Thrombosis Research 133 (2014) 265–271

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Time-dependent hardening of blood clots quantitatively measured *in vivo* with shear-wave ultrasound imaging in a rabbit model of venous thrombosis

Etienne Mfoumou [a], Julien Tripette [a], Mark Blostein [b], Guy Cloutier [a,c,*]

[a] Laboratory of Biorheology and Medical Ultrasonics, University of Montreal Hospital Research Center (CRCHUM), Montreal, QC, Canada
[b] Division of Hematology, Department of Medicine, Sir Mortimer B. Davis Jewish General Hospital, Montreal, QC, Canada
[c] Department of Radiology, Radio-Oncology and Nuclear Medicine and Institute of Biomedical Engineering, University of Montreal, Montreal, QC, Canada

ARTICLE INFO

Article history:
Received 19 July 2013
Received in revised form 22 October 2013
Accepted 5 November 2013
Available online 12 November 2013

Keywords:
Ultrasonography
Elasticity imaging techniques
Young's modulus
Animal experimentation
Venous thrombosis
Ultrasound
Shear wave elasticity imaging
Thrombus aging
Blood clot hardening

ABSTRACT

Objective: Provide *in vivo* blood clot hardening evolution with ultrasound using supersonic imaging of shear waves.
Methods: We conducted a prospective study in flow stasis-induced venous thrombosis within jugular veins of white female New Zealand rabbits. Blood clot elasticity was noninvasively measured *in vivo* using the Young's modulus (in kilopascals), on a 2-hour and a 2-week periods after thrombus induction. Monitoring was followed by a necropsy and *ex vivo* mechanical characterization to validate the existence and elasticity of explanted thrombi.
Results: Stagnant blood in the region of interest underwent clotting and progressive hardening with thrombus aging. The mean Young's moduli varied from 1.0 ± 0.6 kPa (at 10 min) to 5.3 ± 1.6 kPa (at 2 hours), then to 25.0 ± 6.8 kPa (at 14 days) post-surgery. Mean *ex vivo* moduli of 6.2 ± 0.7 kPa at 2 hours and 29.0 ± 2.4 kPa at 2 weeks agreed with *in vivo* measures.
Conclusions: Supersonic imaging of shear waves provides consistent quantitative non-invasive elasticity measurements not available with standard compression ultrasound imaging for diagnosing and following venous thromboembolism. This information translatable to humans could aid in determining whether continued anticoagulant treatment is necessary, especially in the setting of unprovoked venous thromboembolism.

Introduction

Thrombus formation is a complex biological process involving many co-factors [1–4]. Venous thromboembolism (VTE), an important cause of morbidity and mortality in clinical medicine, is typically diagnosed using Doppler ultrasound and compression ultrasonography [5]. VTE is commonly treated with anticoagulants, but the duration of oral anticoagulation therapy (OAT) in patients after a first episode of unprovoked VTE is controversial [6,7]. Indeed, the risk of VTE recurrence after OAT withdrawal is high in this category of patients [7–9]. On the other hand, continuous treatment with OAT assures that recurrent thromboembolism will not occur, albeit at a risk of major bleeding [8–11]. Thus, a diagnostic method is needed to age and determine the nature of a venous thrombus, guide therapy, and predict if VTE events will recur. This could be achieved through a time-dependent monitoring of hardening properties of venous thrombi. In this regard, quantitative ultrasound (US) shear wave elasticity imaging is proposed in this study.

US is widely available, non-invasive and does not result in exposure to radiation. Clinical assessment for venous thrombosis is typically performed in B-mode using compression (henceforth compression US) and with assessment of venous flow using duplex Doppler or colour-Doppler for veins not amenable to direct compression [12]. Veins should easily collapse under an applied external pressure with the US probe, with lack of expected venous collapse indicating the presence of a thrombus, whereas in Doppler US, the absence of flow, specifically in a vein which cannot be subjected to compression suggests the presence of a venous thrombus. However, none of the above mentioned US techniques are able to display quantitative mechanical information that could help to characterize the thrombus formation stage and determine the impact of medication.

Upon its formation, venous thrombi undergo several physiologic changes among which fibrinolytic activation and dissolution, invasion by inflammatory cells, reepithelialisation of the surface of the thrombus in contact with blood, and fibrosis can be seen [3,4]. It is worthwhile noticing that these changes are associated with alterations of blood clot mechanical properties. Indeed, in a cohort of 60 consecutive patients, Heijboer et al. [13] undertook serial compression US after an

* Corresponding author at: Research Center, University of Montreal Hospital, 900 rue St-Denis, Montréal, QC, H2X 0A9, Canada. Tel.: +1 514 890 8000x24703; fax: +1 514 412 7505.
E-mail address: guy.cloutier@umontreal.ca (G. Cloutier).
URL: http://www.lbum-crchum.com (G. Cloutier).

0049-3848/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.thromres.2013.11.001

acute DVT and demonstrated the presence of a residual thrombus in about 50% of patients at 6 to 12 months. This residual thrombus persists indefinitely [14–16] and given that DVT may be contralateral in 50% of patients, residual vein thrombus may be considered a manifestation of an underlying prothrombotic tendency. Hence, an accurate quantitative mechanical characterization of this residual thrombus may offer an opportunity to predict recurrent VTE.

The present study uses, for the first time, the Supersonic Shear-wave Imaging (SSI) technology [17] for the measurement of time-dependent hardening of blood clots *in vivo*. This technique couples high-resolution anatomic images obtained with B-mode US, with mechanical information (Young's modulus) provided by quantitative elastography. Our findings in an animal model support the hypothesis that mechanical characterization of a DVT is possible *in vivo*. Such a quantitative determination of blood clot hardening may define a risk factor, in the clinical setting, for deciding whether to continue oral anticoagulant therapy after an unprovoked venous thromboembolism, which is still controversial.

Materials and Methods

Animals

Experimental procedures, including *ex vivo* sample analyses, were conducted in accordance with guidelines of the Institutional Animal Care Committee of the University of Montreal Hospital Research Center. The investigation conformed with guidelines of the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health (NIH Publication No. 85-23, revised 1996, Assurance Number A5377-01).

Nine healthy female New Zealand white rabbits, of 2.5 to 3.2 kg (mean ± SD, 3.0 ± 0.7 kg) in weight, were used in this study. Animals were purchased from Charles River Canada (St.-Constant, Quebec, Canada). The vendor provided health reports to certify that animals were free of pathogens. All rabbits were housed in separate cages (Lab Products Inc., Seaford, DE) on suspended floor. The holding room was ventilated with filtered 100% outside air at 15 air changes per hour. Room temperature and relative humidity were 22 ± 2 °C and 30-70%, respectively. A 12:12-h light-dark cycle was used. Environmental enrichment consisted in plastic or cardboard boxes, toys (BioServ, Frenchtown, NJ), alfalfa (BHH-1142 J.E. Mondou, Montreal, Quebec) and vegetables. Food (Teklad high fiber rabbit diet 2031, Harlan Laboratories Inc., Madison, WI) and water were provided ad libitum. Animals were acclimated to their environment 7 days before the beginning of the procedure.

Animals' Preparation

All animals were anaesthetized for surgery and euthanized immediately after the short term (2 hours) and long term (2 weeks) monitoring. The general anaesthesia regime for surgery was the same for all animals except for those recovering for anaesthesia (*i.e.*, rabbits followed for the 2-week long term monitoring period). For these animals, a 12 μg fentanyl transcutaneous patch (Fentanyl Transdermal System, Sandoz, Boucherville, QC, Canada) was placed in the ear pinnae twenty hours before surgery. They also received 0.03 mg/kg subcutaneous buprenorphin (Buprenex, Pharmaceutical Limited, Berkshire, United Kingdom) 30 minutes before surgery to ensure postoperative analgesia. All rabbits followed for 2-hour and 2-week periods received a 0.75 mg/kg subcutaneous injection of acepromazine (Atravet, Boehringer Ingelheim, Burlington, ON, Canada) 30 minutes prior to the induction of anaesthesia. A 24-gauge catheter (Surflo, Terumo Medical Co., Elkton, MD) was placed into the marginal ear vein and the anaesthesia was induced by intravenous administration of 5 mg/kg propofol (Propofol 1%, Pharma Science, Montreal, QC, Canada). General anaesthesia was maintained with 1-2% isoflurane (Aerrane, Abbott Laboratories, Montreal, QC, Canada) in oxygen (flow rate 1 L/min) using a 4.5 mm (ID) uncuffed endotracheal tube and a non-rebreathing system (Bain circuit). A second catheter was installed in the saphenous vein for the administration of a 0.12 mg/kg bolus followed by a constant rate infusion (CRI) of 1.2 mg/kg/hr ketamine (Ketalean, Bimeda-MTC Cambridge, Ontario, Canada). The ketamine was diluted in saline and the CRI was calculated to permit fluid maintenance (10 ml/kg/hr).

Each rabbit was then placed in a supine position on a circulating water heating pad (Micro-Temp II Cincinnati Sub-Zero, Cincinnati, OH) and rectal body temperature was monitored and maintained around 38 °C. After shaving and aseptic cleaning of the neck area, the surgical site was infiltrated with 2 mg/kg bupivacaine (Sensorcaine, AstraZeneca, Mississauga, ON, Canada). Haemoglobin oxygen saturation was monitored constantly with a pulse oximeter (Digicare Biomedical Technology Inc., FL, USA) and a clip-on probe placed on finger or toe. Haemoglobin oxygen saturation remained above 96% during the procedure, thus avoiding hypoxia. Heart rate, respiratory rate and indirect blood pressure (Digicare Biomedical Technology Inc., FL, USA) were monitored every 5 minutes. When an eye was not completely closed during anaesthesia, sterile ophthalmic drops were inserted in the eye to minimize corneal drying.

Induction of Venous Thrombosis

The jugular vein was exposed using blunt dissection, including the distal bifurcation (Fig. 1). A slightly modified version of the model from Lorrain et al. [18] and the double-opposing inverted-sutures model [19] were used to induce venous thrombosis in the jugular vein. Two loose sutures were placed 2 cm apart, both proximally and distally on the segment of interest. This technique allows proceeding to the double ligatures without exposing the entire segment of interest. All other vessels connecting the selected jugular segment were ligated. On the segment of interest, the proximal suture on the jugular vein was first closed. A hypercoagulable state was promoted using a syringe, by aspirating the blood trapped in the segment of interest, keeping it 1-2 minutes in the syringe, and re-injecting it back while closing the distal suture, creating a stasis. As a direct result, a venous thrombosis was obtained from the combination of blood-flow stasis and increased coagulability. The incisions were then closed and the thrombus hardening was monitored by echography (SSI imaging), under general anaesthesia. On following days, US recordings were performed without anaesthesia expect for every third day, where animals received subcutaneous acepromazine (0.75 mg/kg) and were briefly anesthetized with isoflurane (2%) in oxygen (1 L/min) delivered with a face mask to permit blood sampling.

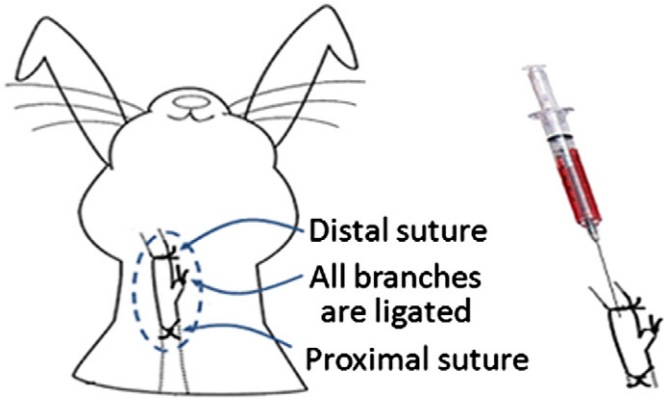

Fig. 1. Venous thrombosis induction in the rabbit jugular vein produced with a combination of blood-flow stasis and the promotion of a hypercoagulable state. (Left): stasis is produced by distal and proximal suture points, as well as ligation of all bifurcation branches. (Right): hypercoagulability is promoted by aspirating the blood trapped in the segment of interest, keeping it about 1 minute in the syringe, and re-injecting it back.

Shear Wave Elastography and Real-time Monitoring of Thrombus Hardening

In shear wave elastography, the scanner generates a remote radiation force through focused ultrasonic beams that induce the propagation of transient shear waves. An ultrafast imaging sequence is then performed to acquire successive radio-frequency (RF) data at a very high frame rate of a few kHz. The tissue displacement due to shear wave propagation is calculated using cross-correlation of successive RF echoes, and the shear wave velocity (and elasticity) is determined using a time-of-flight estimation [20]. In the current study, elastography with SSI was performed using the Aixplorer US scanner (Supersonic Imagine, Aix-en-Provence, France) equipped with a linear array transducer (SuperLinear 15-4). This transducer has a 4-15 MHz frequency band, and was attached to a stand-alone support equipped with a manually adjustable rotary system for appropriate positioning of the probe. This insured no motion of the probe during Doppler and elasticity recordings. Transversal and longitudinal views of the segment of interest were carefully interrogated in colour Doppler mode before and after surgery to insure accurate positioning of the probe on the animal. Images of the vein under analysis and of the adjacent artery were documented prior selecting the region of interest (ROI) for subsequent elasticity data collection.

After a few seconds of US recordings, when a stable colour-coded elasticity map was obtained, the mean value of the Young's modulus within the ROI was recorded by processing the image with a dedicated Matlab software developed in-house (version 7.1, MathWorks Inc., Natick, MA, USA). The time-varying thrombus rigidity was then monitored by assessing its Young's modulus at different stages (the higher is the Young's modulus in kPa, the more rigid is the thrombus). Measurements were triplicated to insure consistency of results. The B-mode images of the Aixplorer scanner were complemented with B-mode images acquired with a high-frequency Vevo 770 US biomicroscope (Visualsonics, Toronto, ON, Canada) equipped with a 35-MHz single-crystal mechanical transducer (RMV-703). The focal length was 10 mm and a field of view of 12 mm × 12 mm was used. The close to four-time higher resolution of the Visualsonics scanner compared with the SSI scanner allowed confirming the identification of the thrombus location seen in elastography; also the higher blood clot echogenicity with frequency [21] helped guiding the thrombus selection. To guarantee comparability between measurements of different vessels, care was taken on maintaining the same US settings throughout experiments.

Typical representations of the ROI are shown for one rabbit in Fig. 2. Colour Doppler maps show the transversal view of the ROI, illustrating the flow inside the jugular vein and in an adjacent artery before (A) and after (B) the thrombus induction, using the Aixplorer SSI scanner. A longitudinal B-mode view of the jugular vein with a ligated end is also shown (C) using the high-frequency Visualsonics biomicroscope, after induction of venous thrombosis. This pre-investigation allowed identifying and documenting all details in a given ROI.

Haematological Analyses

Red blood cell (Rbc), white blood cell (Wbc) and platelet (Pl) counts, haemoglobin (Hb), hematocrit (Ht), prothrombin time (Pt) and fibrinogen concentration (Fib) were measured using standard haematology and clinical laboratory methods (at the University of Montreal Hospital, QC, Canada) on samples obtained by puncture of the central ear artery of the rabbit. Blood sample analyses were performed on the day of the experiment, and on the first and last days and every third day in the case of the 2-week monitoring period.

Euthanasia and Ex Vivo Validation of Explanted Thrombi

At the end of the 2-hour (short term) and 2-week (long term) monitoring periods, animals were euthanized by intravenous injection of 160 mg/kg sodium pentobarbital (Euthanyl, Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada). Immediately after death,

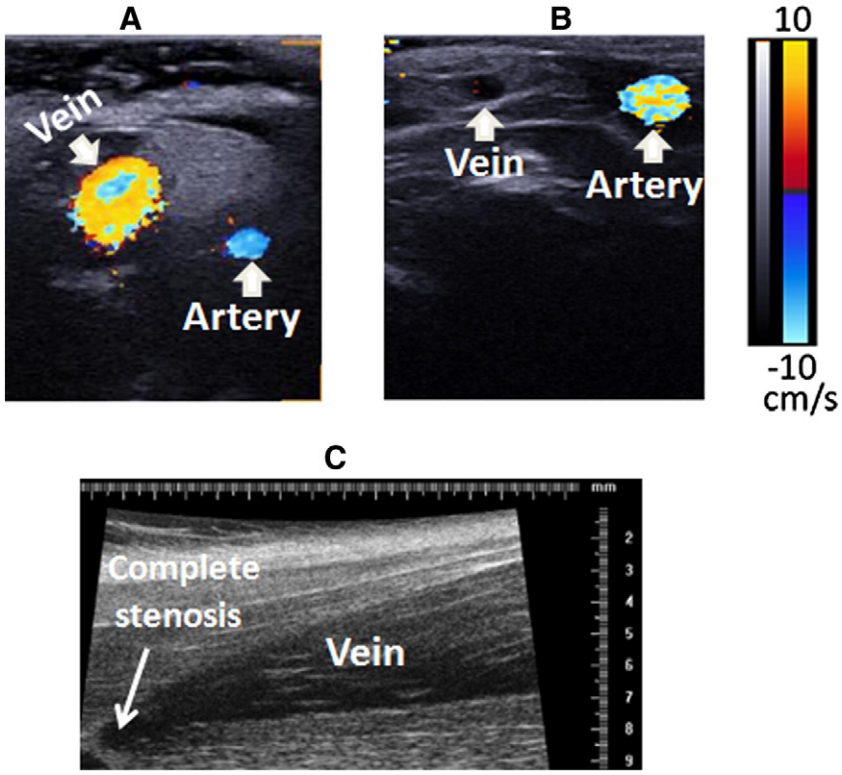

Fig. 2. Typical mapping of a region-of-interest (ROI) showing transversal colour Doppler views of the targeted jugular vein and an adjacent artery of a rabbit before (A) and after (B) thrombus induction, with the Aixplorer scanner (Supersonic Imagine, Aix-en-Provence, France). The surgery in (B) slightly modified anatomical landmarks on the ultrasound image. A longitudinal B-mode view of the vein with a ligated end (C) was obtained with the high-frequency Vevo 770 biomicroscope (Visualsonics, Toronto, ON, Canada).

thrombi were explanted and placed in Krebs solution. Necropsy observations allowed ocular verification of thrombus formation. In addition, to ensure the validity of the technique and because this is the first *in vivo* study on clotting blood shear wave elasticity imaging, the explanted thrombi were immersed in a homogeneous agar (number A-6924; Sigma Chemical, Saint-Louis, MO, USA) and gelatin (number G-2500 type A from porcine skin; Sigma Chemical, Saint-Louis, MO, USA) phantom and tested *ex vivo* with the same Aixplorer scanner, within 1 hour from the time of excision. The aim was to identify potential artefacts attributed to *in vivo* scans by using idealized *ex vivo* scanning conditions.

Results

In Vivo Elasticity Monitoring of Thrombi

Fig. 3 shows a typical evolution of the US elasticity modulus of an *in vivo* thrombus in rabbit #7 for the 2-hour follow-up period after thrombus induction. An increase in the spatially-averaged Young's modulus is observed over time, as it can be observed on the SSI colour maps within the segmented ROI (dashed circles), corresponding to clotting blood. This is accompanied by the B-mode display of the ROI between 20 to 120 min post-surgery. As seen, the B-mode echogenicity did not vary significantly over time.

The mean elasticity curves from measurements on all animals over ROIs selected on thrombi and within a reference region in the surrounding muscle are shown for the 2-hour and 2-week periods in Fig. 4A ($n = 9$) and 4B ($n = 6$), respectively. The Young's modulus varied from 1.0 ± 0.6 kPa (at +10 min) to 5.3 ± 1.6 kPa (at +120 min), then from 7.9 ± 2.8 kPa (at +1 day) to 25.0 ± 6.8 kPa (at +14 days). We tested the rigidity data for changes over time with analyses of variance (ANOVA) computed using the generalized linear model in SPSS (v.17; SPSS Inc, Chicago, IL, USA), with repeated measures. On the first set of data immediately after surgery, measurements at +10 min have been taken as baseline measures, and the rigidity starts to be statistically different from this baseline after 50 min (see Fig. 4A). We did the same analysis for the following days (day #1 to day #14), with day #1 values as baseline, and this shows an increased significant rigidity after 5 days (see Fig. 4B).

The ratio between the blood clots' Young's modulus to surrounding muscle's Young's modulus as a function of clot age is plotted in Fig. 5. Assuming that the muscle maintains its elastic properties over time (see Fig. 4), this ratio may be used in future studies to subdivide the thrombosis into acute, sub-acute and chronic stages for therapy planning (see Discussion).

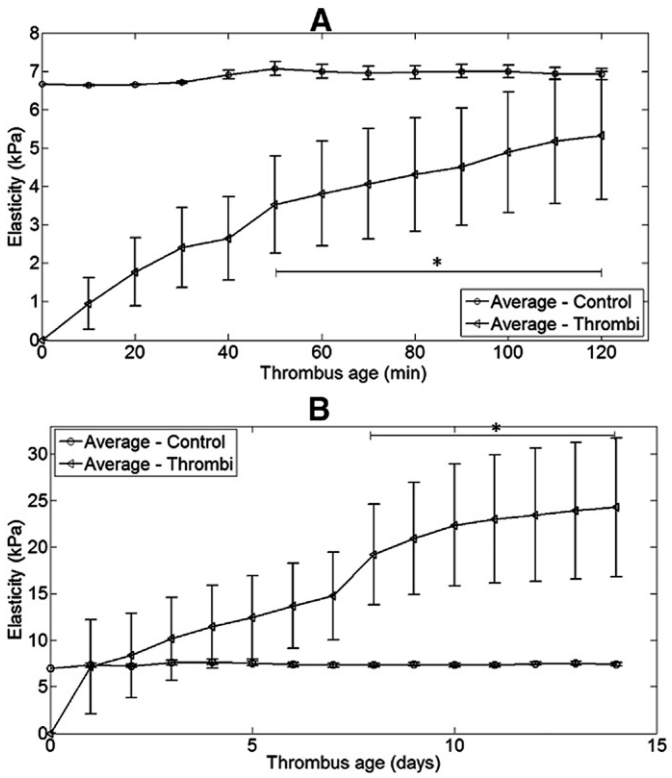

Fig. 4. Average elasticity modulus variation within 2 hours (A) and 2 weeks (B) after thrombus induction. Data are expressed as mean ± SD with $n = 9$ (A) and $n = 6$ (B) cases for the control surrounding muscle (open circles) and thrombi (open triangles). Significant differences ($^*p < 0.001$) were found for the rigidity between measurements at +10 min after surgery and those after 50 min (A), as well as between those at day #1 and those after 8 days (B).

Post-mortem Observations

Thrombi were confirmed at necropsy in all rabbits. A typical appearance of a thrombus is shown *in situ* in Fig. 6A, along with its excised appearance, B-mode and SSI images at the 2-hour (B- left) and 2-week (B- right) post-surgery. US images were acquired with thrombi

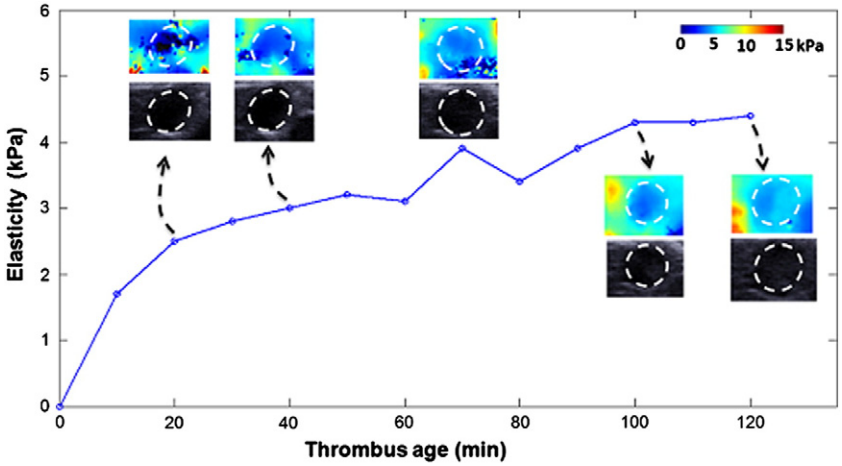

Fig. 3. Typical correspondence of ultrasound elasticity imaging to determine the mechanical property of a thrombus in rabbit #7. The Young's modulus in the ROI increases with time. B-mode (bottom boxes, dynamic range 50 dB) and elasticity (upper boxes, on a scale 0 to 15 kPa) maps over 2 mm × 1 mm ROI are displayed on the graph at 20, 40, 70, 100 and 120 min after thrombus induction. The clot boundary is approximately outlined by a white dashed-lined ellipse in each image. The thrombus hardening correlates with the gradual colour change from dark blue to light blue within the thrombus in the elasticity map.

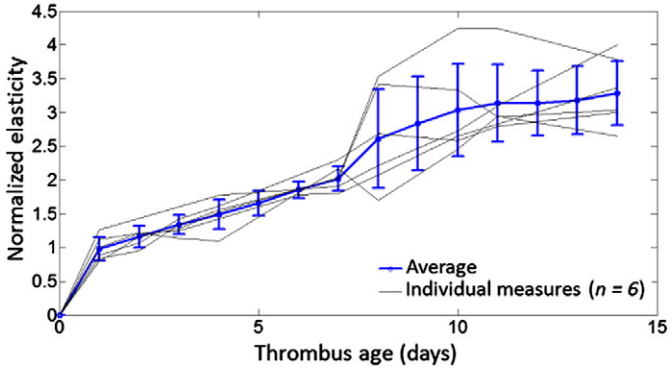

Fig. 5. Normalized elasticity modulus curves and their average plot for the 2-week monitoring period. Each normalized data represents the ratio of the clot's Young's modulus and the one of a surrounding muscle at the measurement time. The average curve represents the mean of $n = 6$ samples and error bars are the standard deviations on these samples between animals.

embedded in agar-gelatin phantoms. Explanted thrombi were measured with SSI and the average Young's modulus on day #14 ($n = 6$) was 29.0 ± 2.4 kPa *ex vivo*, which is comparable to *in vivo* measures of 25.0 ± 6.8 kPa (see Fig. 4). Similarly, for the 2-hour monitoring period, we obtained an average Young's modulus of 6.2 ± 0.4 kPa ($n = 4$) *ex vivo,* which is close to *in vivo* values of 5.3 ± 1.6 kPa ($n = 9$).

Haematological Parameters

All haematological parameters, *i.e.* Rbc, Wbc, Pl, Hb, Ht, Pt and Fib were within the normal range of experimental rabbits reported by Mitruka and Rawnsley [22]. Their mean values are presented in Table 1.

Discussion

The risk of recurrent thrombosis in patients with unprovoked VTE has been estimated to be close to 30%, after eight years of discontinuation of anticoagulation treatment [9]. This risk can be abrogated through prolonged use of anticoagulants. Given that the risk of haemorrhage, for patients on anticoagulants, is approximately 1-2% per year [23–25], clearly the risk of thrombosis recurrence is of greater concern and has prompted recent guidelines, published by the American College of Chest Physicians (ACCP), to recommend that patients with unprovoked VTE, in the absence of risk factors for haemorrhage, be treated with indefinite anticoagulant therapy [26]. However, despite these recommendations, it is clear that the identification of patients particularly susceptible to recurrent VTE be identified. Indeed, the risk of recurrent thrombosis must be weighed against the risk of haemorrhage in deciding which patients should continue to receive anticoagulants. Two recent major clinical trials have attempted to identify such clinical risk factors [7, 27]. As a result, patients whose risk of rethrombosis would be greater than the risk of haemorrhage would benefit from continued anticoagulation. More importantly, other clinical data examining residual venous thrombosis by standard US, as a potentially important risk factor for recurrent VTE, has been published [28]. Therefore, this novel technology can be used as a "risk factor" for recurrent thrombosis and therefore aid in the decision as to which patients should remain on anticoagulants.

According to reported SSI ultrasound results of the current work, the Young's modulus may become a new parameter to help clarify important properties of venous thrombi that could predict recurrent VTE. A monotonically increasing trend was observed in the first two weeks following thrombus induction. This can be explained by a combination of factors, among which the organization and transformation of the blood clot from a platelet-rich to dominant fibrin composition, with possible fibrin cross-linking [29]. In addition, because the clot rigidity increases monotonically with the fibrin content [30], it appears reasonable to use US elastography to characterize venous clots. Ultimately, a new and non-invasive parameter for assessing recurrent thrombosis risk can be developed. In addition, the SSI technology can likely enhance venous compression US [31] for non-invasive, quantitative and less operator dependent clinical assessment of biomechanical properties of thrombi.

The animal protocol investigated in this work revealed that the quantitative SSI method is capable of reflecting the existence and evolution of all analysed thrombi on a 2-hour and 2-week periods. It suggests that SSI can identify the blood clot regions. Further, close scrutiny of our data (Fig. 5) suggests that the variation of thrombus elasticity progresses in two different stages: one running from day #0 to day #7 (with few variances between Young's modulus of different measures), and another phase from day #8 to day #14 (with larger variability likely associated with the heterogeneous nature of aged thrombi) [32]. Hence, day #7 appears to be a transition point between acute and chronic thrombi in rabbits. This is in agreement with existing transition points reported in the literature for mouse venous thrombosis (day #6) [33], rats (day #6) [34], and between acute and sub-acute thrombosis in humans (mean age of 5.7 days) [35]. Note that the proposed animal model was designed to create a blood clot incarcerated between two ligatures, thus inhibiting restoration of flow. We recognize this limitation for studying the effect of flow mediated agents on clot hardening and

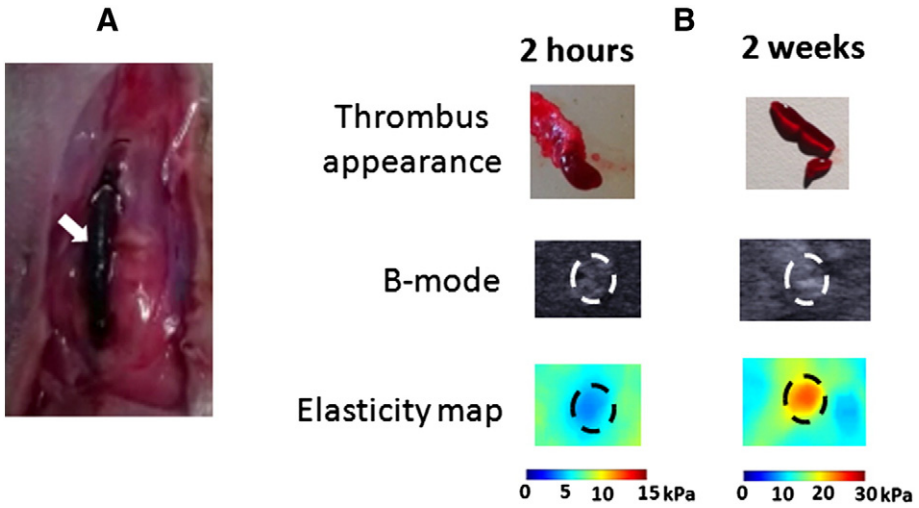

Fig. 6. Evidence of thrombus formation after necropsy and *ex vivo* validation. (A) Typical thrombus appearance *in situ*. (B) Appearance of the explanted 2-hour old (left) and 2-week old (right) thrombi, together with conventional B-mode images (dynamic range 50 dB) and SSI elasticity mapping; the mean Young's moduli of the explanted thrombi are 6.2 ± 0.4 kPa (at +2 hours) and 29.0 ± 2.4 kPa (at +2 weeks).

Table 1
Haematological parameters (mean ± SD) recorded immediately before the initial monitoring on all rabbits.

Rbc count	Wbc count	Pl count	Hb	Ht	Pt	Fib
[$x10^{12}$ L^{-1}]	[$x10^{9}$ L^{-1}]	[$x10^{9}$ L^{-1}]	[g L^{-1}]	[V/V]	[s]	[g L^{-1}]
5.6 ± 0.3	5.9 ± 1.9	303.8 ± 72.7	118.4 ± 7.2	0.34 ± 0.02	8.2 ± 0.3	1.2 ± 0.2

thrombolysis. The latter objectives could be addressed with the use of the animal model we proposed in [36].

There is a growing interest in the use of residual vein obstruction (RVO) to guide the duration of oral anticoagulant therapy (OAT) for unprovoked DVT, toward recurrent VTE prophylaxis [9–11]. While OAT has been shown to be effective for reducing the risk of recurrent VTE in the context of continued treatment, this advantage appears to be reduced in the case of treatment discontinuation [10]. However, the advantage of OAT is counteracted by major bleeding, which is the main adverse event for this therapy [10,11, 37]. Therefore, given that RVO is time dependent and that the duration of anticoagulation might influence the risk of recurrent VTE, a real-time, non-invasive and quantitative assessment of the thrombus rigidity appears potentially useful in helping to tailor clinical decision making. For example, thrombus rigidity after three months of anticoagulation, a major time when decisions are made regarding continuing anticoagulation or not, may help in guide that decision, as recommended in the 2012 ACCP guidelines. It is unlikely that RVO will aid in the acute situation when medical thrombolysis or medical thrombectomy is used. Prospective studies are thus required to assess the importance of measuring the hardening of a thrombus with SSI in the context of a RVO, as an independent risk factor to evaluate the risk/benefit ratio of anticoagulation treatment in patients.

In conclusion, the present study is the first to monitor *in vivo* thrombus hardening using a non-invasive dynamic elastography technique, namely the SSI technology. We followed the thrombus formation during two weeks after its induction, and observed a constant increase of clot hardening. *In vivo* and *ex vivo* measurements were in acceptable agreement, demonstrating the validity of the SSI method in the context of VTE, especially with regard to its ability to potentially predict recurrent thrombosis.

Keypoints

a. Ultrasound elastography with supersonic shear wave imaging can monitor blood clot hardening *in vivo*
b. Quantification of blood clot hardening can monitor the age of venous thrombosis
c. Supersonic shear wave imaging, because it is a technique used in radiology imaging, can be translated to human diagnosis and treatment follow-up

Authors' Contributions

E. Mfoumou, J. Tripette and G. Cloutier conceived and designed experiments. E. Mfoumou and J. Tripette performed experiments and analyzed data. G. Cloutier directed this research, contributed to reagents/materials/analysis tools and equipments. E. Mfoumou, J. Tripette, M. Blostein and G. Cloutier wrote the paper.

Conflict of Interest Statement

None to declare.

Acknowledgement

This work was supported by the Canadian Institutes of Health Research (MOP-84358). The authors gratefully acknowledge Dr H. Héon, Dr S. Qi, Dr L. Allard and Mr B. Chayer for helping with animal procedures.

References

[1] von Brühl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med 2012;209:819–35.
[2] Manly DA, Boles J, Mackman N. Role of tissue factor in venous thrombosis. Annu Rev Physiol 2011;73:515–25.
[3] Esmon TC. Basic mechanisms and pathogenesis of venous thrombosis. Blood Rev 2009;23:225–9.
[4] Furie B, Furie BC. Mechanisms of thrombus formation. N Engl J Med 2008;359:938–49.
[5] Dupras D, Bluhm J, Felty C, Hansen C, Johnson T, Lim K, et al. Venous thromboembolism diagnosis and treatment. Institute for Clinical Systems Improvement; 2013 [https://www.icsi.org/_asset/5ldx9k/VTE0113.pdf, Update January 2013].
[6] Baglin T, Luddington R, Brown K, Baglin C. Incidence of recurrent venous thromboembolism in relation to clinical and thrombophilia risk factors: prospective cohort study. Lancet 2003;362:523–6.
[7] Rodger MA, Kahn SR, Wells PS, Anderson DA, Chagnon I, Le Gal G, et al. Identifying unprovoked thromboembolism patients at low risk for recurrence who can discontinue anticoagulant therapy. Can Med Assoc J 2008;179:417–26.
[8] Agnelli G, Prandoni P, Santamaria MG, Bagatella P, Iorio A, Bazzan M, et al. Warfarin Optimal Duration Italian Trial Investigators. Three months versus one year of oral anticoagulant therapy for idiopathic deep venous thrombosis. N Engl J Med 2001;345:165–9.
[9] Prandoni P, Noventa F, Ghirarduzzi A, Pengo V, Bernardi E, Pesavento R, et al. The risk of recurrent venous thromboembolism after discontinuing anticoagulation in patients with acute proximal deep vein thrombosis or pulmonary embolism. A prospective cohort study in 1,626 patients. Haematologica 2007;92:199–205.
[10] Ridker PM, Goldhaber SZ, Danielson E, Rosenberg Y, Eby CS, Deitcher SR, et al. Long-term, low-intensity warfarin therapy for the prevention of recurrent venous thromboembolism. N Engl J Med 2003;348:1425–34.
[11] Agnelli G, Prandoni P, Becattini C, Silingardi M, Taliani MR, Miccio M, et al. Warfarin Optimal Duration Italian Trial Investigators. Extended oral anticoagulant therapy after a first episode of pulmonary embolism. Ann Intern Med 2003;139:19–25.
[12] Baarslag HJ, van Beek EJ, Koopman MM, Reekers JA. Prospective study of color duplex ultrasonography compared with contrast venography in patients suspected of having deep venous thrombosis of the upper extremities. Ann Intern Med 2002;136:865–72.
[13] Heijboer H, Jongbloets LM, Buller HL, Lensing AW, ten Cate JW. Clinical utility of real-time compression ultrasonography for diagnostic management of patients with recurrent venous thrombosis. Acta Radiol 1992;4:297–300.
[14] Prandoni P, Lensing AW, Prins MH, Bernardi E, Marchiori A, Bagatella P, et al. Residual venous thrombosis as a predictive factor for recurrent venous thromboembolism. Ann Intern Med 2002;137:955–60.
[15] Piovella F, Crippa L, Barone M, Vigano D`Angelo S, Galli L, Beltrametti C, et al. Normalization rates of compression ultrasonography in patients with a first episode of deep vein thrombosis of the lower limbs: association with recurrence and new thrombosis. Haematologica 2002;87:515–22.
[16] Siragusa S, Mariani G. Residual vein thrombosis assessment establishes the optimal duration of oral anticoagulants in patients with idiopathic or provoked deep vein thrombosis: a randomised, controlled trial [abstract]. Blood 2003;102:55a.
[17] Bercoff J, Tanter M, Fink M. Supersonic shear imaging: A new technique for soft tissue elasticity mapping. IEEE Trans Ultrason Ferroelectr Freq Control 2004;51:396–409.
[18] Lorrain J, Millet L, Lechaire I, Lechot S, Ferrari P, Visconte C, et al. Antithrombotic properties of SSR182289A, a new, orally active thrombin inhibitor. J Pharmacol Exp Ther 2002;304:567–74.
[19] Shieh SJ, Chiu HY, Shi GY, Wu CM, Wang JC, Chen CH, et al. A novel platelet-rich arterial thrombosis model in rabbits. Simple, reproducible, and dynamic real-time measurement by using double-opposing inverted-sutures model. Thromb Res 2001;103:363–76.
[20] Clover GH, Sharp JC. Reconstruction of ultrasound propagation speed distributions in soft tissue: time-of-flight tomography. IEEE Trans Sonics Ultrason 1977;24:229–34.
[21] Libgot-Callé R, Ossant F, Gruel Y, Lermusiaux P, Patat F. High frequency ultrasound device to investigate the acoustic properties of whole blood during coagulation. Ultrasound Med Biol 2008;34:252–64.
[22] Mitruka BM, Rawnsley HM. Clinical, biochemical and haematological reference values. Normal experimental animals. New York: Masson Pub. Inc.; 1977. p. 21–84.
[23] Oden A, Fahlen M. Oral anticoagulation and risk of death: a medical record linkage study. Br Med J 2002;325:1073–5.
[24] Palareti G, Leali N, Coccheri S, Poggi M, Manotti C, D`Angelo A, et al. Bleeding complications of oral anticoagulant treatment: an inception-cohort, prospective collaborative study (ISCOAT). Lancet 1996;348:423–8.
[25] Go AS, Hylek EM, Chang Y, Phillips KA, Henault LE, Capra AM, et al. Anticoagulation therapy for stroke prevention in atrial fibrillation: how well do randomized trials translate into clinical practice? J Am Med Assoc 2003;290:2685–92.
[26] Kearon C, Akl EA, Comerota AJ, Prandoni P, Bounameaux H, Goldhaber SZ, et al. Antithrombotic therapy for VTE disease. Chest 2012;141:e419S–94S.

[27] Tosetto A, Iorio A, Marcucci M, Baglin T, Cushman M, Eichinger S, et al. Predicting disease recurrence in patients with unprovoked venous thromboembolism: a proposed prediction score (DASH). J Thromb Haemost 2012;10:1019–25.
[28] Prandoni P, Lensing AW, Prins MH, Bernardi E, Marchiori A, Bagatella P, et al. Residual venous thrombosis as a predictive factor of recurrent venous thromboembolism. Ann Intern Med 2002;137:955–60.
[29] Browse NL, Burnand KG, Irvine AT, Wilson NM. Deep vein thrombosis: Pathology. In: Browse NL, Burnand KG, Wilson NM, editors. Disease of the veins. 2nd ed. London: Arnold; 1999. p. 249–89.
[30] Roberts WW, Lorand L, Mockros LF. Viscoelastic properties of fibrin clots. Biorheology 1973;10:29–42.
[31] Cronan JJ. History of venous ultrasound. J Ultrasound Med 2003;22:1143–6.
[32] Kim DE, Kim JY, Nahrendorf M, Lee KS, Ryu JH, Kim K, et al. Direct thrombus imaging as a means to control the variability of mouse embolic infarct models: the role of optical molecular imaging. Stroke 2011;42:3566–73.
[33] Diaz JA, Obi AT, Myers Jr DD, Wrobleski SK, Henke PK, Mackman N, et al. Critical review of mouse models of venous thrombosis. Arterioscler Thromb Vasc Biol 2012;32:556–62.
[34] Wakefield TW, Strieter RM, Wilke CA. Venous thrombosis-associated inflammation and attenuation with neutralizing antibodies to cytokines and adhesion molecules. Arterioscler Thromb Vasc Biol 1995;15:258–68.
[35] Rubin JM, Xie H, Kim K. Sonographic elasticity imaging of acute and chronic deep venous thrombosis in humans. J Ultrasound Med 2006;25:1179–86.
[36] Yu FTH, Armstrong JK, Tripette J, Meiselman HJ, Cloutier G. A local increase in red blood cell aggregation can trigger deep vein thrombosis: evidence based on quantitative cellular ultrasound imaging. J Thromb Haemost 2011;9:481–8.
[37] Kearon C, Ginsberg JS, Kovacs MJ, Anderson DR, Wells P, Julian JA, et al. Comparison of low-intensity warfarin therapy with conventional-intensity warfarin therapy for long-term prevention of recurrent venous thromboembolism. N Engl J Med 2003;349:631–9.