


# Endothelial barrier protective properties of low molecular weight heparin: A novel potential tool in the prevention of cancer metastasis?

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## Abstract

**Background:** One of the key events in the progression of cancer metastasis is the trans-endothelial migration of circulating tumor cells. Moreover, inhibition of tumor-induced vascular permeability has been shown to inhibit metastasis in vivo. Low molecular weight heparin (LMWH) appears to confer a survival benefit in cancer but the underlying mechanisms are poorly understood.

**Objective:** To characterise LMWH-mediated endothelial barrier protection and to explore strategies to limit the LMWH-associated haemorrhagic risk in this setting.

**Methods:** Endothelial barrier function was assessed using in vitro assays of endothelial permeability and tumor cell trans-endothelial migration. Thrombin-mediated activation of PAR-1 signalling was assessed by flow cytometry and western blotting. LMWH anticoagulant activity was assessed by calibrated automated thrombography and plasma anti-factor Xa activity assay.

**Results:** LMWH tinzaparin enhanced endothelial barrier function and reduced tumor cell trans-endothelial migration (73.9±5.7% of baseline;  $P < .05$ ). Tinzaparin-mediated attenuation of thrombin-induced permeability was not mediated through an inhibition of thrombin proteolytic activity. In addition, fractions of LMWH with diminished anticoagulant activity retained endothelial barrier protective properties and a marked synergistic effect on barrier function was observed using combinations of sub-anticoagulant concentrations of tinzaparin with simvastatin (which exhibits endothelial barrier protective properties in vitro), with almost complete protection against agonist-induced endothelial barrier permeability achieved (7.9±0.2% of baseline;  $P < .05$ ).

**Conclusion:** Collectively, these results suggest that LMWH supports endothelial barrier function in a manner which does not appear to be dependent on its anticoagulant activity. If replicated in vivo, these findings could represent a novel therapeutic approach to the suppression of metastasis.

## KEYWORDS

anticoagulants, endothelium, haemorrhage, heparin, metastasis

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### Essentials

- Inhibition of tumor cell trans-endothelial migration inhibits metastasis formation.
- Low molecular weight heparin (LMWH)-mediated endothelial barrier protection was investigated.
- LMWH enhanced endothelial barrier activity and attenuated tumor cell transmigration.
- The cytoprotective activity of LMWH did not appear to be dependent on anticoagulant activity.

## 1 | INTRODUCTION

One of the key events in the progression of cancer metastasis is the migration of circulating tumor cells through vascular endothelium.<sup>1–4</sup> Tumors induce abnormal endothelial permeability by various mechanisms in order to promote this process of cancer cell invasion and recently inhibition of tumor-induced vascular permeability has been shown to attenuate metastasis *in vivo*.<sup>5–9</sup>

Several clinical studies have suggested that low molecular weight heparin (LMWH) may confer a survival benefit in sub-groups of patients with cancer.<sup>10–14</sup> This benefit appears to be restricted to patients with early-stage disease, suggesting that it may be mediated through suppression of metastasis. LMWH influences cell signalling pathways implicated in tumor dissemination *in vitro* and has been shown to attenuate abnormal vascular permeability in experimental models of sepsis and acute lung injury.<sup>15–22</sup>

The risk of bleeding associated with LMWH precludes its use in cancer in settings other than the treatment or prevention of thrombosis.<sup>23</sup> Consequently, LMWH could only be utilized for prevention of metastasis if the associated bleeding risk can be addressed.

In the present study, we aimed to characterize the effects of LMWH in enhancing the barrier function of vascular endothelium and to explore the molecular mechanism underlying this phenomenon. We also aimed to investigate the relative anticoagulant and cytoprotective properties of a fraction of LMWH tinzaparin composed primarily of short heparin polysaccharide chains. In addition, we explored the potential additive or synergistic effects on barrier function which are observed when sub-anticoagulant concentrations of LMWH are combined with a statin (a lipid-lowering agent which has also been shown to stabilize endothelial barrier function *in vitro*).<sup>24,25</sup>

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

EA.hy926 (a PAR-1 expressing human umbilical vein endothelial cell line) was a kind gift from Dr. C. Edgell (University of North Carolina, Chapel Hill, North Carolina, USA). DU145, a metastatic prostate carcinoma cell line was from ATCC (Manassas, VA, USA). Polyethylene terephthalate (PET) membrane trans-well inserts (Millicell Hanging Cell Culture Inserts; 6-well plate, 3.0  $\mu\text{m}$  pore size, 4.5  $\text{cm}^2$  surface area; 24-well plate, 8.0  $\mu\text{m}$  pore size, 0.3  $\text{cm}^2$  surface area) were purchased from Merck Millipore Corporation (Billerica MA, USA).

LMWH tinzaparin and a 2.8 kDa tinzaparin-derived LMWH fraction were from Leo Pharma (Cork, Ireland). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and RPMI-1640 cell culture media were purchased from Invitrogen (Carlsbad, CA, USA). RWJ56110, a selective PAR-1 antagonist was from R&D Systems, Inc. (Minneapolis, MN, USA). Rabbit anti phospho-MLC-2 (Thr18/Ser 19) primary antibody was purchased from Cell Signalling Technology (Danvers, MA, USA). Human alpha-thrombin was purchased from Haematologic Technologies (Essex Junction, VT, USA). Thrombin generation reagents (thrombin calibrator, PPP-reagent, FluCa thrombin substrate) were purchased from Thrombinoscope BV (Maastricht, The Netherlands). HaemosIL Liquid anti-Xa reagent and HaemosIL pooled normal control plasma were from Instrumentation Laboratory (Richmond, MN, USA). Recombinant human VEGF<sub>165</sub>, simvastatin, anti-PAR-1 receptor antibody (ATAP2), PAR-1 activating peptide (Ser-Phe-Leu-Leu-Arg-Asn-amide; SFLLRN), heparinase III (from *Flavobacterium heparinum*) and all other chemicals and reagents, unless otherwise stated, were purchased from Sigma Aldrich (St Louis, MO, USA).

### 2.2 | Endothelial barrier permeability assay

An *in vitro* assay of endothelial barrier function was established as previously described.<sup>26,27</sup> Briefly, EA.hy926 human endothelial cells were seeded on PET membrane trans-well inserts (3.0  $\mu\text{m}$  pore size) and cultured in DMEM supplemented with 10% FBS. At 72 hours, the confluent EA.hy926 monolayers were washed in phosphate buffered saline (PBS), and re-incubated in serum-free DMEM. An Evans Blue (0.67 mg/mL)-conjugated bovine serum albumin (BSA, 4%) solution was added to the inner chamber of each trans-well and aliquots of cell culture media from the outer chamber of the trans-well plate were then sampled at 2-minute intervals. The permeability of the endothelial cell layers was determined through the spectrophotometric measurement of the increase in absorbance in the sampled media as a result of flux of the albumin solution through the endothelial cell layer over time.

### 2.3 | Tumor cell trans-endothelial migration assay

Endothelial monolayers were grown on PET trans-well membrane inserts (8  $\mu\text{m}$  pore size) as outlined above. At 72 hours, the inserts were washed in sterile PBS and returned to wells containing FBS-supplemented DMEM. A calcein blue AM-labelled DU145 prostate carcinoma cell suspension was incubated with the endothelial

monolayer and at 3 hours the extent of DU145 cell trans-endothelial migration was assessed by measuring the increase in fluorescence absorbance of the culture media in the outer chamber of the trans-well as a consequence of migration of the DU145 cells through the endothelial monolayer.

## 2.4 | Characterisation of PAR-1 expression on the endothelial cell surface

EA.hy926 endothelial cells were incubated with the ATAP2 murine anti-PAR-1 receptor antibody or a murine IgG control (2 µg/mL) for 60 minutes on ice. The EA.hy926 cells were then incubated with a fluorescent secondary antibody (AlexaFluor488 goat anti-mouse IgG; 1:1000) for 30 minutes on ice and the expression of PAR-1 determined by the measurement of the cellular fluorescence intensity by flow cytometry.

## 2.5 | Cleavage of endothelial cell surface heparan sulphate proteoglycans (HSPG)

Monolayers of EA.hy926 endothelial cells grown on PET membrane trans-well inserts were incubated with heparinase III (1 U/mL) for 2 hours at 37°C. The cell culture supernatant was aspirated and the monolayers washed three times with PBS and re-incubated with fresh cell culture media.

## 2.6 | Characterisation of endothelial cell myosin light chain-2 phosphorylation status

EA.hy926 cell monolayers were incubated with a sample lysis buffer (125 mmol/L TRIS pH 6.8, 4% SDS w/v, 0.1% Bromophenol blue w/v, 20% Glycerol v/v, 100 mmol/L dithiothreitol (DTT), 1% v/v protease and phosphatase inhibitor cocktail) and cell lysates were generated by scraping. The lysates were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and western blotting using a rabbit anti-phospho-MLC-2 (Thr18/Ser19) antibody. The degree of MLC-2 diphosphorylation observed in endothelial lysates was quantified by particle densitometry (ImageJ software; <https://imagej.net/Welcome>) with the numerical arbitrary units generated for each band in control and LMWH-treated cells expressed as a percentage of the mean maximum particle density observed in bands representing thrombin-induced MLC-2 diphosphorylation.

## 2.7 | Assessment of parameters of plasma thrombin generation and plasma anti-FXa activity

Plasma thrombin generation was assessed by calibrated automated thrombography using a Fluoroskan Ascent Plate Reader (ThermoLab Systems, Helsinki, Finland) in conjunction with Thrombinoscope software (Thrombinoscope BV) as previously described.<sup>28,29</sup> Briefly, 80 µL aliquots of normal pooled plasma were incubated with 20 µL of platelet-poor-plasma reagent (PPP-reagent) containing 5 pmol/L

TF and 4 µmol/L phospholipids (composed of 60% phosphatidylcholine, 20% phosphatidylserine, and 20% phosphatidylethanolamine). Thrombin generation was initiated by the automatic dispensation of a fluorogenic thrombin substrate and CaCl<sub>2</sub> into each well. Plasma anti-factor Xa activity was measured using the ACL TOP 500 haematology analyser (Instrumentation Laboratory) in conjunction with the HaemosIL liquid Anti-Xa reagent.

## 2.8 | Statistical analysis

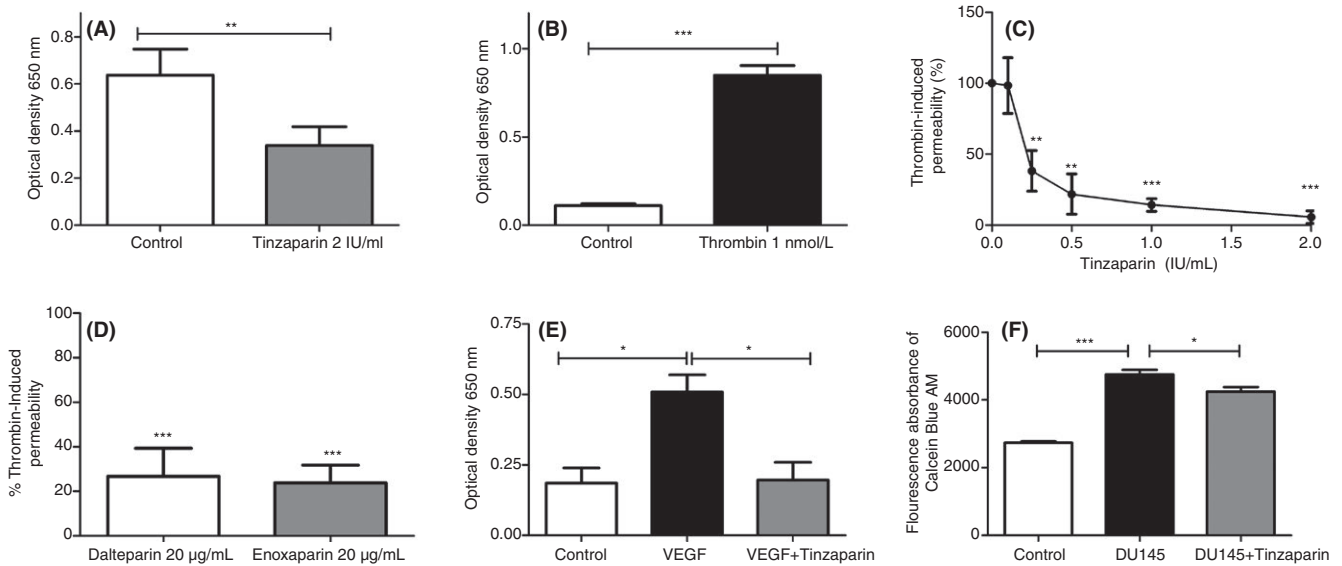
All experiments were performed at least in duplicate with results expressed as the mean±SEM of at least three independent experiments. The results generated were analyzed using either the students unpaired *t* test, Mann-Whitney test, or one-way ANOVA with Bonferroni multiple comparisons test, with a *P*<.05 considered to represent statistical significance. All statistical analysis was performed using GraphPad Prism software (Version 5.0; La Jolla, CA, USA).

## 3 | RESULTS

### 3.1 | LMWH supports endothelial barrier function, attenuates tumor cell trans-endothelial migration and inhibits agonist-induced endothelial permeability

EA.hy926 monolayers formed a barrier which was, initially, virtually impermeable to the albumin solution, with minimal flux through the intact monolayer detected during the first 10 minutes of incubation (culture media optical density at 650 nm [OD 650 nm], 0.17±0.04). However, there was a progressive increase in endothelial permeability over time, with an almost 6-fold increase in outer-chamber absorbance from baseline observed at 40 minutes (OD 650 nm, 0.63±0.1) (data not shown). Remarkably, following a 3-hour period of incubation with LMWH tinzaparin (2 IU/mL) prior to exposure to the albumin solution, the monolayers exhibited enhanced barrier activity with the degree of albumin flux observed to be significantly reduced relative to that which was observed in the untreated monolayers (OD 650 nm at 40 minutes, 0.34±0.1; *P*<.01), suggesting that LMWH enhances basal endothelial barrier activity (Figure 1A).

Agonist-induced endothelial barrier dysfunction is a hallmark of several physiological and pathological processes including inflammation and tumor metastasis. Exposure of endothelial monolayers to thrombin (1 nmol/L, 10 minute incubation; a potent inducer of vascular permeability) led to an 8-fold increase in barrier permeability from baseline (*P*<.001; Figure 1B) but pre-treatment with LMWH tinzaparin at concentrations within a clinically relevant range for 3 hours prior to exposure to thrombin significantly attenuated thrombin-induced permeability. At tinzaparin concentrations of 0.25, 0.5, 1 and 2 IU/mL, thrombin-induced endothelial permeability was suppressed to 38.2±14.4% (*P*<.01), 21.9±14.2% (*P*<.01), 14.3±4.4% (*P*<.001) and 5.7±4.3% (*P*<.001) of baseline permeability respectively (Figure 1C). Similarly, incubation of EA.hy926 monolayers with enoxaparin and dalteparin (at equivalent concentrations) also led to a significant attenuation of thrombin-induced endothelial permeability (Figure 1D).



**FIGURE 1** Low molecular weight heparin (LMWH) supports endothelial barrier function and attenuates tumor cell trans-endothelial migration. Incubation of endothelial monolayers with LMWH tinzaparin (2 IU/mL) led to a significant reduction in the trans-endothelial migration of an Evans blue-conjugated albumin solution (A). Incubation of endothelial monolayers with thrombin (1 nmol/L) led to a marked increase in the permeability of the endothelial cell layers to the albumin solution (B) however LMWH tinzaparin significantly attenuated thrombin-induced permeability within a clinically relevant LMWH concentration range (C). A similar endothelial barrier protective effect was observed in endothelial monolayers pre-treated with LMWH enoxaparin and LMWH dalteparin (20 µg/mL, approx. equivalent to 2 IU/mL anti-FXa activity) (D). Pre-treatment of endothelial monolayers with tinzaparin (2 IU/mL) also significantly attenuated VEGF (1 nmol/L)-induced endothelial permeability (E) and tumor cell trans-endothelial migration (F) (\* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ )

Incubation of confluent EA.hy926 monolayers with VEGF (1 nmol/L; 30-minute incubation) led to a 3-fold increase in monolayer permeability (0 nmol/L VEGF, OD 650 nm:  $0.1 \pm 0.1$ ; 1 nmol/L VEGF, OD 650 nm  $0.3 \pm 0.05$ ;  $P < .05$ ). However, pre-incubation of the endothelial monolayers with LMWH tinzaparin (2 IU/mL) for 3 hours prior to VEGF treatment also significantly attenuated VEGF-induced permeability, suggesting that the LMWH-mediated attenuation of agonist-induced endothelial barrier dysfunction is not limited to the inhibition of the activity of a single agonist (Figure 1E).

The endothelial barrier protective activity of LMWH was also reflected in diminished tumor cell trans-endothelial migration, with a reduction in the migration of DU145 carcinoma cells through confluent endothelial monolayers to  $73.9 \pm 5.7\%$  of baseline observed following incubation of the endothelial cell layers with LMWH (tinzaparin 2 IU/mL;  $P < .05$ ) (Figure 1F).

## 3.2 | LMWH-mediated suppression of thrombin-induced endothelial permeability is not mediated through a direct inhibition of thrombin proteolytic activity

### 3.2.1 | LMWH inhibits thrombin-induced endothelial cell MLC-2 diphosphorylation

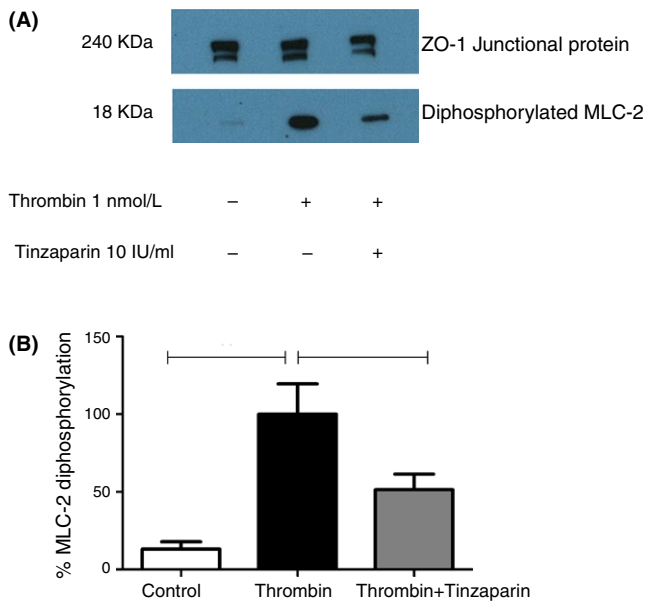
Agonists such as thrombin, which induce endothelial cell contraction, activate signalling pathways which lead to the

diphosphorylation and activation of myosin light chain-2 (MLC-2, the primary regulator of actin cytoskeleton contraction) and actin cytoskeleton activation.

As expected, minimal MLC-2 diphosphorylation was observed in untreated endothelial cells in the absence of stimulation with thrombin (Figure 2A and B). Stimulation with thrombin (1 nmol/L; 10 minutes) led to a marked increase in endothelial MLC-2 diphosphorylation, but this was significantly attenuated in endothelial cells which had been pre-treated with tinzaparin (10 IU/mL; 3-hour incubation) prior to incubation with thrombin (mean maximum thrombin-induced MLC-diphosphorylation reduced from  $100 \pm 19.5\%$  to  $51.4 \pm 9.9\%$ ,  $P < .03$ ; Figure 2A and B), suggesting that LMWH suppresses the activity of thrombin-activated signalling pathways which promote MLC-2 phosphorylation and actin cytoskeleton activation.

### 3.2.2 | Thrombin-mediated endothelial permeability is entirely PAR-1 dependent

In the presence of a cleavage-blocking anti-PAR1 antibody (ATAP2, thrombin receptor antibody; 20 µg/mL), thrombin-induced endothelial permeability was completely abolished. Similarly, a PAR-1 inhibitory peptide (RWJ56110; 20 µmol/L) also inhibited thrombin-induced permeability, confirming that thrombin-mediated endothelial barrier permeability is entirely dependent on PAR-1 cleavage and the subsequent activation of PAR-1 intracellular signalling (Figure 3A).



**FIGURE 2** Low molecular weight heparin (LMWH) attenuates thrombin-induced endothelial cell MLC-2 diphosphorylation. Endothelial cell lysates were prepared and analysed by SDS-PAGE and western blotting using a loading control antibody (anti-zona occludens-1, a membrane associated junctional protein; ZO-1) and an anti-phospho-MLC-2 (Thr18/Ser19) antibody (A). MLC-2 diphosphorylation was significantly enhanced following incubation with thrombin (1 nmol/L; 10 minutes), however this was attenuated in cells which had undergone pre-treatment with LMWH tinzaparin (10 IU/mL; supra-therapeutic LMWH concentration utilised in order to facilitate the detection of a LMWH effect in the context of the sensitivity of the assay utilised) (B) (\* $P < .05$ ; \*\* $P < .01$ )

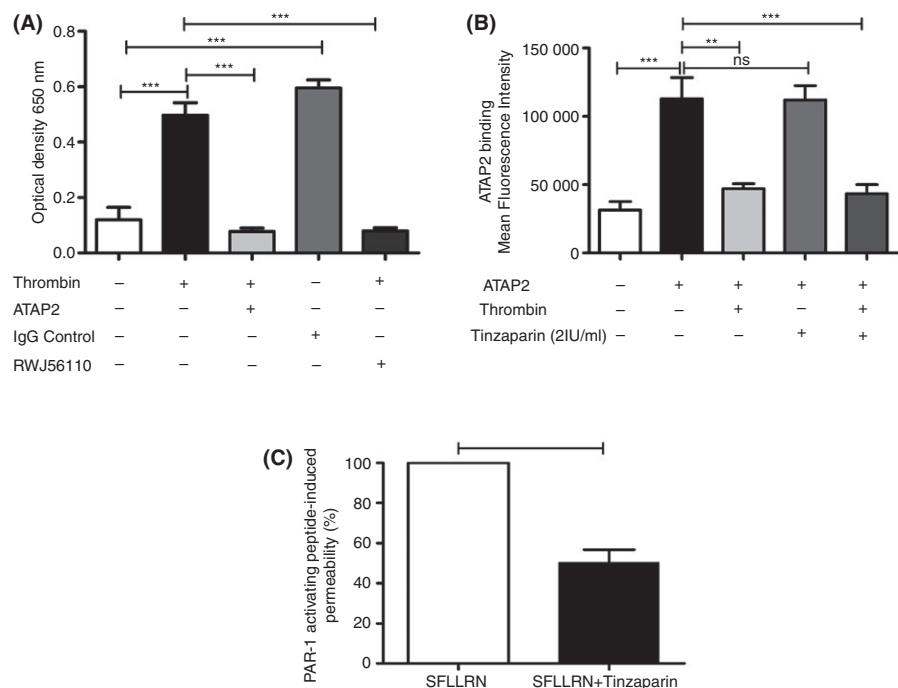
### 3.2.3 | LMWH-mediated attenuation of thrombin-induced endothelial permeability is not achieved through inhibition of thrombin-mediated PAR-1 cleavage

Predictably, a significant reduction in mean fluorescence intensity (as determined by flow cytometry using an antibody detecting PAR-1 activation) was observed following incubation of EA.hy926 endothelial cells with thrombin, as a consequence of thrombin-mediated activation of the PAR-1 extracellular domain (Figure 3B).

Incubation of EA.hy926 cells with tinzaparin (2 IU/mL; 3-hour incubation) did not significantly alter the measured fluorescence intensity, suggesting that LMWH does not influence endothelial PAR-1 expression. Moreover, the fluorescence intensity measured in cells incubated with thrombin (1 nmol/L; 10 minutes) following pre-incubation with tinzaparin was similar to that measured in cells treated with thrombin alone, suggesting that LMWH does not impair thrombin-mediated activation of PAR-1 (Figure 3B).

Furthermore, incubation of endothelial monolayers with tinzaparin (2 IU/mL) prior to incubation with a thrombin receptor agonist peptide (Ser-Phe-Leu-Leu-Arg-Asn-amide, SFLLRN; 50  $\mu$ mol/L) which activates PAR-1 and PAR-2 signalling independent of PAR cleavage also led to a significant attenuation of PAR-1/PAR-2 mediated endothelial barrier permeability (Figure 3C). Collectively, these results suggest that LMWH suppresses PAR-1 mediated signalling but that this cytoprotective effect is not mediated through the direct inhibition of PAR-1 cleavage. The finding that the protective effect of LMWH in opposing thrombin-mediated permeability is independent of thrombin proteolytic activity supports the hypothesis that the cytoprotective activity of LMWH is not dependent on its anticoagulant activity.

**FIGURE 3** Low molecular weight heparin-induced suppression of PAR-1-mediated endothelial barrier dysfunction is not mediated through an inhibition of PAR-1 cleavage and activation. Inhibition of PAR-1 cleavage (anti-PAR-1 receptor antibody, ATAP2; 20  $\mu$ g/mL) or inhibition of PAR-1 signalling (selective PAR-1 antagonist, RWJ56110; 20  $\mu$ mol/L) abolished thrombin-induced endothelial permeability suggesting that thrombin mediated endothelial barrier dysfunction is entirely PAR-1 dependent (A). Tinzaparin does not inhibit PAR-1 expression on endothelial cells and does not inhibit thrombin-mediated PAR-1 activation (B). PAR-1/PAR-2 mediated endothelial permeability is attenuated by tinzaparin independent of PAR-1 cleavage (C) (\* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ )



### 3.2.4 | The endothelial barrier protective effects of LMWH are not mediated through an interaction with endothelial cell surface heparan sulphate proteoglycans

Endothelial cell surface heparan sulphate proteoglycans (HSPG) have been shown to exhibit co-factor activity in supporting the effects of various agonists including pro-metastatic agonists such as thrombin, VEGF and fibroblast growth factor-2.<sup>30–32</sup> Endogenous HSPGs are structurally diverse but bear some similarity to pharmacological heparin formulations.<sup>33</sup> In order to characterize the role of cell surface HSPG in modulating thrombin-induced endothelial permeability and to investigate whether LMWH-mediated attenuation of thrombin-induced endothelial barrier permeability arises through an interaction with HSPG, EA.hy926 monolayers were treated with heparinase III (derived from *Flavobacterium Heparinum* and which specifically cleaves heparan sulphates) prior to assessing monolayer permeability.

As expected, thrombin-induced endothelial barrier permeability was diminished following HSPG cleavage (Figure 4A). However, the relative attenuation of endothelial barrier permeability by LMWH in heparinase III-treated and untreated monolayers was similar (Figure 3B), as evidenced by a LMWH/thrombin OD ratio of  $0.46 \pm 0.1$  and  $0.3 \pm 0.1$  ( $P=.14$ ) respectively. This finding suggests that the barrier protective properties of LMWH are not mediated through an interaction with cell surface HSPG.

### 3.3 | A 2.8 KDa LMWH fraction with limited in vitro anticoagulant activity exhibits endothelial barrier protective properties

A concentration-dependent suppression of plasma thrombin generation was observed in plasma incubated with LMWH tinzaparin (Figure 5A). At a concentration of 0.5 IU/mL (within the target plasma range for thrombosis therapy in patients) a profound attenuation of thrombin generation was observed with peak thrombin generation just reaching  $4.2 \pm 0.7$  nmol/L from a baseline of  $308.2 \pm 20$  nmol/L ( $P<.001$ ), representing a tinzaparin-mediated attenuation of peak plasma thrombin generation of 98.6%. Similarly, 0.5 IU/mL

tinzaparin suppressed plasma endogenous thrombin potential (ETP) to  $110.5 \pm 17.1$  nmol/L\*min from a baseline of  $2229 \pm 136$  nmol/L\*min ( $P<.001$ ), representing an attenuation of ETP of approximately 95%.

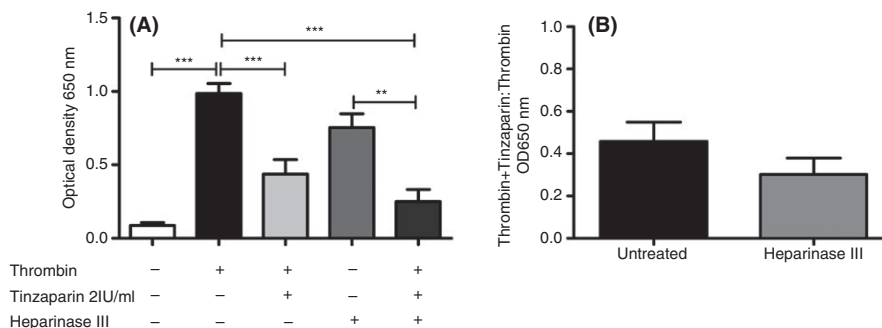
At a concentration range of 0–5 µg/mL, which is approximately equivalent to a LMWH tinzaparin concentration range of 0–0.5 IU/mL, the 2.8 KDa fraction attenuated plasma thrombin generation but to a lesser extent than that observed with tinzaparin (Figure 5B). At a 2.8 KDa LMWH plasma concentration of 5 µg/mL (equivalent to 0.5 IU/mL tinzaparin), mean ETP was only attenuated from  $2184 \pm 65.4$  nmol/L\*min to  $1532 \pm 123$  nmol/L\*min ( $P<.01$ ) and no significant difference in any other parameter of plasma thrombin generation within this concentration range was observed.

Similarly, in the plasma anti-FXa activity assay, the 2.8 KDa fraction exhibited reduced anticoagulant activity (Figure 5D) relative to standard tinzaparin (Figure 5C). At a plasma concentration of 5 µg/mL, the fraction exhibited  $0.18 \pm 0.01$  IU/mL of anti-FXa activity, a value which would not be expected to confer a significant bleeding risk (Figure 5D).

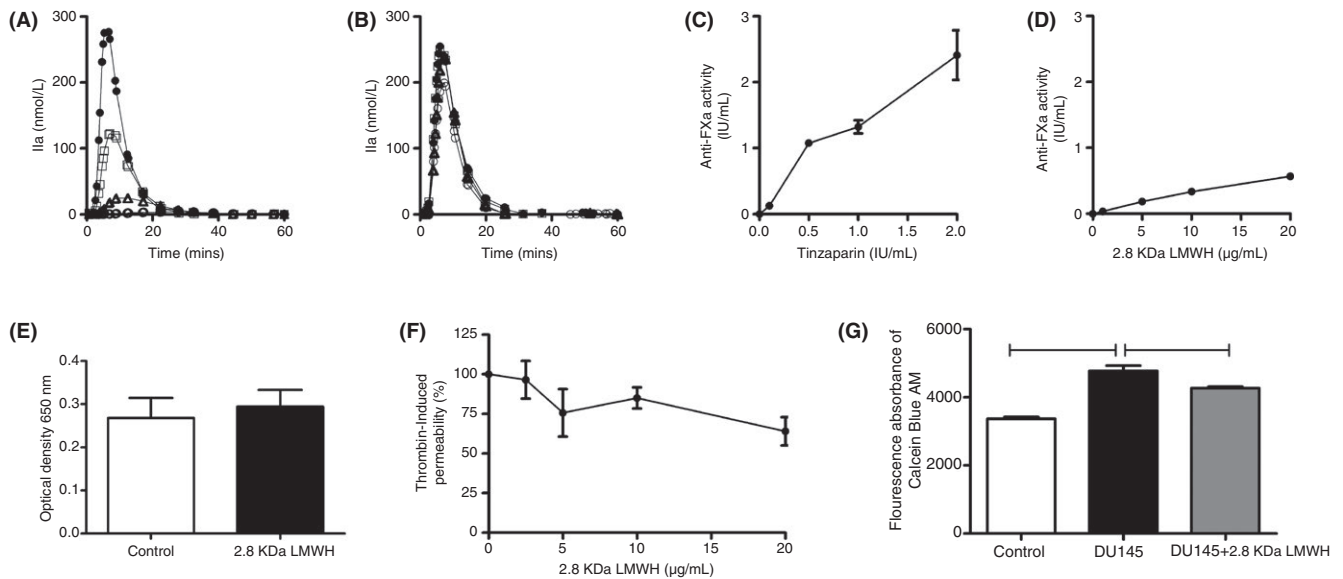
However, despite diminished in vitro anticoagulant activity, the 2.8 KDa LMWH fraction did exhibit endothelial barrier protective properties. At a concentration of 20 µg/mL, basal albumin permeability of the endothelial monolayer was unchanged (Figure 5E), however, thrombin-induced endothelial monolayer permeability was significantly attenuated, although to a lesser extent than that observed with the standard tinzaparin formulation ( $64 \pm 9\%$ ,  $P<.05$ ; Figure 5F) and, moreover, tumor cell trans-endothelial migration ( $63.9 \pm 3\%$  of baseline,  $P<.001$ ; Figure 5G) was also significantly attenuated.

### 3.4 | Simvastatin attenuates endothelial permeability and exhibits synergistic inhibitory effects on agonist-induced endothelial permeability when co-incubated with sub-anticoagulant concentrations of LMWH

Following incubation of EA.hy926 monolayers with simvastatin, at a concentration range similar to the predicted plasma statin concentrations achieved in clinical practice, a modest concentration-dependent attenuation of thrombin-induced endothelial permeability was observed (Figure 6A).



**FIGURE 4** Low molecular weight heparin (LMWH)-mediated endothelial barrier protection is not mediated through an interaction with cell surface heparan sulphate proteoglycans. Heparan sulphate proteoglycans cleavage attenuated thrombin-induced endothelial permeability (A). The relative attenuation of thrombin-induced endothelial permeability following incubation with LMWH was similar in heparinase III treated and untreated endothelial monolayers (B) (\*\* $P<.01$ ; \*\*\* $P<.001$ )



**FIGURE 5** A 2.8 kDa low molecular weight heparin (LMWH) fraction derived from tinzaparin exhibits diminished anticoagulant activity *in vitro* relative to that observed with standard tinzaparin but retains endothelial barrier protective properties. Plasma thrombin generation in pooled normal plasma is suppressed to a greater extent in the presence of tinzaparin (A; 0 IU/mL, ●; 0.1 IU/mL, □; 0.25 IU/mL, △; 0.5 IU/mL, ○) relative to that observed in the presence of the equivalent concentrations of the 2.8 kDa LMWH fraction (B; 0 µg/mL, ●; 1 µg/mL, □; 2.5 µg/mL, △; 5 µg/mL, ○). The 2.8 kDa fraction also exhibits diminished anti-factor Xa activity (D) relative to that observed with equivalent concentrations of standard tinzaparin (C). Incubation of endothelial monolayers with the 2.8 kDa LMWH fraction did not appear to enhance baseline endothelial barrier function (E) but did attenuate thrombin induced endothelial permeability (F) and reduced DU145 cell trans-endothelial migration (G) (\* $P < .05$ ; \*\* $P < .01$ , \*\*\* $P < .001$ )

A sub-anticoagulant concentration of tinzaparin (0.1 IU/mL) did not attenuate thrombin-induced endothelial permeability but remarkably following incubation of statin-treated monolayers with tinzaparin at a range of LMWH concentrations, a synergistic effect on endothelial barrier protection was observed and at the sub-anticoagulant tinzaparin concentration of 0.1 IU/mL, the thrombin-induced permeability of EA.hy926 monolayers treated with simvastatin (20 nmol/L) was reduced to just  $7.9 \pm 0.2\%$  of baseline ( $P < .05$ ; Figure 6A).

An effect, although less marked, was observed when EA.hy926 monolayers were incubated with tinzaparin at a concentration range of 0–0.5 IU/mL following a 24-hour incubation period with a fixed simvastatin concentration (5 nmol/L), although the protective effect against thrombin-induced permeability only reached statistical significance at the tinzaparin concentration of 0.5 IU/mL and a statistically significant difference between 0.5 IU/mL of tinzaparin used in isolation and 0.5 IU/mL in combination with simvastatin 5 nmol/L was not detected (Figure 6B).

An additive effect was also observed following co-incubation of endothelial cells with increasing concentrations of simvastatin in the presence of the 2.8 kDa LMWH fraction although the magnitude of barrier protective effect relative to simvastatin alone did not reach statistical significance (Figure 6C).

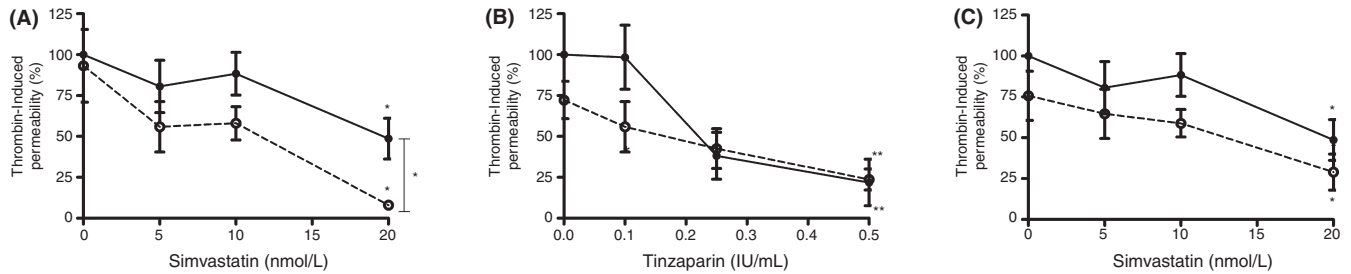
## 4 | DISCUSSION

In the present study, we have demonstrated *in vitro* evidence to suggest that the exposure of endothelium to LMWH leads to enhanced endothelial barrier function. Moreover, we have described

the endothelial barrier protective properties of a tinzaparin-derived LMWH fraction with limited anticoagulant activity and we have characterized the additive endothelial protective effect which is observed following exposure of endothelium to combinations of LMWH and simvastatin.

Tumor cells which enter the circulation must attach to the vascular wall and extravasate through the impermeable barrier presented by normal endothelium in order for metastasis to proceed.<sup>2,34</sup> The vital importance of the barriers to metastasis present at the stage of extravasation is reflected in the observation that only a tiny minority of circulating cancer cells (<0.01%) successfully establish secondary tumors.<sup>2,3,34–36</sup> Therefore, therapeutic strategies which directly enhance the activity of these physiological barriers to tumor cell extravasation would be predicted to attenuate cancer metastasis.<sup>5,34,36</sup>

A number of investigators have described several distinct mechanisms through which malignant tumors induce vascular permeability leading to enhanced metastasis. The expression of specific sets of genes or metastatic gene “signatures” in cells of a primary tumor, the release of specific tumor-derived exosomes expressing specific oncoproteins or pro-metastatic microRNA as well as the enhanced expression of pro-metastatic factors such as TGF $\beta$  in the tumor microenvironment have been shown to promote enhanced permeability and tumor cell extravasation in the vasculature of the target organ.<sup>6,8,34,35,37,38</sup> Moreover, at the interface between the tumor cell and the vascular endothelium, the secretion of factors such as VEGF and tumor-derived SPARC protein as well as the enhanced generation of thrombin also serve to induce loss of barrier integrity.<sup>9</sup> Crucially,



**FIGURE 6** Co-incubation of endothelial monolayers with simvastatin and low molecular weight heparin (LMWH) leads to a marked suppression of thrombin-induced endothelial permeability. Simvastatin attenuates thrombin-induced permeability at clinically-relevant concentrations although to a lesser extent than that observed with concentrations of tinzaparin within the clinical range. Incubation of endothelial monolayers with sub-anticoagulant concentrations of tinzaparin (0.1 IU/mL) does not protect against thrombin-induced endothelial barrier permeability ( $98.45 \pm 19.6\%$  of baseline permeability) but co-incubation with simvastatin (20 nmol/L) leads to marked barrier protection relative to tinzaparin 0.1 IU/mL alone ( $7.9 \pm 2\%$  of baseline permeability;  $P = 0.02$ ) or relative to simvastatin 20 nmol/L alone ( $P = 0.02$ ) (A). Similarly, co-incubation of endothelial cells with a fixed concentration of simvastatin (5 nmol/L) with a range of LMWH concentrations also appeared to lead to enhanced barrier protection against thrombin-induced permeability relative to tinzaparin alone however this combination only reached statistical significance at a tinzaparin concentration of 0.5 IU/mL with simvastatin 5 nmol/L and this was not significantly different to the effect observed with tinzaparin 0.5 IU/mL alone (B). Co-incubation of endothelial cells with simvastatin also appeared to potentiate the endothelial barrier protective properties of the 2.8 kDa LMWH fraction at the non-anticoagulant concentration of 5 μg/mL, although this effect was not significantly different to the effect observed with the 2.8 kDa fraction or simvastatin in isolation (C) (\* $P < 0.05$ ; \*\* $P < 0.01$ )

inhibition of these various mechanisms in animal models of metastasis has been shown to attenuate tumor-mediated vascular permeability and metastasis formation.<sup>6,9,37,38</sup>

The observation that LMWH attenuates endothelial permeability induced by multiple agonists and also attenuates tumor trans-endothelial migration suggests that LMWH may mediate a supportive effect on barrier function through a general mechanism involved in maintaining barrier integrity. Thrombin is one of the most potent physiological inducers of vascular permeability and the molecular mechanism underlying its activity in this regard is well characterized.<sup>39,40</sup> Moreover, thrombin is the key effector protease of the blood coagulation cascade and the inhibition of thrombin generation and thrombin proteolytic activity forms the basis of anticoagulant therapy.<sup>41,42</sup> Given that the risk of hemorrhage associated with anticoagulation represents the main disadvantage of LMWH use for the purposes of its anti-metastatic effect, we specifically investigated the mechanism underlying LMWH-mediated inhibition of thrombin-induced endothelial permeability in order to ascertain if it might be possible to separate the anticoagulant and cytoprotective properties of LMWH in a manner which would facilitate the safe administration of this agent to patients with cancer at high risk of bleeding. Interestingly, the barrier protective properties of LMWH in inhibiting thrombin-induced endothelial permeability did not appear to be mediated through the inhibition of its activation of PAR-1 nor did it appear to be mediated through an effect on HSPG cofactor activity in facilitating agonist-induced signalling. These findings, in conjunction with the observation that exposure of endothelial monolayers to LMWH also directly attenuates cellular trans-endothelial migration, suggest that LMWH mediates its cytoprotective effect on barrier function by independently signalling on the endothelial cell surface (a hypothesis which is supported by recent reports describing for the first time the expression of a specific heparin receptor on the endothelial cell surface which appears to modulate inflammatory responses including

actin cytoskeleton rearrangement).<sup>43</sup> Crucially, LMWH appears to attenuate thrombin-induced endothelial permeability in a manner which is not dependent on inhibition of thrombin-proteolytic activity a finding which suggests that the cytoprotective and anticoagulant properties of LMWH may not be inter-dependent.<sup>42–44</sup>

We have described the relative anticoagulant and cytoprotective of a fraction of LMWH derived from standard commercial tinzaparin but consisting primarily of shorter heparin polysaccharides than those which would be predicted to be observed in the commercial formulation. As predicted, the 2.8 kDa fraction exhibited diminished in vitro anticoagulant activity relative to that observed at equivalent concentrations of standard tinzaparin. Remarkably, this non-anticoagulant heparin fraction retained cytoprotective signalling activity, although to a lesser extent than that observed with the standard preparation in the albumin permeability assay. The 2.8 kDa fraction exhibited an effect on tumor cell trans-endothelial migration which appeared to be equivalent to that observed with standard tinzaparin (the albumin permeability assay represents a more sensitive measure of changes in barrier function than the cellular trans-migration assay given the smaller size of albumin molecules relative to intact tumor cells, moreover, it is possible that the LMWH fraction may exhibit additional properties in the context of tumor cell migration such as an inhibition of tumor cell adhesion to the endothelium, a key step in tumor cell trans-migration). This finding suggests that the potency of LMWH in mediating endothelial barrier protection may be related to heparin polysaccharide chain length while not necessarily being dependent on LMWH anticoagulant activity.

The statins have been previously shown to exhibit endothelial barrier protective properties in vitro and in vivo in models of hyperglycaemia and thrombin-induced endothelial barrier dysfunction (although at concentrations which are in excess of the therapeutic range in humans). The actions of statins in regulating endothelial barrier function appear to be mediated through the inhibition of the biosynthesis of specific lipid metabolites which are involved in regulating endothelial barrier



function, such as components of the RhoA GTPase signalling pathway. Statin-mediated inhibition of these processes appears to diminish agonist-induced endothelial barrier dysfunction and endothelial inflammatory responses.<sup>24,25</sup> We demonstrated that simvastatin attenuates thrombin-induced endothelial barrier dysfunction in vitro, although this observation only reached statistical significance at a simvastatin concentration at the upper limit of the therapeutic range.<sup>45,46</sup> However, we also demonstrated that a synergistic effect on endothelial barrier function is observed when simvastatin and LMWH are co-incubated with endothelial cell monolayers and that almost complete protection against thrombin-induced endothelial barrier dysfunction can be achieved in vitro when combining a concentration of LMWH tinzaparin which would not be predicted to confer a risk of hemorrhage in vivo (0.1 IU/mL) and a concentration of simvastatin within the therapeutic range.

This study has some limitations: firstly, the putative receptor and signalling pathway for LMWH-mediated endothelial barrier protection remain to be fully characterized. Secondly, in vivo data supporting a synergistic role for LMWH and simvastatin in enhancing the endothelial barrier are currently lacking, although future work is planned. In addition, with regard to the clinical implications of our study, the interpretation of our findings is somewhat limited by the conflicting reports from clinical studies to date, some of which failed to detect a survival benefit following LMWH administration to cancer patients.<sup>47,48</sup> However, in contrast to animal models of metastasis (which have consistently demonstrated a LMWH-mediated anti-tumor effect), a significant proportion of participants in clinical trials would be predicted to have already developed clinically undetectable micro-metastases by the time of recruitment, particularly in the sub-groups of patients with more advanced disease. We would hypothesise that LMWH is likely to only be effective in preventing metastasis in the sub-group of patients with very early, limited-stage tumors prior to development of established micrometastases.<sup>2</sup>

In conclusion, the role of LMWH therapy in the prevention of cancer metastasis in clinical practice remains to be determined and the hypothesis that LMWH confers a definite survival benefit has yet to be definitively proven in an adequately powered, prospective clinical trial (although several such studies, such as the TILT study, are currently in progress). However, in keeping with existing data from in vitro and pre-clinical studies which have suggested that LMWH mediates an anti-cancer effect, we have demonstrated that LMWH supports endothelial barrier function and attenuates agonist-induced endothelial permeability, pathways which are key to the progression of metastasis. Crucially, we have also described strategies which would be predicted to limit the risk of LMWH-associated haemorrhage without diminishing these LMWH-mediated cytoprotective activities; findings which, if replicated in vivo, would be of significant translational value.

## AUTHOR CONTRIBUTION

B.K. conducted the experiments, analysed data and wrote the paper; K.E., S.A., E.N. and A.L. conducted experiments; P.M. contributed to the concept of the study; F.N.A. developed the concept of the study and edited the final draft of the manuscript.

## RELATIONSHIP DISCLOSURES

Dr. Kevane has a patent null pending. Dr. Ní Áinle has a patent Optimization of endothelial protective properties of low molecular weight heparin (or fractions thereof) by combination with a statin pending.

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