate, 20 µl plasma and 80 µl of the working solution were added to each well. The solutions were mixed for 25 s using the plate-shaking function of the fluorescence reader. Serial readings were performed at 1, 4, 7 and 15 min to monitor the quenching reaction. The means of the readings at 7 and 15 min were used in the analysis ('mean fluorescence'). The excitation wavelength and emission wavelength were 544 nm and 610 nm, respectively, using a spectral bandwidth of 13.5 nm and read height of 8 nm. To obtain a standard curve, pooled plasma from healthy donors was spiked with enoxaparin (Klexane® 100 mg/ml, Sanofi AB, Stockholm, Sweden) in the concentration range of 0–10 µg/ml and analysed in parallel in duplicate. The standard curves were comparable between the two batches. The intra-assay and interindividual variations have previously been reported by coefficients of variation of <5% [14] and 8% [13], respectively.

2.5. Statistical analysis

Data were analysed using the statistics programs SPSS version 22, STATA 15.1 and R version 3.5.1. For the Heparin Red standard curve, the mean of the healthy controls at each known concentration was used, and linear regression was applied. To obtain the equivalent concentrations of enoxaparin in the patient samples, the 'mean fluorescence' was applied to the linear regression. Owing to the skewed distribution with negative concentrations, we used fluorescence data in the statistical analyses, unless stated otherwise. For correlation and comparison of biomarker levels, Spearman's rank correlation and Kruskal–Wallis tests were performed. Patients receiving therapeutic LMWH owing to a VTE diagnosis before cycle 3 were only included in the assay validation analysis but excluded from the adherence assessments. The cut-off value for anti-FXa activity and Heparin Red mean fluorescence, respectively, was determined at the values maximising the product of sensitivity and specificity, and ROC analysis was performed. The Kaplan–Meier method and Cox regression analysis were used to estimate survival probabilities. Proportional hazards assumptions were checked graphically, and the relative effects on mortality were found to vary with follow-up time. Hence, the estimated hazard ratios (HRs) should be cautiously interpreted as average effects over the follow-up time. Adherence rates over time were estimated using the so-called running means function in STATA.

3. Results

3.1. Description of the cohort

The plasma samples taken before chemotherapy cycle 3 were available for 258 patients. Five patients had initiated therapeutic anticoagulation before cycle 3 and were only included in the assay validation analysis. A total of 253 patients were included in the final adherence analysis (Supplementary Fig. S1), 128 and 125 in the control and treatment arms, respectively. The study arms were well balanced in terms of baseline demographics (Supplementary Table S1).

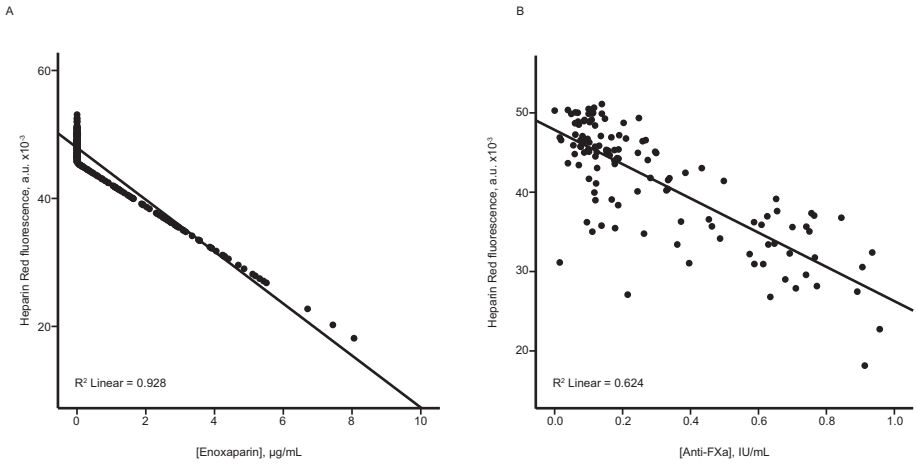

Fig. 1. **Correlations between anti-FXa activity levels and Heparin Red fluorescence.** (A) Fitted regression line for Heparin Red fluorescence and enoxaparin LMWH concentration in the study population ($N = 253$), as calculated from the standard curve (see Fig. S2, Supporting information). In 151 cases, corresponding concentrations were <0, which were replaced by 0. (B) Scatter plot correlation between anti-FXa activity and Heparin Red fluorescence in the LMWH treatment arm ($N = 125$). anti-FXa, anti–factor Xa; LMWH, low-molecular-weight heparin.

3.2. Validation of the Heparin Red assay

The Heparin Red assay detected and quantified the presence of LMWH in the patient samples at fluorescence ranges of 53,100–18,100, corresponding to enoxaparin concentrations of 0–8.1 µg/mL (Fig. 1A and Supplementary Fig. S2). Heparin Red fluorescence strongly correlated to the 'gold standard' anti-FXa assay in an inverse relationship, that is, increased anti-FXa activity correlated with increased quenching of the Heparin Red fluorescent probe (Spearman's rho $= -0.724$, $P < 0.001$) (Fig. 1B). Both assays demonstrated a dose–response relationship across the therapeutic spectrum, and significant differences were seen in anti-FXa, Heparin Red fluorescence and equivalent LMWH concentrations, when comparing patients receiving prophylactic LMWH and therapeutic LMWH and patients in the control arm receiving no LMWH (Fig. 2 and Table 1).

3.3. Assessment of adherence

Using ROC analysis, we obtained an area under the curve of 0.93 for anti-FXa (Fig. 3A) and 0.78 for Heparin Red (Fig. 3B). For anti-FXa, the cut-off value of 0.082 gave a sensitivity of 84.8% and specificity of 91.4%. In the LMWH arm, 106 (85%) patients were considered to be adherent ($LMWH_{adh}$), and 19 (15%) were considered non-adherent ($LMWH_{non\text{-}adh}$). For the Heparin Red assay, a cut-off value of 45,913 was identified, yielding a sensitivity of 68.0% and specificity of 82.0%. Eighty-five (68%) and 40 (32%) patients were considered to be adherent *vs* non-adherent, respectively.

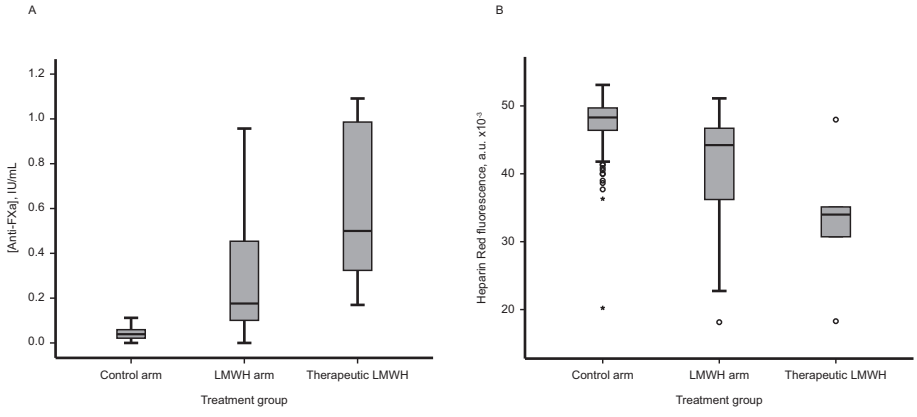

Fig. 2. **Anti-FXa activity and Heparin Red fluorescence assays are dose dependent.** Boxplots illustrating dose–response relationship of anti-FXa activity (A) and Heparin Red fluorescence (B) based on treatment groups: control arm (not receiving any LMWH, $N = 128$), LMWH arm (receiving prophylactic LMWH, $N = 125$), therapeutic LMWH (receiving therapeutic dosages of LMWH due to prior VTE, $N = 5$). LMWH, low-molecular-weight heparin; VTE, venous thromboembolism; anti-FXa, anti–factor Xa.

Table 1
Assay results by the treatment arm.

Assay	Control arm $N = 128$ Median (IQR)	LMWH arm $N = 125$ Median (IQR)	Therapeutic LMWH $N = 5$ Median (IQR)
Anti-FXa, IU/mL	0.04 (0.02–0.06)	0.18 (0.10–0.46)	0.50 (0.25–1.04)
Heparin red Fluorescence, a.u	48,300 (46,400–49,700)	44,200 (36,100–46,700)	34,000 (24,500–41,600)
LMWH concentration, μg/mL	0.0 (0.0–0.0)	0.4 (0.0–2.8)	3.4 (1.5–6.2)

Comparison of assay results between the treatment groups using the Kruskal–Wallis test revealed P-values <0.001 for anti-FXa, Heparin Red fluorescence and LMWH concentrations, respectively.
anti-FXa, anti–factor Xa; LMWH, low-molecular-weight heparin; IQR, interquartile range; a.u., arbitrary units.

Although the two methods showed a strong correlation, the definition of adherence did not show full concordance (Fig. 3C). Seventy-eight (62%) patients were defined as adherent and 12 (10%) were defined as non-adherent in both assays, whereas 28 (22%) and 7 (6%) patients were considered non-adherent only according to anti-FXa or Heparin Red, respectively. Hence, the cohorts from the respective assay were handled separately in further analyses.

3.4. Description of subgroups

We did not observe any major deviations of baseline characteristics in the adherent and non-adherent subjects, with the exception of a tendency towards

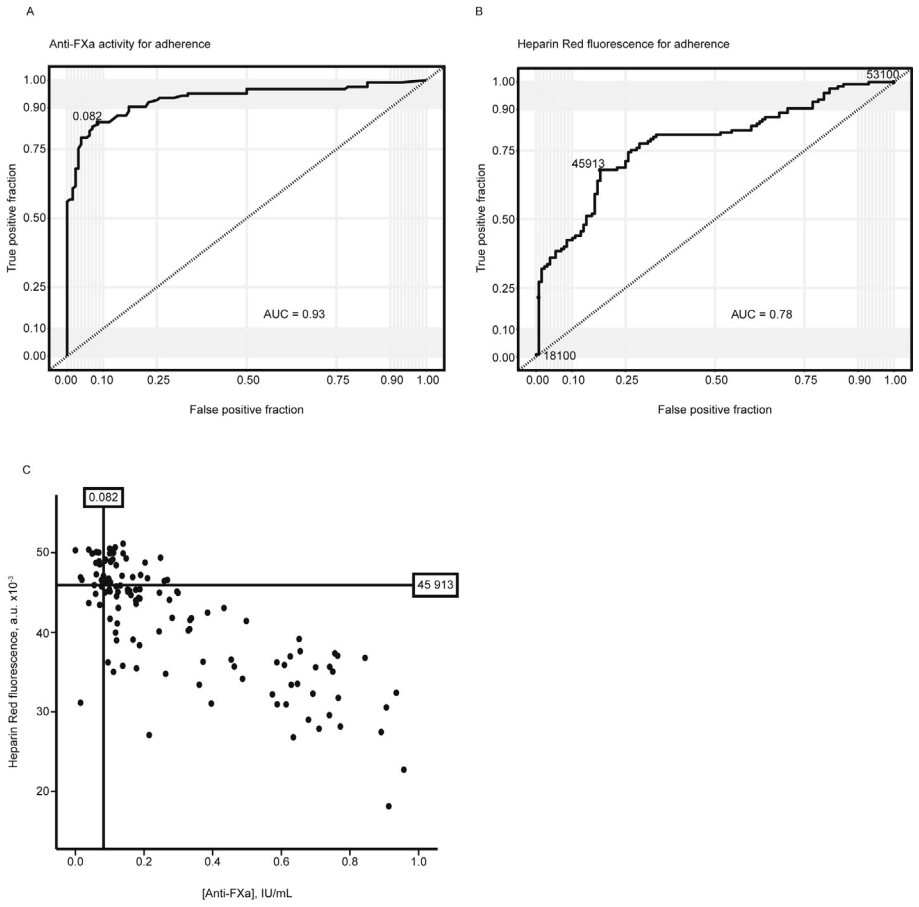

Fig. 3. **Receiver Operating Characteristic (ROC) curves for adherence to LMWH according to anti-FXa activity (A) and Heparin Red fluorescence (B).** The fluorescence cut-off point of 45,913 in (B) corresponds to an enoxaparin concentration of 0 μg/mL. (C) Scatter plot correlation between anti-FXa activity and Heparin Red fluorescence demarcating cut-off points for adherence estimation for each assay in the LMWH arm ($N = 125$). Seventy-eight (62%) patients were defined as adherent and 12 (10%) patients were defined as non-adherent in both assays. Twenty-eight (22%) and 7 (6%) patients were considered adherent only by the anti-FXa or Heparin Red assays, respectively. LMWH, low-molecular-weight heparin; anti-FXa, anti–factor Xa.

better performance status in the non-adherent cohorts (Table 2).

3.5. Patient-reported adherence

At the study visit, in conjunction with cycle 3, temporary or permanent cessation of LMWH administration was reported in 21 (17%) cases in the LMWH arm. The majority were of short duration (<7 days), main reasons being forgetfulness ($N = 7$), side-effects from chemotherapy ($N = 3$) or before minor surgical interventions ($N = 3$). Because the timing of the discontinuations in relation to the blood sampling was not recorded, correlations between patient-reported adherence and assay results were not performed.

3.6. Correlation between adherence and the clinical outcome

We next set out to investigate how adherence, as defined by anti-FXa and Heparin Red, respectively, correlated with patient survival. There were no significant survival differences when comparing the control arm and the LMWH arm, when the non-adherent subpopulations were excluded. The median overall survival (OS) was 12.4 months in the control arm and 11.2 months in the $LMWH_{adh}$ subgroup, as defined by anti-FXa (HR: 1.26; 95% confidence interval [CI]: 0.95–1.67; $P = 0.105$) (Fig. 4A). The median OS was 12.7 months in the $LMWH_{adh}$ subgroup (HR: 1.19; 95% CI: 0.89–1.60; $P = 0.248$), as defined by Heparin Red (Fig. 4B). Further subgroup analysis by disease stage (limited *vs* extensive) did not reveal any significant differences (Supplementary Fig. S3), but there was a non-significant trend towards reduced survival in the $LMWH_{adh}$ groups. Moreover, when comparing the $LMWH_{adh}$ and the $LMWH_{non\text{-}adh}$ groups, we could not demonstrate any differences in survival (Supplementary Fig. S4).

Table 2
Baseline characteristics of the adherent and non-adherent subpopulations in the LMWH arm defined by the anti-FXa and Heparin Red assays.

		Anti-FXa	Heparin Red
Adherent, *N* (%)		106 (85)	85 (68)
Non-adherent, *N* (%)		19 (15)	40 (32)
Mean age, years ± SD			
Adherent		66 ± 7.8	66 ± 7.8
Non-adherent		65 ± 9.4	66 ± 8.5
Gender, *N* (%)			
Adherent	Female	63 (59)	49 (58)
	Male	43 (41)	36 (42)
Non-adherent	Female	11 (58)	25 (62)
	Male	8 (42)	15 (38)
Disease stage, *N* (%)			
Adherent	Limited	41 (39)	33 (39)
	Extensive	75 (61)	52 (61)
Non-adherent	Limited	8 (42)	16 (40)
	Extensive	11 (58)	24 (60)
Performance status, *N* (%)			
Adherent	0-1	77 (73)	60 (71)
	2-3	29 (27)	25 (29)
Non-adherent	0-1	15 (79)	32 (80)
	2-3	4 (21)	8 (20)

LMWH, low-molecular-weight heparin; anti-FXa, anti–factor Xa; SD, standard deviation.

3.7. Adherence over time

Considering the eight-year enrolment period of the RASTEN trial, we analysed the anti-FXa activity and Heparin Red fluorescence levels over time (Supplementary Fig. S5). The results did not reveal any significant changes based on the inclusion date, suggesting that the enoxaparin concentrations do not decrease with prolonged storage periods. Focussing on anti-FXa, we noted a trend towards higher adherence rates during the first compared with the second half of the enrolment period, but this was non-significant ($P = 0.167$).

4. Discussion

It is well known that adherence to medication varies greatly and is difficult to measure objectively [16]. Adequate levels of adherence are pivotal when assessing the efficacy of a study treatment, and patient-reported adherence may not always reflect patient behaviour [17,18]. In the RASTEN trial, we could not demonstrate any survival benefit with the addition of enoxaparin, despite promising data from previous, smaller studies and meta-analyses, which had suggested highly significant improvement of survival in patients with SCLC receiving LMWH [4,19,20]. Motivated by the concern of inadequate adherence to a study drug administered by s.c. injections, we set out to examine if the trial outcome could in fact be explained by poor adherence in the LMWH arm. We have not found evidence to suggest improved survival in a subpopulation of patients with objectively proven levels of LMWH. Thus, the potentially tumour-inhibiting effects of LMWH could not be confirmed, and the argument that the negative outcome of the RASTEN trial is due to deficient adherence can be disregarded.

To objectively measure the concentration of enoxaparin, we established the Heparin Red assay that was compared with the indirect anti-FXa assay. Our study shows that the Heparin Red assay can detect clinically relevant concentrations of enoxaparin in plasma from patients receiving LMWH, and the levels were significantly correlated to the current, ‘gold standard’ anti-FXa assay. Notably, the anti-FXa activity measured in the LMWH study arm was in the lower range of previously reported activities during LMWH treatment [15], which is consistent with the subtherapeutic dose

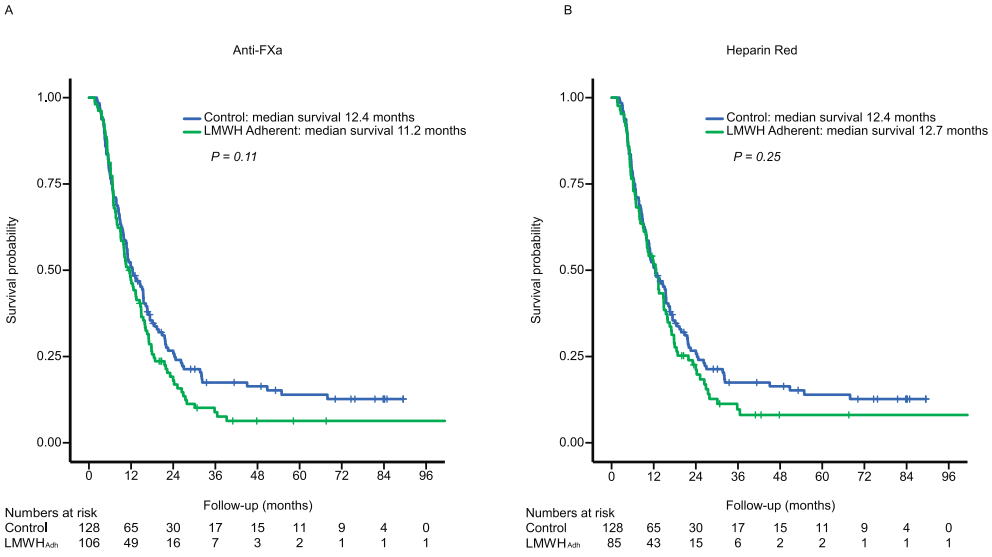

Fig. 4. **Overall survival in the control arm and the $LMWH_{Adh}$ subgroup.** Adherence defined by anti-FXa activity (A) and Heparin Red fluorescence (B). LMWH, low-molecular-weight heparin; anti-FXa, anti–factor Xa.

in the RASTEN study. More importantly, we demonstrate that both assays may be used to define a subpopulation of adherent *vs* non-adherent patients. We were not able to demonstrate a survival benefit in the subpopulation considered to be adherent, which raises the question as to whether LMWH actually has tumour-inhibiting effects in the clinical scenario and why this conflicts with the large body of evidence from experimental studies showing remarkable inhibitory effects of LMWH on tumour development and metastasis [21–27]. It is conceivable that the pleiotropic interactions and functions of LMWH in the complex environment of patient tumours are poorly mimicked by the cell and animal models used in the experimental setting. Clearly, several questions remain unresolved with regard to the optimal dosing, timing and duration of LMWH treatment to achieve inhibition of tumour growth.

Adherence rates were estimated to 85% using the anti-FXa assay, which is consistent with a study by Lemke *et al.* [28], reporting adherence rates of 81% in patients with cancer completing a 4-week course of prophylactic enoxaparin following pancreatic or liver resection. In the literature, adherence rates of ≥80% are often considered acceptable [29–31], which supports the conclusion that the participants in the RASTEN trial were adequately adherent to LMWH. However, according to the Heparin Red analysis, adherence rates were only 68%, and thus, there was some discordance between the assays. Our results suggest that anti-FXa has a higher predictive value than Heparin Red in assessing adherence to enoxaparin. Although the reason for this will have to be determined by further studies, the two assays clearly differ; although Heparin Red provides a direct measure of the concentration of LMWH based on the quenching of a cationic fluoroprobe, anti-FXa measures enzyme activity based on a chromogenic assay, providing an indirect assessment of LMWH that depends on the availability of antithrombin in the patient's own plasma. As Heparin Red is a novel assay, further protocol optimisation should improve the performance by increased sensitivity and specificity.

A potential limitation of the present study is the lack of information concerning the timing of LMWH injections and the collection of plasma samples, which may overestimate the non-adherent cohort when considering the limited half-life of LMWH in the circulation. Moreover, the analyses were performed in two batches, three months apart, which could be a source of technical bias. To limit this risk, the assays were performed by the same personnel and using the same equipment. In the context of the present study, it would have been of interest to correlate LMWH adherence with thrombotic events. However, the limited number of venous thrombotic events in the LMWH arm ($N = 1$) precluded any meaningful analysis. A strength of this study is the use of two separate assays that measure different aspects of LMWH activity in a large, homogenous population of patients with newly diagnosed SCLC within a randomised controlled trial.

In summary, this is the first study where plasma levels of the anticoagulant LMWH have been measured quantitatively in the context of the survival outcome within a clinical trial. We conclude that LMWH cannot be recommended in the general management of patients with SCLC, and further studies are warranted to elucidate the role of Heparin Red prospectively in cancer populations.

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Conflict of interest statement

R. Krämer holds shares in Redprobes UG, Münster, Germany. The other authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.06.015.

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