Dabigatran aggravates topoisomerase I peptide-loaded dendritic cells-induced lung and skin fibrosis

H. Mehta[1], S. Mashiko[1], P.O. Goulet[1], J. Desjardins[1], G. Pérez[2], M. Koenig[2], J.L. Senécal[2], M. Sarfati[1]

[1]*Immunoregulation Laboratory and* [2]*Laboratory for Research in Autoimmunity, Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montréal, Québec, Canada.*

Heena Mehta, PhD
Shunya Mashiko, BPharm
Philippe-Olivier Goulet, MSc
Jade Desjardins, MSc
Gemma Pérez, BSc
Martial Koenig, MD
Jean-Luc Senécal, MD
Marika Sarfati, MD, PhD

Please address correspondence to:
Dr Marika Sarfati,
Immunoregulation Laboratory,
CRCHUM, Tour Viger (R12.424),
900 rue St Denis,
Montréal, Québec H2X 0A9, Canada.
E-mail: m.sarfati@umontreal.ca

Received on September 27, 2016; accepted in revised form on January 20, 2017.

Clin Exp Rheumatol 2017; 35 (Suppl. 106): S35-S39.

Key words: systemic sclerosis, animal models, inflammation, fibrosis, thrombin

Funding: This work was supported by the Canadian Institutes of Health Research (operating grant MOP-142211) and also by research grants from Sclérodermie Québec, Scleroderma Society of Ontario and Scleroderma Society of Canada. Donations were given by Mrs Gisele Sarrazin-Locas in support of the Laboratory for Research in Autoimmunity. J.L. Senécal holds the University of Montreal Scleroderma Research Chair.

Competing interests: none declared.

ABSTRACT

Objective. *Dysregulated coagulation cascade has been implicated in development of fibrosis in systemic sclerosis (SSc). Thrombin, a key mediator of the coagulation pathway, has both proinflammatory and procoagulant properties. Here, we evaluated the efficacy of oral dabigatran, a direct thrombin inhibitor, on topoisomerase I dendritic cells (TOPOIA DCs)-induced lung and skin fibrosis, an experimental model of SSc.*

Methods. *Mice were repeatedly immunised with TOPOIA DCs. Dabigatran was administered in food either during the onset of fibrotic (late treatment) or inflammatory (early treatment) phase.*

Results. *Early administration of dabigatran caused an aggravation of pulmonary fibrosis associated with signs of severe perivascular inflammation while late treatment was not protective when compared to the untreated TOPOIA DCs group. Thrombin was increased in lungs of TOPOIA DCs immunised group and, paradoxically, further augmented by administration of dabigatran to immunised mice. As in lungs, early and not late drug administration exacerbated skin fibrosis. Moreover, early dabigatran treatment induced a profibrotic and inflammatory skin gene expression signature with upregulated expression of Col5a1, Timp1, Tweakr, Vwf, Il6, Il33, Il4 and Ifng.*

Conclusion. *Dabigatran aggravated lung and skin fibrosis in a TOPOIA DCs-induced model of SSc-like disease. Therefore, our results argue against the use of dabigatran to treat patients with SSc.*

Introduction

The pathophysiology of systemic sclerosis (SSc) is complex but evidence suggests that the coagulation cascade together with the immune system are involved in the development of pulmonary fibrosis (1). Activation of thrombin is one of the earliest events following tissue injury and subsequent inflammation. Thrombin modulates tissue repair responses as well as induces secretion of several pro-immune and pro-fibrotic factors (1). Furthermore, thrombin promotes lung fibroblast differentiation into a myofibroblast phenotype resistant to apoptosis (1). Thrombin has been reported to be elevated in bronchoalveolar lavage fluid of SSc patients with lung fibrosis (2). Therefore, blocking thrombin could arrest development of pulmonary fibrosis in SSc.

Dabigatran, an oral direct inhibitor of thrombin, suppresses thrombin-induced collagen production in SSc lung fibroblasts, blocks development of the myofibroblast phenotype from thrombin-activated normal lung fibroblasts and reverses the myofibroblast phenotype expressed by lung fibroblasts from patients with SSc-interstitial lung disease (ILD) (3). These *in vitro* observations suggest that dabigatran could interfere with late events that lead to fibrosis. Dabigatran was effective in inhibiting bleomycin-induced pulmonary fibrosis in mice when given at the start of bleomycin injection or during ongoing disease (4). A clinical trial to test the safety of administration of this drug to patients with SSc-ILD is currently underway (Clinical Trial Registration Number NCT02426229). Given that bleomycin-induced fibrosis does not hold promise as a unique translational pre-clinical model, it has been proposed that new therapies/drugs for SSc should be evaluated in more than one animal model to ensure increased success rate of clinical trials. Therefore, we asked whether dabigatran could interfere with the development of lung and skin fibrosis when admin-

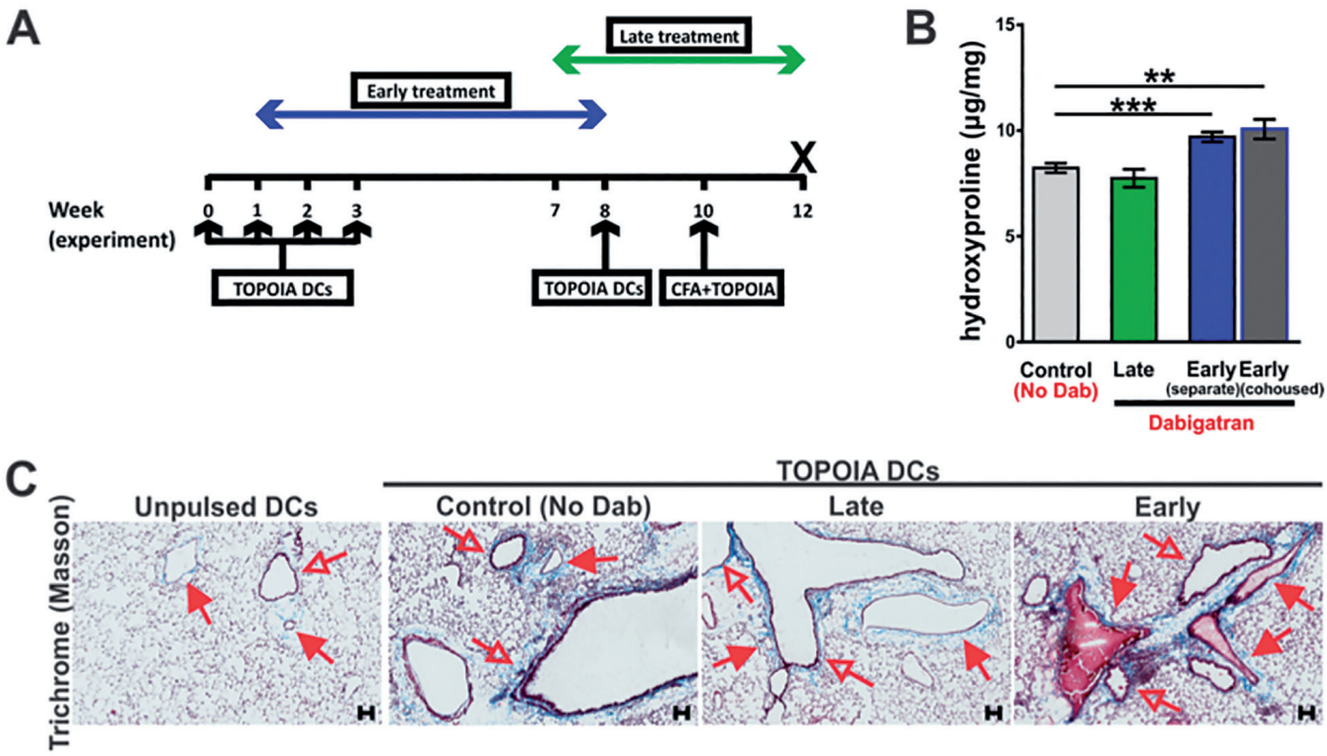

Fig. 1. Dabigatran aggravates lung fibrosis during early treatment.
A Schematic of immunisation and dabigatran administration plan. At week 12, B fibrosis was quantified by hydroxyproline assay, and C lung cryosections (5 μm) were assessed for peribronchial (open arrows) and perivascular (closed arrows) collagen and inflammation by trichrome (Masson) stain. Magnification 20x. Scale bar=100 µm. Data shown are mean±SEM and a pool of at least two experiments with n=9 control (no dabigatran), n=6 late, n=6 early (separate) and n=8 early (cohoused).
$^{**}p<0.01$; $^{***}p<0.001$.

istered orally either during the inflammatory or fibrotic phase of disease in topoisomerase I peptide loaded dendritic cells (TOPOIA DCs)-induced experimental SSc (5).

Materials and methods

TOPOIA DCs model of fibrosis and administration of dabigatran etexilate

Syngenic Balb/c mice were housed and bred in specific pathogen free facility of the CRCHUM and immunised at 5-7 weeks of age. All animal procedures were approved by the institutional committee for protection of animals of CHUM (CIPA-Comité Institutionnel de Protection des Animaux du CHUM). CIPA regulations are in accordance with the Canadian Council on Animal Care. Rationale for selection of $TOPOIA_{10-26}$ peptide (CanPeptide, Montreal, Canada) from TopoI protein, preparation of murine bone marrow derived DCs, and immunisation protocol have been described in (5). A standard concentration of 10 mg/g dabigatran etexilate (4) (Pradaxa from Boehringer Ingelheim, Burlington, Canada) was incorporated in rodent chow (Envigo, Madison, WI). Pradaxa capsules were opened, granules were weighed and sent to Envigo to be ground and incorporated in to the normal rodent diet used in the animal facility at CRCHUM. The amount of dabigatran etexialte in the drug was taken as 35.4% of the weight of the contents of the capsule. Mice were given normal or dabigatran etexilate chow *ad libitum*, and no weight loss or dehydration was observed during the study. Fibrosis in lungs and skin was quantified by the hydroxyproline assay, and tissue sections were examined by trichrome (Masson) stain as described in (5).

Lung explant culture and thrombin measurement

Lung explant culture was done as described by Mehta *et al*. (5). Thrombin was measured in culture supernatants using the thrombin fluorometric assay kit (K373-100 BioVision, Milpitas, CA). Plates were read for one hour at 460 nm on the Victor^3 V fluorometer (PerkinElmer, Waltham, MA).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Shaved 6 mm punch skin biopsy sample from near the site of injection was put in RNA*later* (Qiagen, Valencia, CA) for 24 hours and then stored at -80°C. RNA was extracted using RNeasy Fibrous Tissue Kit (Qiagen) and then converted to cDNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). For real time PCR, 100 ng of cDNA was used for each reaction along with TaqMan Fast Advanced Master Mix (Applied Biosystems). TaqMan probes for *Col1a1*, *Col5a1*, *Acta2* (*αSMA*), *Tnfrsf12a (Tweakr)*, *Ifng*, *Il4*, *Il5*, *Il6*, *Il13*, *Il17a*, *Il22*, *Il33*, *Tgfb1*, *Tslp*, *Mmp12*, *Serpine1*, *Vwf*, *Timp1* and *Hprt* (housekeeping gene) were purchased from Thermo Fisher (Waltham, MA). Real time PCR reaction was run on the Quant Studio 7 Flex Real Time PCR system (Thermo Fisher). Relative

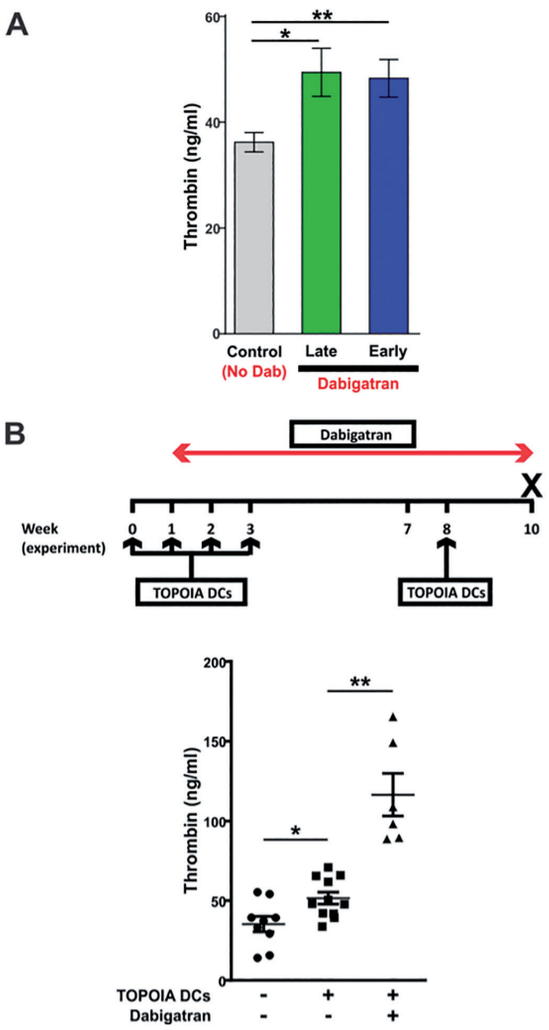

Fig. 2. Administration of dabigatran results in increased pulmonary thrombin levels.
Thrombin levels in lung explant culture supernatants at A week 12. Data shown are mean±SEM and a pool of at least two experiments with n=6-9 mice/group as in Fig. 1, and B week 10 according to dabigatran administration plan. Data shown are scatter plots with mean±SEM and a pool of 2 experiments with n=9 naive (no TOPOIA DCs and no dabigatran), n=11 TOPOIA DCs only and n=6 TOPOIA DCs + dabigatran. *p<0.05; **p<0.01.

expression was calculated using the $2^{(-\Delta CT)}$ method and multiplied by 1000 for graphical representation.

Statistical analysis

Prism 6 (Graphpad, San Diego, CA) was used to compare group means by unpaired Student's *t*-test with Welch's correction. A *p*-value of <0.05 was considered significant.

Results

In the TOPOIA DCs-induced model of SSc-like disease, the peak of pulmonary inflammation occurs at week 10 prior to development of pronounced fibrosis at week 12 (5) (Fig. 1A). Hence, in a late intervention protocol, dabigatran was administered one week before DC boost and continued until experimental endpoint (ongoing inflammation and onset of fibrosis) (Fig. 1A). As reported by other groups, the concentration of dabigatran used in this study did not cause bleeding abnormalities (4, 6). For the early intervention protocol, dabigatran was given after the second TOPOIA DCs immunisation and stopped prior to DC boost (onset of inflammatory response) (Fig. 1A). In both protocols, mice were sacrificed at week 12 and the effect of dabigatran on fibrosis was evaluated against untreated TOPOIA DCs (control; no dabigatran) immunised group. We first showed that late intervention offered no protection against development of pulmonary fibrosis since collagen content, as quantified by lung hydroxyproline levels, was similar to the control group (Fig. 1B). However, early intervention significantly exacerbated pulmonary fibrosis (Fig. 1B). The aggravation in fibrosis was not affected by cohousing TOPOIA DCs mice with unpulsed DCs mice (mixed cages). Qualitative analysis of lung sections stained for collagen deposition corroborated the hydroxyproline data (Fig. 1C). Fibrosis was observed around airways and blood vessels. Early dabigatran treatment appeared to severely aggravate histological pulmonary inflammation and haemorrhage in female mice, even though the level of exacerbation of pulmonary fibrosis was similar between males and females.

Given the lack of protection versus aggravation of pulmonary fibrosis in mice treated with dabigatran, we next assessed its effect on thrombin levels in pulmonary tissue at experimental endpoint. Thrombin was paradoxically increased in both early and late dabigatran-treated mice when compared to untreated control group (Fig. 2A). Notably, during early intervention, dabigatran administration was terminated at week 8 and thrombin levels were still significantly augmented 4 weeks later (week 12). Given these unexpected findings, thrombin levels were examined in lungs of untreated TOPOIA DCs immunised mice when compared with naive mice as well as in mice that were started on dabigatran after the second TOPOIA DCs immunisation (early treatment), but continued until week 10 (Fig. 2B). Immunisation increased thrombin levels, which were further augmented by dabigatran therapy, indicating a procoagulant status in lungs (Fig. 2B).

Finally, we evaluated the impact of dabigatran administration on skin disease. Skin hydroxyproline content was significantly increased in the early but not late dabigatran treated mice relative to control group, with no gender bias as was seen in lungs (Fig. 3A and B). Skin gene expression of *Col1a1* and *Il13* was significantly increased by 2.1-fold (p<0.032) and 1.9-fold (p<0.042) respectively, in TOPOIA DCs when compared to unpulsed DCs immunised mice, corroborating the observed skin fibrosis in control (no Dab) group (5). However, aggravated skin pathology observed in the early dabigatran group was associated with upregulated expression of *Col5a1*, and profibrotic genes such as *Il33*, *Tweakr* and *Il6* when compared to control group (Fig.

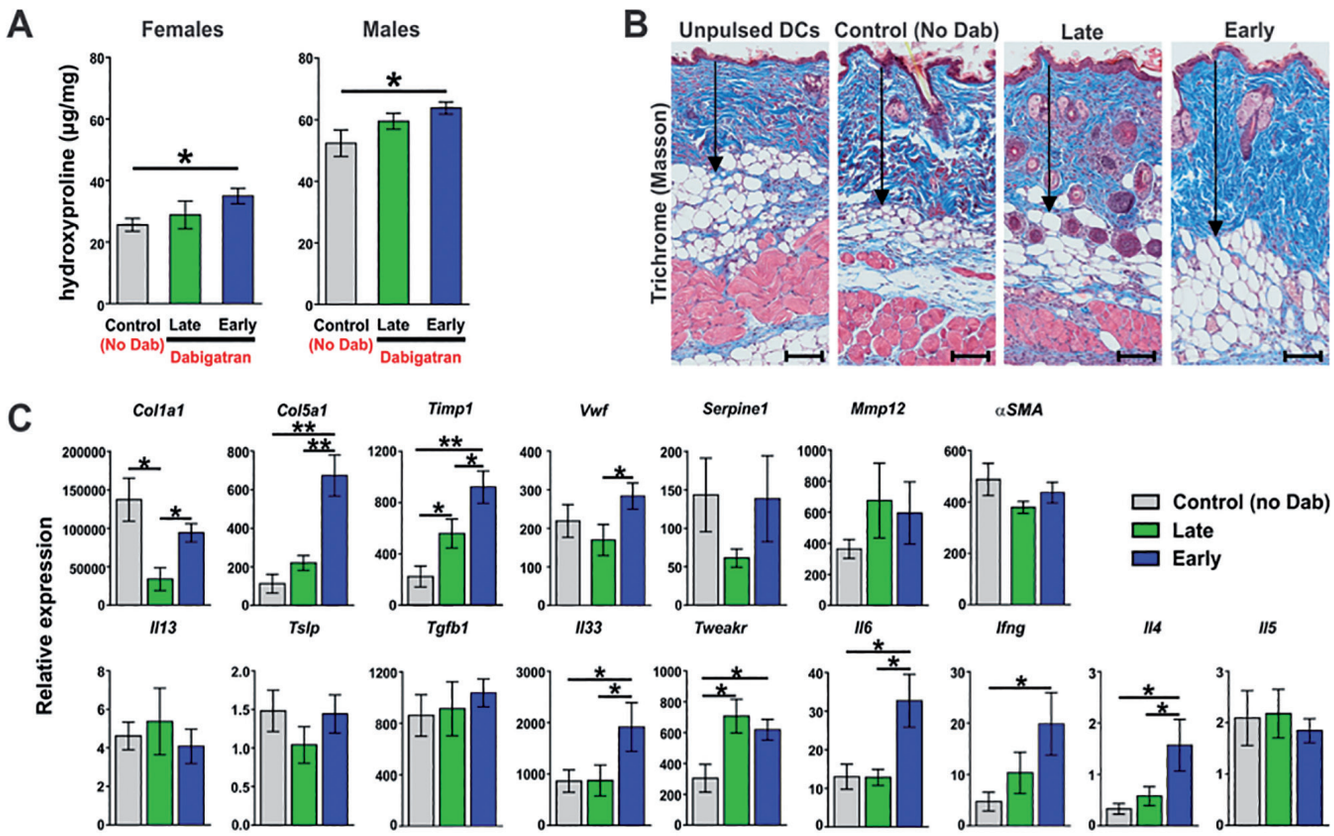

Fig. 3. Dabigatran treatment does not protect against skin fibrosis.
At week 12, A fibrosis was quantified by hydroxyproline assay, B skin paraffin sections (5 μm) were assessed for collagen deposition and inflammation by trichrome (Masson). Magnification 20x. Scale bar=100 µm, and C comparison of relative skin mRNA gene expression by qRT-PCR in control (no Dab) and dabigatran treated groups. (A) Data shown are mean±SEM and a pool of at least two experiments with n=13 control (no dabigatran), n=13 late and n=14 early. (B-C) Histology pictures shown are from males and qRT-PCR data are from male skin samples from at least 2 independent experiments with n=7 control (no Dab), n=5 late and n=8 early. *p<0.05; **p<0.01.

3C). Early treated group also showed signs of increased vasculopathy and inflammation since *Vwf*, *Timp1* and *Ifng* were significantly increased. *Il4* was also elevated but the overall expression was 10 times lower than *Ifng*. In case of the late treatment group, only *Timp1* and *Tweakr* were significantly upregulated relative to the control group. *Il17a* and *Il22* expression was very low or undetectable for all groups.

Discussion

In the present study, we demonstrated that dabigatran did not protect against the development of lung and skin fibrosis in TOPOIA DCs-induced model of SSc. On the contrary, dabigatran administered during the inflammatory phase worsened pulmonary and skin fibrosis, and induced a profibrotic and inflammatory skin gene expression signature. Furthermore, a paradoxical increase in pulmonary thrombin levels was observed. At steady state, thrombin complexes with thrombomodulin (TM) and activates protein C which in turn degrades Factor Va to prevent the generation of excess thrombin. *In vitro* studies have reported that direct thrombin inhibitors like dabigatran can disrupt this negative feedback by inhibiting protein C activation and subsequent Factor Va degradation resulting in enhanced thrombin generation (7). Moreover, dabigatran has a greater affinity for thrombin-TM complex than free thrombin (8), thereby preventing the generation of activated protein C. This suggests that a high concentration of dabigatran, with a concomitant increased risk of bleeding, would be required to block thrombin-TM and free thrombin as well in our experimental model. Notably, in a recent report, dabigatran caused alveolar haemorrhage in a patient with interstitial pulmonary fibrosis (9).

IL-33, IL-6, Tweak R, Timp-1 as well as vWF have been reported to be increased in SSc serum and affected organs (10-12) and in the present study, were shown to be upregulated in the skin of early dabigatran treated mice with exacerbated skin fibrosis. During haemostasis, vWF is bound to Factor VIII to form a stable complex and serves as a cofactor to thrombin to activate Factor VIII (Factor VIIIa) (13). Factor VIIIa can then further accelerate the generation of thrombin. Furthermore, global gene expression analysis of skin demonstrated that *TWEAKR* is associated with both inflammatory and fibroproliferative SSc patient subsets (10). Interestingly, both IL-33 and IL-6 can induce Timp-1 expression (14, 15). In conclusion, our findings raise concerns about the safety of dabigatran therapy in SSc patients, especially during the inflammatory pathophysiological phase of their disease.

Acknowledgments

We thank all staff members of the CR-CHUM molecular pathology core facility and the histology platform of Goodman Cancer Research Center, McGill University for staining of lung and skin sections, and scanning microscopy.

References

1. LUDWICKA-BRADLEY A, SILVER RM, BOGATKEVICH GS: Coagulation and autoimmunity in scleroderma interstitial lung disease. *Semin Arthritis Rheum* 2011; 41: 212-22.
2. OHBA T, McDONALD JK, SILVER RM, STRANGE C, LEROY EC, LUDWICKA A: Scleroderma bronchoalveolar lavage fluid contains thrombin, a mediator of human lung fibroblast proliferation via induction of platelet-derived growth factor alpha-receptor. *Am J Respir Cell Mol Biol* 1994; 10: 405-12.
3. BOGATKEVICH GS, LUDWICKA-BRADLEY A, SILVER RM: Dabigatran, a direct thrombin inhibitor, blocks differentiation of normal fibroblasts to a myofibroblast phenotype and demonstrates anti-fibrotic effects on scleroderma lung fibroblasts. *Arthritis Rheum* 2009; 60: 3455-64.
4. BOGATKEVICH GS, LUDWICKA-BRADLEY A, NIETERT PJ, AKTER T, VAN RYN J, SILVER RM: Antiinflammatory and antifibrotic effects of the oral direct thrombin inhibitor dabigatran etexilate in a murine model of interstitial lung disease. *Arthritis Rheum* 2011; 63: 1416-25.
5. MEHTA H, GOULET PO, NGUYEN V *et al*.: Topoisomerase I peptide-loaded dendritic cells induce autoantibody response as well as skin and lung fibrosis. *Autoimmunity* 2016; 49: 503-13.
6. SPARKENBAUGH EM, CHANTRATHAM-MACHART P, MICKELSON J *et al*.: Differential contribution of FXa and thrombin to vascular inflammation in a mouse model of sickle cell disease. *Blood* 2014; 123: 1747-56.
7. KAMISATO C, FURUGOHRI T, MORISHIMA Y: A direct thrombin inhibitor suppresses protein C activation and factor Va degradation in human plasma: Possible mechanisms of paradoxical enhancement of thrombin generation. *Thrombosis Res* 2016; 141: 77-83.
8. MATTSSON C, MENSCHIK-LUNDIN A, NYLANDER S, GYZANDER E, DEINUM J: Effect of different types of thrombin inhibitors on thrombin/thrombomodulin modulated activation of protein C in vitro. *Thrombosis Res* 2001; 104: 475-86.
9. HUSARI A, BEYDOUN A, SHEIK AMMAR A, MAAKARON JE, TAHER A: The untold story of Dabigatran etexilate: alveolar hemorrhage in an elderly patient with interstitial pulmonary fibrosis. *J Thromb Thrombolysis* 2013; 35: 81-2.
10. SARGENT JL, LI Z, ALIPRANTIS AO *et al*.: Identification of Optimal Mouse Models of Systemic Sclerosis by Interspecies Comparative Genomics. *Arthritis Rheumatol* 2016; 68: 2003-15.
11. KUROIWA K, ARAI T, OKAZAKI H, MINOTA S, TOMINAGA S: Identification of human ST2 protein in the sera of patients with autoimmune diseases. *Biochem Biophys Res Commun* 2001; 284: 1104-8.
12. PATTANAIK D, BROWN M, POSTLETHWAITE BC, POSTLETHWAITE AE: Pathogenesis of systemic sclerosis. *Front Immunol* 2015; 6: 272.
13. REININGER AJ: Function of von Willebrand factor in haemostasis and thrombosis. *Haemophilia* 2008; 14 (Suppl. 5): 11-26.
14. RANKIN AL, MUMM JB, MURPHY E *et al*.: IL-33 induces IL-13-dependent cutaneous fibrosis. *J Immunol* 2010; 184: 1526-35.
15. SILACCI P, DAYER JM, DESGEORGES A, PETER R, MANUEDDU C, GUERNE PA: Interleukin (IL)-6 and its soluble receptor induce TIMP-1 expression in synoviocytes and chondrocytes, and block IL-1-induced collagenolytic activity. *J Biol Chem* 1998; 273: 13625-9.