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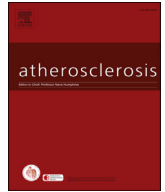
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Imatinib treatment attenuates growth and inflammation of angiotensin II induced abdominal aortic aneurysm

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ABSTRACT

Background: Abdominal aortic aneurysm (AAA) is characterized by vascular remodeling with increased infiltration of inflammatory cells and apoptosis/modulation of vascular smooth muscle cells (SMCs). Imatinib is a selective inhibitor of several tyrosine kinases, including PDGF receptors, Abl, and c-kit. The objective of this study was to characterize the potential protective role of imatinib on AAA development and the molecular mechanisms involved.

Methods: Male *ApoE*^{−/−} mice were infused with angiotensin (Ang) II (1000 ng/kg/min) for 4 weeks to induce AAA or saline as controls. Daily treatment with 10 mg/kg imatinib, or tap water as control, was provided via gavage for 4 weeks.

Results: Treatment with imatinib was found to decrease the aortic diameter and vessel wall thickness, mediated by multiple effects. Imatinib treatment in AngII infused mice resulted in a reduced cellular infiltration of CD3ε positive T lymphocytes by 86% and reduced gene expression of *mast cell chymase* by 50% compared with AngII infused mice lacking imatinib. Gene expression analysis of SMC marker *SM22α* demonstrated an increase by 48% together with a more intact medial layer after treatment with imatinib as evaluated with SM22α immunostaining.

Conclusion: Present findings highlight the importance of tyrosine kinase pathways in the development of AAA. Our results show, that imatinib treatment inhibits essential mast cell, T lymphocyte and SMC mediated processes in experimental AAA. Thus, our results support the idea that tyrosine kinase inhibitors may be useful in the treatment of pathological vascular inflammation and remodeling in conditions like AAA.

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1. Introduction

Abdominal aortic aneurysm (AAA) is a chronic inflammatory disease resulting in vascular remodeling. AAA is associated with infiltration of leukocytes, such as T lymphocytes, mast cells, neutrophils and macrophages, which produce various matrix-degrading proteases. Phenotypic modulation and increased

apoptosis of smooth muscle cells (SMCs) and degradation of the extracellular matrix (ECM) also occurs [1–4]. In normal blood vessels, the predominant vascular SMC phenotype is the contractile phenotype that regulates vessel diameter and blood flow [5]. Phenotypic modulation of SMCs is critical in regulating vascular function in health and disease. The switch from the contractile to the migratory and proliferative phenotype – known as the dedifferentiated synthetic phenotype – takes place during tissue repair in response to vascular injury. In this phenotype, SMCs increase their rate of cell proliferation and migration in response to growth factors and/or chemoattractant and this process plays a critical role in maintaining the integrity of the vessel [6]. SMCs are one of the

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major producers of medial matrix collagen, providing tensile strength to the aortic wall. Collagen synthesis increases during the early stages of aneurysm formation suggesting a repair process while in later stages of AAA collagen degradation, due to protease produced by infiltrated leukocytes, exceeds its synthesis which may eventually lead to rupture of the aortic wall [7].

Imatinib is a small molecule with potent and selective inhibitory activity against several tyrosine kinases, including: Abl, c-Kit and platelet-derived growth factor receptor (PDGFR) α and β [8,9]. Imatinib has good oral bioavailability and has become the standard of care for patients with chronic myelogenous leukemia and gastrointestinal stromal tumors, because it inhibits the growth of malignant cells [8]. However, the mechanism of action of imatinib may be broader than originally expected, and might also be targeting non-malignant disorders such as pulmonary arterial hypertension, hyperlipidemia, hypercholesterolemia and atherosclerosis [10–12]. Furthermore, imatinib may inhibit T lymphocytes proliferation and activation [13] as well as inhibit mast cell activation by inducing apoptosis [14]. Both cells play an evident role in the development and progression of AAA [15,16].

Given the fact that imatinib may inhibit key pathways in formation of AAA, i.e. T lymphocytes, mast cells, SMC proliferation and PDGF signaling, the objectives of this study was to characterize the potential protective role of imatinib on AAA development and the molecular mechanisms involved.

2. Material and methods

2.1. Human subjects

This study included 12 patients in whom preoperative computed tomography had demonstrated an eccentric intraluminal thrombus and who were scheduled for elective surgery for infrarenal AAA at Karolinska University Hospital, Stockholm, Sweden. During surgery the intraluminal thrombus was removed from the aortic vessel wall. The intima/media and adventitia of AAA were separated by adventicectomy. Patients were included after informed, written, and signed consent, and the studies were performed with approval from the local ethical committee at Karolinska Institutet in Stockholm, Sweden.

Control ascending aorta samples (layers not separated) for RNA studies were obtained from 8 organ donors, and infrarenal control aortas for histology were collected from 14 medicolegal autopsies without clinical or macroscopic signs of aortic atherosclerosis or aneurysm. Autopsies of control aorta samples were performed in the Department of Forensic Medicine, University of Helsinki. The sections were immediately fixed in 4% formaldehyde for light microscopy or snap frozen in liquid nitrogen for RNA isolation. The use of organ donor and autopsy tissues was approved by The National Authority for Medicolegal Affairs of Finland.

2.2. AngII-induced AAA in mice

AngII-induced aneurysm is an inflammation-driven model that is frequently used to experimentally induce AAA [17]. Male hypercholesterolemic *ApoE*^{−/−} mice were obtained from Taconic (Bomholt, Denmark). At eight weeks of age, AAA was induced by chronic infusion of 1000 ng/kg/min AngII (Cat.no.9525, Sigma Aldrich, St. Louis, USA) via mini-osmotic pumps (Model 1004, Alzet, CA, USA) as described previously [18]. A group of *ApoE*^{−/−} mice were infused with 0.9% NaCl and were used as control mice. In order to study the significance of tyrosine kinase signaling in AAA development, imatinib, a tyrosine kinase inhibitor was administered. Oral gavage treatment with 10 mg/kg imatinib mesylate (STI571, Novartis, Stockholm, Sweden) was given daily throughout

the study starting two days before implantation of the mini-osmotic pumps. Administration of tap water was given orally to *ApoE*^{−/−} mice which served as control mice. Mice were divided into four groups, two groups obtained mini-osmotic pumps releasing AngII and two groups obtained mini-osmotic pumps releasing 0.9% NaCl. From each exposure one group either obtained 10 mg/kg imatinib or tap water orally. Groups were divided as follow; NaCl and tap water (n = 7), AngII and tap water (n = 9), NaCl and imatinib (n = 9) and AngII and imatinib (n = 8). Standard Chow diet and water was allowed *ad libitum* throughout the whole study and mice were monitored daily for signs of discomfort. After 28 days, mice were sacrificed. The aorta was removed and fixated in RNA-later for 24 h thereafter frozen in −70 °C for gene expression analysis and plasma was taken for cholesterol measurements which were performed using Total cholesterol + HDL + Glucose Panel, CardioChec (Mediastore, Stockholm, Sweden). The study was approved by the local ethical committee 2014-09-04, 68-14 in Linköping, Sweden.

2.3. Quantitative real-time PCR

Human and mouse aortas were homogenized with trizol and chloroform in Lysing Matrix D tubes (MP Biomedicals, Illkirch, France) using FastPrep. Total RNA from human and mice aortas were isolated with RNeasy mini kit (Qiagen, Hilden, Germany) and reversely transcribed with random primers and Superscript II (Invitrogen, Carlsbad, USA). cDNA (human 0.5 µg; mouse 0.21 µg) was amplified by RT-PCR with 1× TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, USA) on a ABI 7700 Real-time PCR Sequence Detector, run in duplicates as previously described [18]. All probes were obtained from Applied Biosystems (Supplementary table 1), and the results were normalized to expression levels of human *RPLP0* or mouse *Gapdh*.

2.4. Immunohistochemistry

Paraffin-embedded human and mouse abdominal aortas were sectioned (5 µm) and rehydrated in several changes of ethanol and Tissue-Clear[®] (Sakura Finetek, Leiden, The Netherlands). Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide for 5 min followed by incubation in 5% blocking bovine serum albumin solution. Sections were then incubated with primary antibodies against mouse CD3ε (1 µg/ml, Cat. no. BS3476, Bioworld Technology, St Louis Park, USA), SM22α (0.6 µg/ml, Cat. no. ab14106, Abcam, Cambridge, UK) and PDGFR-β (phospho-Tyr751, 10 µg/ml, Cat. no. LS-C178098, LSBio, Seattle, USA) or human PDGF-D (0.5 µg/ml, Cat. no. AF1159) and PDGFR-β (1 µg/ml, Cat. no. AF385) (RnD Systems, Minneapolis, USA), Von Willebrand factor (0.2 µg/ml, Cat. no. A0082, Dako, Glostrup, Denmark), CD68 (0.1 µg/ml, Cat. no.NCL-CD68-KP1, Leica Microsystems, Newcastle, UK) and α-actin (0.6 µg/ml, Cat. no. A5228, clone 1A4, Sigma-Aldrich) at 4 °C overnight followed by secondary biotinylated goat anti-rabbit IgG or goat anti-mouse IgG (Dako) antibody. Isotype-matched immunoglobulin was used in the same concentration as corresponding primary antibody in order to assess the level of non-specific binding (Cat. no. ab27472, Abcam). Avidin-biotin peroxidase complexes (Dako) were added followed by visualization with 3,3'-diaminobenzidine tetrahydrochloride (Dako). All sections were counterstained with Mayer's hematoxylin (Histolab Products, Göteborg, Sweden). Sections were studied under light microscopy (Zeiss, Jena, Germany). Quantification of CD3ε positive cells in aortas was defined as number per mm². A scoring system from 1 to 4 was used for quantification of phosphor-specific PDGFR-β expression, with 1 defined as no or low expression, 2 as expression in aortic media, 3 as expression in aortic media and

adventitia, and 4 as strong expression in all layers of the aorta and data is expressed in arbitrary units (AU). Quantifications were performed blinded.

2.5. Masson Trichrome staining

Paraffin-embedded sections (5 μm) were rehydrated in several changes of ethanol and Tissue-Clear® (Sakura Finetek). Sections were then incubated in Bouin's solution (Sigma-Aldrich) at room temperature overnight, counterstained with Weigert's hematoxylin working solution (HistoLab) for 10 min following by staining with the Masson Trichrome kit (Sigma-Aldrich) containing Biebrich's Scarlet-acid Fuchsin solution for 15 min, Phosphomolybdic-Phosphotungstic acid solution for 10 min and Aniline Blue solution for 5 min. Sections were differentiated in 1% acetic acid, washed in distilled water, dehydrated in tissue clear and several changes of ethanol before mounted with mounting medium. Sections were studied under light microscopy. Collagen displayed blue and cytoplasm and muscle fibers stained red. Quantification of collagen was performed blinded. A scoring system from 1 to 4 was used with 1 defined as low amount of collagen in all layers of the aorta, 2 as low amount of collagen in aorta media or in aorta adventitia, 3 as high amount of collagen in aorta media or in aorta adventitia, and 4 as high amount of collagen in all layers of the aorta. Aortic diameter was measured as the leading edge to leading edge measurement calculated from the inner luminal diameter and outer adventitial diameter based on respective circumference and vessel wall thickness. An aneurysm was set as a 1.5-fold enlargement of the vessel wall. As there occurs vascular remodeling, aortic vessel wall thickness was also measured.

2.6. Toluidine blue staining

Paraffin-embedded sections (5 μm) were deparaffinized and hydrated in several changes of ethanol and Tissue-Clear® (Sakura Finetek). Sections were then stained in toluidine blue working solution with pH 2.3 for 3 min followed by three washes in distilled water, dehydrated in tissue clear and several changes of ethanol before mounted with mounting medium. Sections were studied under light microscopy. Quantification of the mast cells, stained dark purple, was performed blinded and normalized to aortic vessel wall area (mm^2) and total numbers per aorta since they were few in numbers.

2.7. Verhoeff's Van Gieson staining

Paraffin-embedded mice aortas were stained with Verhoeff's hematoxylin for 1 h, differentiated in 2% ferric chloride for 2 min, and counterstained with Van Gieson's stain for 5 min to identify elastic fibers in the aortic tissue stained black. Quantification of elastin was performed blinded using a scoring system from 1 to 4 was used with 1 defined as intact elastin, 2 as low degradation of elastin, 3 as intermediate degradation of elastin, and 4 as high degradation of elastin.

2.8. Statistical analysis

The statistical analysis was performed with the IBM SPSS Statistics 22. All measurements are shown as median and standard deviations. Two-group comparisons of quantitative data were performed using the non-parametric Mann–Whitney *U* test or the parametric Student *T*-test, and correlation between genes was performed using Pearson's Rank Correlation. Multiple test correction was performed using Holm–Bonferroni. *P*-values < 0.05 were considered statistically significant where * indicates *P* < 0.05, **

indicates *P* < 0.01 and *** indicates *P* < 0.001. Quantitative results represented in bar as mean values and error bars as standard deviation.

3. Results

3.1. Imatinib inhibits vascular remodeling in AngII infused mice

To elucidate the potential role of imatinib and corresponding pathway in aneurysmal formation, mice were administrated imatinib in the drinking water under infusion of AngII by mini-osmotic pumps. Infusion of AngII for four weeks led to a significant increase in the aortic diameter (0.47 ± 0.09 mm; *P* < 0.01) and vessel wall thickness by 3.2-fold (0.08 ± 0.03 mm; *P* < 0.001) and displayed an aneurysmal phenotype compared with saline-infused control aortas (0.34 ± 0.04 mm; Fig. 1A, B and C). Imatinib inhibited growth of the aortic diameter by 50% when compared with AngII infused mice lacking imatinib treatment (0.40 ± 0.07 mm; *P* < 0.05, Fig. 1A and B) and displayed a less aneurysmal phenotype. Imatinib was further found to decrease the vessel wall thickness by 50% as compared with mice administrated with only AngII (0.04 ± 0.01 mm; *P* < 0.001, Fig. 1A and C). After AngII infusion, 9/13 (69%) of the mice administrated with tap water developed an aneurysm but only 2/9 (22%) of the imatinib administrated mice developed such lesions (*P* < 0.05). Aneurysmal aortas displayed a more disorganized medial layer and extracellular matrix components such as increased production of collagen and cellular composition compared with imatinib treated mice. Number of ruptures was 17% in AngII infused mice administrated with tap water while in mice administrated with imatinib rupture did not occur. Histological staining and scoring of elastin in aorta media demonstrated a variation in the degree of elastin degradation in all mice infused with AngII compared to saline-infused control aortas when stained with Verhoeff's van Gieson (Supplementary Fig. 1). However there were no differences in elastin degradation between imatinib administrated mice and tap water administrated mice after infusion with AngII.

Further, levels of total cholesterol was decreased (*P* = 0.01) in AngII infused mice administrated with imatinib compared to its control at the same time HDL levels were increased (Supplementary Table 2).

3.2. Imatinib inhibit infiltration of T-cells and mast cell in the aortic wall

Cellular infiltration of inflammatory leukocytes is one of the hallmarks of vascular remodeling leading to aneurysmal development. Administration of imatinib in AngII infused mice resulted in a reduction of CD3e positive T lymphocytes (*P* < 0.05) to normal levels as compared with mice infused with only AngII (Fig. 2A and B) as demonstrated with immunohistological staining. However, imatinib did not change the expression of CD3e on gene level (Fig. 2C).

Histological staining of mast cells showed few mast cells in general (Fig. 3A) with a decreased number of mast cells per aorta in imatinib treated AngII mice (*P* < 0.05, Fig. 3A and C). However we could not observe any effects of imatinib on the number of mast cells/ mm^2 in the vessel wall (Fig. 3A and B). Gene expression analysis on aortas of imatinib treated AngII infused mice demonstrated decreased expression of *mast cell chymase* (*P* < 0.05) by 50% as compared with AngII infused mice that were not treated with imatinib (Fig. 3D).

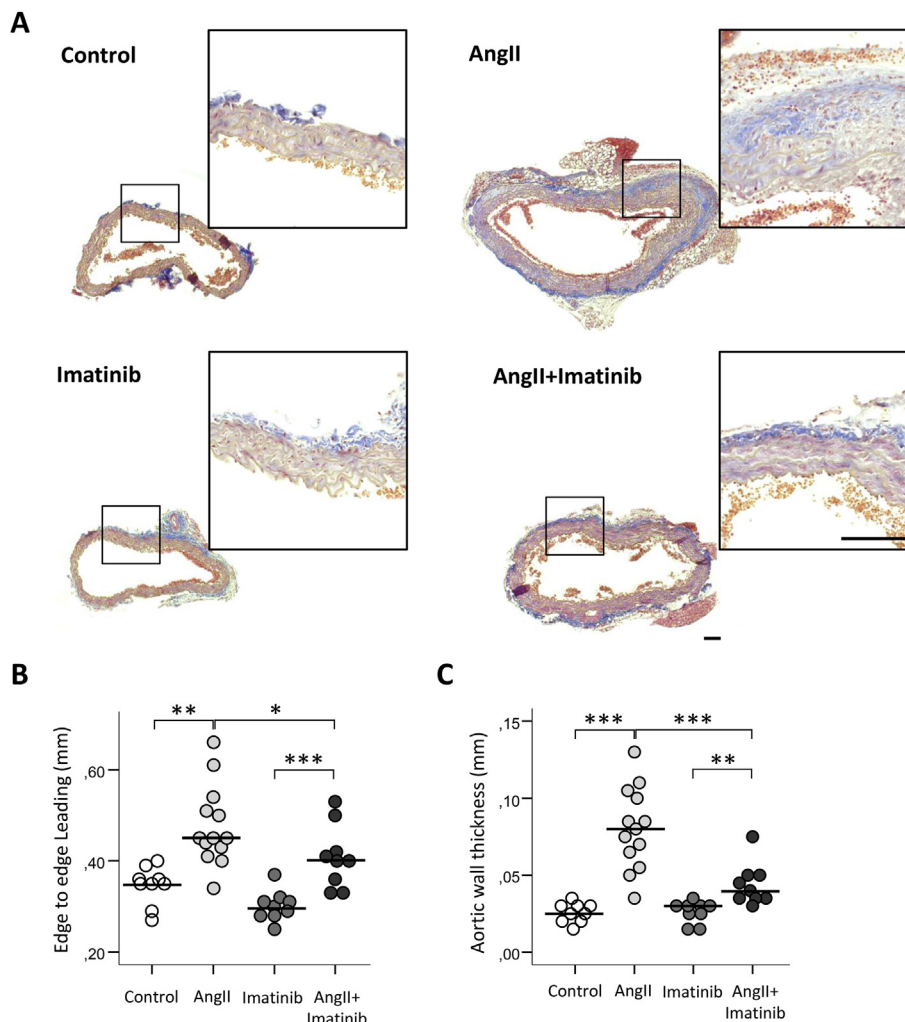


Fig. 1. Abdominal aortic aneurysm development. (A) show representative images of the histological analyses performed by Masson Trichrome staining. Measurements of the edge to edge leading aortic diameter (B) and the aortic wall thickness (C). Horizontal lines represent median of each group. Statistical significance was established using non-parametric Mann Whitney *U* test where * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$ after adjusting for multiple testing. Control $n = 10$; AngII $n = 13$; Imatinib $n = 9$; AngII + Imatinib $n = 9$. The images are taken with $10\times$ and $40\times$ magnification. Scale bar represents $50\ \mu\text{m}$.

3.3. Effects on inflammation

To further determine the effects of imatinib in vascular inflammation and matrix degeneration, changes in expression levels of proinflammatory cytokines, chemokines and proteases were measured in the aortas by semi-quantitative real-time PCR. Overall, the increased expression of AngII tended to decrease in the imatinib treated mice (Supplementary Fig. 2). Chemokine (C–C motif) ligand 5 (*CCL5*), a T lymphocytes chemokine, was significantly higher expressed in both AngII groups as compared with their controls and imatinib tended to reduce the increase by 50% compared to AngII infused mice without imatinib. AngII infusion lacking imatinib treatment significantly increased gene expression of *TNF- α* by 15-fold ($P < 0.001$) and *IL-6* by 76-fold ($P < 0.001$), an increase that was inhibited and not significantly increased in imatinib treated animals.

3.4. Imatinib preserves SMCs

We next examined the impact of imatinib on SMCs content and gene expression. Immunohistochemical staining demonstrated that SMCs in mice treated with imatinib after AngII infusion were

localized to the intima/media as observed in control mice while aneurysmal AngII infused mice administrated with tap water demonstrated an intimal/medial thickening by SMCs (Fig. 4A). With quantitative real-time PCR imatinib administrated mice were found to express *SM22 α* to levels equal to control mice and 48% higher ($P < 0.01$) as compared with AngII infused mice (Fig. 4B). Furthermore, staining of *SM22 α* revealed that the reduced amount of *SM22 α* seen on gene level in AngII infused mice administrated with tap water not only is caused by loss of SMCs due to apoptosis, but might also suggest a phenotypical switch of contractile SMC phenotype to the more proliferative and migratory synthetic SMC phenotype [19].

SMCs are one of the main producers of collagen to the vessel wall. We identified an increased compensatory production of collagen in mice infused with AngII lacking imatinib compared with control aortas ($P < 0.01$). AngII infused mice administrated with imatinib, which displayed a more normal aorta, did not increase the production of collagen as compared with AngII infused mice lacking imatinib ($P < 0.05$, Fig. 4C).

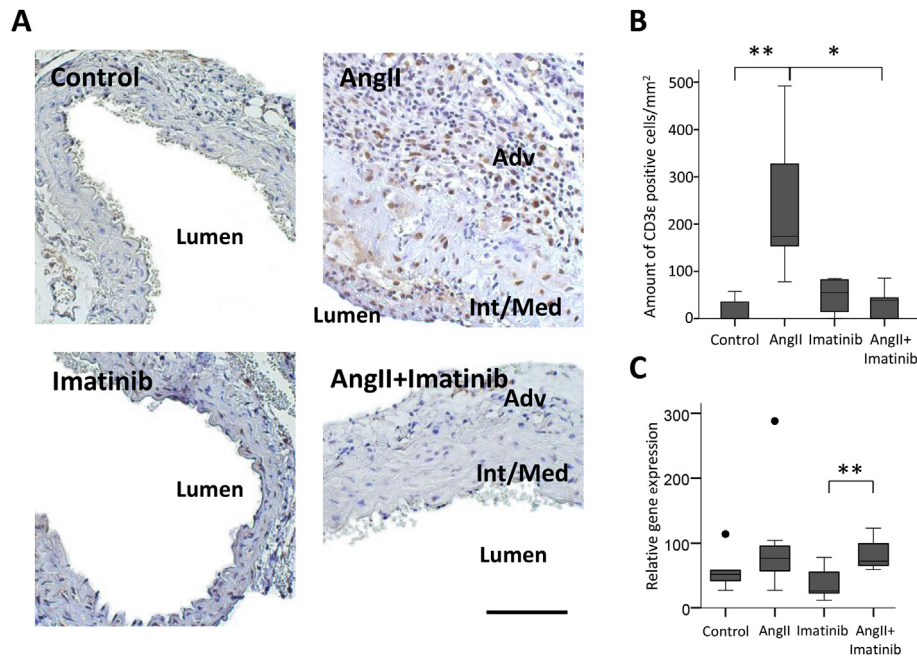


Fig. 2. Effect of Imatinib on T-cells. (A) immunohistochemical staining of CD3ε in the different mice groups stained in brown. (B) Quantification of CD3ε positive cells in aortas per mm². (C) Gene expression of the lymphocyte marker CD3ε relative to *Gapdh* as measured by qRT-PCR. Results are presented in box plot using median with the 25th and 75th percentiles. T-bars indicate outliers extend to 1.5 times the height of the box. "●" outlier greater than 3 times the interquartile range. Statistical significance was established using non-parametric Mann Whitney *U* test where * indicates $P < 0.05$ and ** indicates $P < 0.01$. The images is taken with 40× magnification. Scale bar represents 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

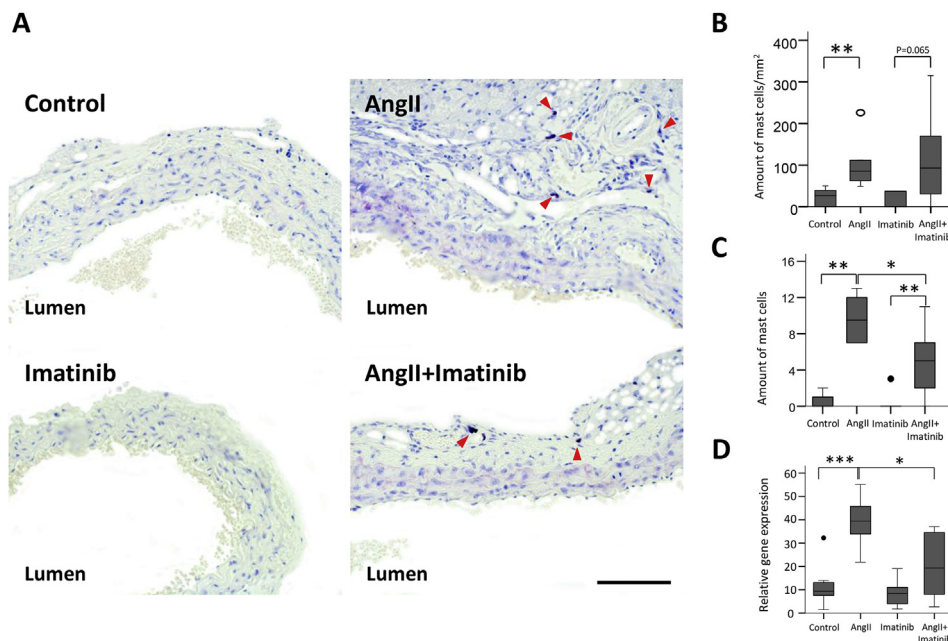


Fig. 3. Toluidine blue staining of mast cell in the aortas of each treatment group (A). Positive cells are stained in dark purple and are indicated with red arrows. Quantification of mast cells in the aortas per mm² (B) and the total number of cells per aorta (C). The mRNA expression of *mast cell tryptase* (D) relative to *Gapdh* as measured by qRT-PCR. Results are presented in box plot using median with the 25th and 75th percentiles. T-bars indicate outliers extend to 1.5 times the height of the box. "○" Outlier greater than 1.5 times the interquartile range. "●" outlier greater than 3 times the interquartile range. Statistical significance was established using non-parametric Mann Whitney *U* test where * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$. The images is taken with 40× magnification. Scale bar represents 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Decreased expression of PDGF-D in mouse and human AAA

Effects of imatinib in vascular integrity are mediated through several tyrosine kinases, also including PDGF receptors. Gene

expression of PDGFR-β tended to decrease after imatinib treatment, but no significant differences were observed between AngII infused aortas and saline-infused control aortas (Fig. 5A). The expression and localization of the receptors as well as most of their ligands

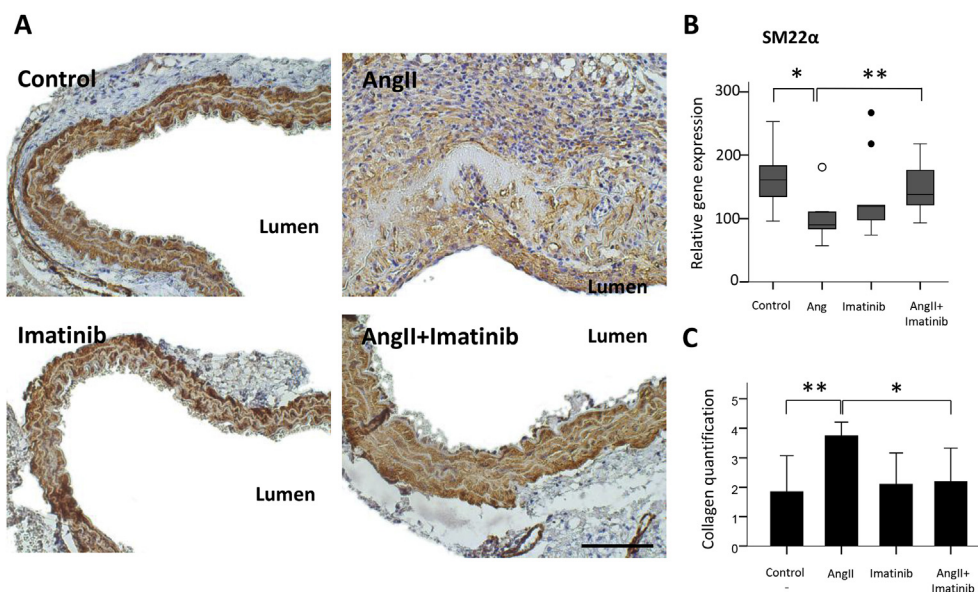


Fig. 4. Immunohistochemical staining of SM22 α in the different treatments; control, AngII, Imatinib, and AngII + Imatinib (A). Immunohistochemical staining of positive cells are stained in brown. The mRNA expression of smooth muscle cell marker SM22 α (B) relative to *Gapdh* as measured by qRT-PCR. Results are presented in box plot using median with the 25th and 75th percentiles. T-bars indicate outliers extend to 1.5 times the height of the box. "○" Outlier greater than 1.5 times the interquartile range. "●" Outlier greater than 3 times the interquartile range. (C) Histological quantification of collagen performed on Masson trichrome stained aortas. Statistical significance was established using non-parametric Mann Whitney *U* test where * indicates $P < 0.05$ and ** indicates $P < 0.01$. The images are taken with 40 \times magnification. Scale bar represent 50 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have been examined in AAA before, however no studies to date have investigated the latest discovered member, PDGF-D, in AAA. In addition, gene expression analysis of *PDGF-D* showed decrease by 52% in AngII mice compared to control mice ($P < 0.001$, Fig. 5B). At the same time, treatment with imatinib was found to normalize the *PDGF-D* gene expression to levels observed in control mice and as compared to AngII infused mice ($P < 0.05$). The mRNA expression of *PDGF-D* and *PDGFR- β* correlated strongly to SM22 α expression ($r = 0.87$; $P < 0.001$ and $r = 0.80$; $P < 0.01$). Immunohistochemical analysis of phosphorylated PDGFR- β revealed a heterogeneous expression localized dominantly to SMCs (Fig. 5C). Imatinib significantly ($P < 0.05$) reduced the expression in the aortas of AngII infused mice (2.5 ± 0.5 AU) compared to AngII infused mice not given the drug (3.3 ± 0.9 AU).

While expression of *PDGFR- β* was unaffected in murine AAA, the expression of *PDGFR- β* was significantly lower in all layers in human aortas with AAA as compared with non-aneurysmal control aortas (Fig. 5D). In agreement with what was observed in mouse AAA, expression of *PDGF-D* was also reduced in all layers of the AAA samples with a 68% decrease in the aneurysmal wall tissues compared to non-aneurysmal control aortas (Fig. 5F). The mRNA expression of *PDGF-D* correlated strongly to *PDGFR- β* expression ($r = 0.84$; $P < 0.001$). Immunohistochemical analysis of PDGF-D and PDGFR- β confirmed the gene expression pattern on a protein level. In non-aneurysmal control aortas, the staining of both PDGF-D and its receptor PDGFR- β were, in general, strong and mainly localized to SMCs, luminal endothelial cells, and neovessels in the adventitia. Staining of PDGF-D and PDGFR- β , however, was weaker and localized to SMCs and neovessels in the aneurysmal adventitia (Fig. 5E and G) and, to some extent, to macrophages (data not shown).

4. Discussion

By identifying key cellular and molecular mechanisms that promote aneurysmal expansion we could get a better insight into

the disease progression and thereby identify new therapeutic targets used for future treatment. Inhibition of key features using imatinib may represent a novel therapeutic target in cardiovascular disease such as AAA. This is particularly of interest because imatinib has been shown to have minimal side effects when used in cancer therapy in humans [20]. Overall, imatinib is a well-tolerated drug and clinical side effects mainly appear as moderate. Some patients experience nausea, diarrhea, edema, weight loss, muscle cramps, and headache. In some rare occasions patients might develop more severe pathological events such as cardiac toxicities which are associated with long-term treatment [21]. The majority of patients who develop cardiotoxicity, heart failure or myocardial infarction are generally older (>65 years) with existing or pre-existing risk factors for cardiac disease. Other cardiac complications associated with imatinib are hypo/hypertension, tachycardia and pericarditis however the prevalence appears to be less than 1% [22,23]. These side effects are also dose dependent and could be reduced by lowering the dosage. One beneficial side effect is that imatinib lowers plasma LDL cholesterol, and increases HDL, which we also could observe in our animal model. Imatinib is currently a Food and Drug Administration (FDA) approved drug for treatment of chronic myelogenous leukemia and gastrointestinal stromal tumors due to its inhibitory effect on growth of malignant cells, but recent studies have suggested that imatinib might be useful not only against tumors, as it has also shown vascular protective properties [24].

It is well-established that there is an increased infiltration of inflammatory cells into the aortic wall due to artery injury, including AAA. Furthermore, it is known that tyrosine kinases play a prominent role in T lymphocyte receptor signaling [13] and mast cell survival [14], suggesting that imatinib may interfere with this process. With histological staining of mouse aortas, we demonstrated that treatment with imatinib decreased the amount of CD3 ϵ positive T lymphocytes and mast cells significantly in the arterial wall of mice infused with AngII, compared with aneurysmal mice aortas without imatinib treatment. Our findings were shared by a recent study published by Seggewiss and co-workers where they

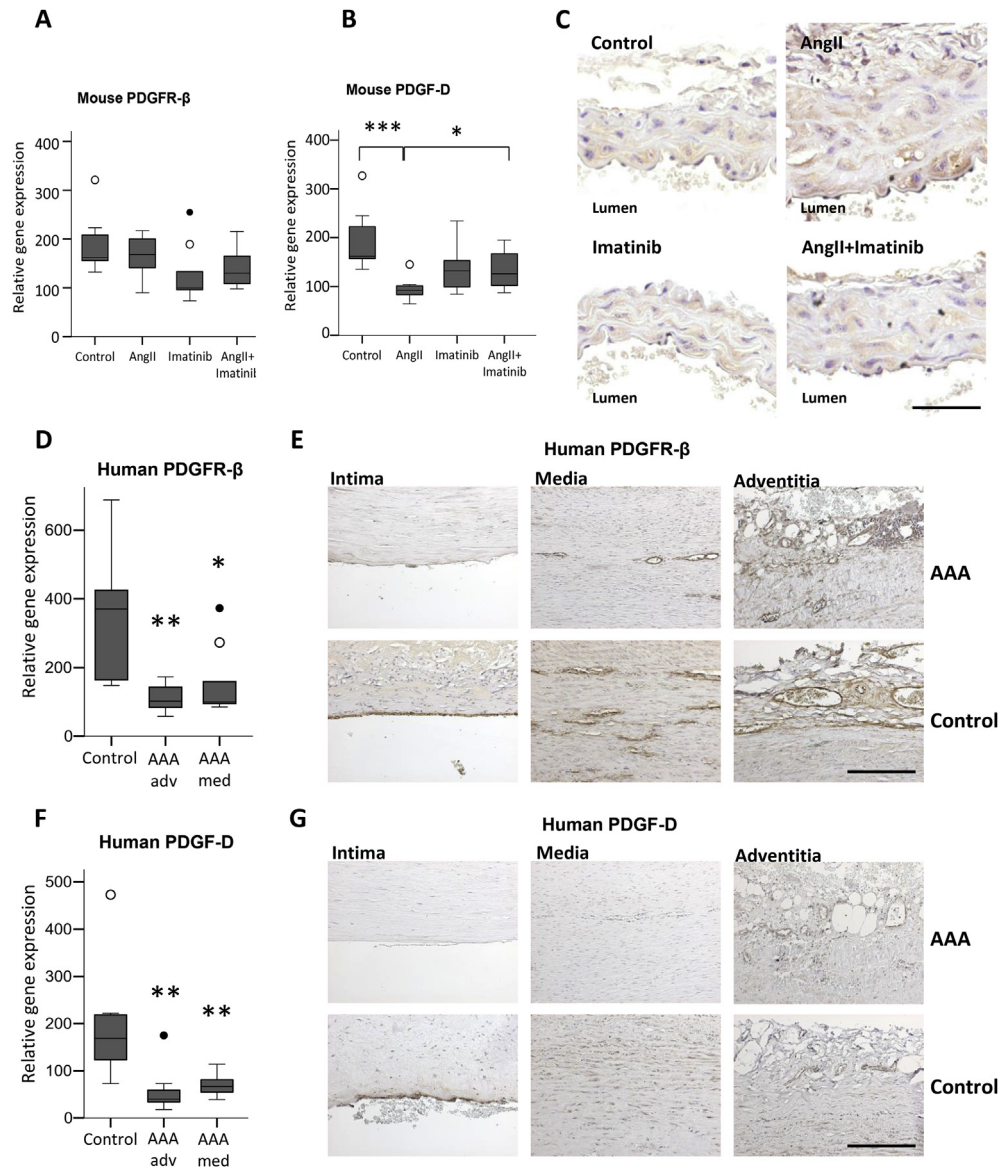


Fig. 5. Gene expression of mouse *PDGFR-β* (A) and *PDGF-D* (B) relative to *Gapdh* and immunohistochemical staining of phospho-PDGFR-β in mice aortas (C). Gene expression of human *PDGFR-β* (D) and *PDGF-D* (F) relative to *RPLP0* in non-aneurysmal control aortas and AAA samples from the intima/media or adventitia ($n = 8$ or 9 in each group) as determined by real-time PCR. Immunohistochemical staining of PDGFR-β (E) and PDGF-D (G) in the tunica intima, media, and adventitia of AAA samples and non-aneurysmal control aortas, stained in brown. Scale bar represents $50 \mu\text{m}$. Results are presented in box plot using median with the 25th and 75th percentiles. T-bars indicate outliers extend to 1.5 times the height of the box. * $P < 0.05$, ** $P < 0.01$ vs. non-aneurysmal aortas (Mann-Whitney U test). "○" Outlier greater than 1.5 times the interquartile range. "●" Outlier greater than 3 times the interquartile range. Abbreviations: adv, adventitia; med, intima/media. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

presented that imatinib inhibits T lymphocyte proliferation *in vitro*, in response to different kinds of mitogens, by direct inhibition of TCR signaling through inhibition of the tyrosine kinases such as Abl which is known to be involved in T lymphocyte activation [13,25]. They showed that both the overall percentage of all T lymphocytes undergoing cell division and the average number of divisions completed were significantly reduced. Furthermore, imatinib does not induce apoptosis in T lymphocytes and the reduced proliferation observed is therefore not contributed by increased apoptosis [13]. We further observed that imatinib decreased the expression of *CCL5*, a chemokine important for the recruitment of T lymphocytes to the aneurysmal wall. Several studies have also shown the importance and involvement of mast cells in many aspects of AAA formation [15,26]. Sun and coworkers demonstrated increased

accumulation of mast cells within the AAA lesion after inducing AAA with elastase perfusion. These cells were shown to produce cytokines IL-6 and IFN- γ which in turn affected matrix degrading proteases such as MMPs and cathepsins. We observed that imatinib treatment inhibited the induction of IL-6 in our animal model which could to some degree depend on the inhibition of mast cells and T lymphocytes within the vessel wall but also to loss and phenotypic changes of SMCs. They also suggested that mast cell-derived IL-6 and IFN- γ might regulate SMC apoptosis [15]. Mast cells are known to express c-kit which is essential for its survival. Further, Juurikivi and coworkers treated cultured mast cell with imatinib which resulted in an induced mast cell apoptosis through inhibition of c-kit [14]. This is in agreement with our findings where we demonstrated that imatinib reduces the number of mast

cells per aorta. Fewer mast cells contribute less to the inflammation that potentially could lead to fewer inflammatory cells such as T lymphocytes, having an essential role on aneurysmal progression. Furthermore, since mast cells may modulate SMC responses in experimental aneurysm making it even more critical to consider this aspect.

The chronic inflammation occurring during disease development activity leads to phenotypic modulation and apoptosis of vascular SMCs and degradation of the ECM network, including collagen. Our *in vivo* study on aneurysmal mice also demonstrated a decrease in the expression level of the SMC marker *SM22 α* which could indicate SMC differentiation from contractile to synthetic SMC and/or apoptosis which is in agreement with other studies [27]. With our immunostaining we could demonstrate a more disorganized aortic wall with migrating SMC. Nevertheless, we report that administration of imatinib during AngII infusion blocked the repression of *SM22 α* as well as having a more normal SMC morphology reflecting an aortic wall resembling the normal aorta. In response to injury SMC actively produces collagen, which is in agreement with the compensatory production of collagens found in human AAA [7]. We could also observe this effect in our aneurysmal *in vivo* model whereas an increased amount of collagen was demonstrated after infusion with AngII in order to stabilize the vessel wall, which was inhibited by imatinib since the disease progression was dampened. Lassila and coworkers demonstrated a minimal expression of C-abl kinase in the vasculature indicating that the effects seen with imatinib administration *in vivo* has very low or no effect via this protein [12]. Contradictory, studies has demonstrated c-kit as highly present in dedifferentiated vascular SMCs in the aortic wall suggesting that effects of imatinib partially could be mediated via c-kit inhibition [28,29]. These findings resulted in a decreased aortic diameter as well as thickness indicating the importance of imatinib on vascular remodeling and progression of AAA through regulation of T lymphocyte and mast cell infiltration within the aneurysmal aorta as well as SMC content and collagen production.

Further evidence suggests that effects of imatinib in vascular integrity and atherosclerosis are primarily via its ability to control PDGF receptor activation [9]. By forming a complex with PDGFR- β , Boucher and co-workers could demonstrate that low density lipoprotein receptor-related protein 1 (LRP-1) has a pivotal role in protecting vessel wall integrity and preventing atherosclerosis and thereby controlling its activation. They also demonstrated that treatment with imatinib blocked activation of LRP-1–induced phosphorylation of PDGFR- β and reduced the size and area of the atherosclerotic lesions [9]. In our experiments, PDGFR- β seem to be differently regulated in human and murine AAA and especially by the SMCs [30–32]. Prior to the present study, the expression and localization of PDGF-D in AAA disease is unknown. In human aortas PDGF-D was the most dramatically changed family member and its expression was decreased significantly in the aneurysmal wall of both human and mice. We found that PDGF-D was predominantly localized to medial SMCs and endothelial cells, and to some extent in macrophages, which is in agreement with what is observed in atherosclerotic aortas [33,34]. The expression of PDGFR- β is found to be particularly high in aortic media localized and expressed by aortic SMC [32,34,35]. Expression of PDGF-D and PDGFR- β was strongly

correlated to SMC marker *SM22 α* in human samples. In AAA, not only does phenotypical differentiation of SMC occur [4], but apoptosis is another important hallmark in the disease progression. The reduced expression of PDGF-D and PDGFR- β in the aortic wall could, to some part, be an outcome of SMC loss due to apoptosis. Upregulated PDGF-D expression in imatinib treated mice may reflect a more preserved SMC content.

5. Conclusion

Taken together, our findings identify imatinib as a novel potential drug in aneurysmal treatment by affecting several key features important in aneurysmal formation. Through inhibition of T lymphocytes and mast cells recruitment and by preserving the medial layer of SMC, imatinib could play a part in attenuating pathological vascular inflammation and remodeling and thereby AAA development. Our data supports the fact that blockage of tyrosine kinase signaling using imatinib may be extended as treatment of cardiovascular diseases, such as AAA, due to its potential to impair features associated with AAA development. In order to investigate potential biological significance and implications clinical trials are needed.

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Disclosures

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.atherosclerosis.2016.04.006>.

References

- [1] J.B. Michel, J.L. Martin-Ventura, J. Egido, et al., Novel aspects of the pathogenesis of aneurysms of the abdominal aorta in humans, *Cardiovasc Res.* 90 (1) (2011) 18–27.
- [2] M. Kazi, J. Thyberg, P. Religa, et al., Influence of intraluminal thrombus on structural and cellular composition of abdominal aortic aneurysm wall, *J. Vasc. Surg.* 38 (6) (2003) 1283–1292.
- [3] A. Lopez-Candales, D.R. Holmes, S. Liao, M.J. Scott, S.A. Wickline, R.W. Thompson, Decreased vascular smooth muscle cell density in medial degeneration of human abdominal aortic aneurysms, *Am. J. Pathol.* 150 (3) (1997) 993–1007.
- [4] G. Ailawadi, C.W. Moehle, H. Pei, et al., Smooth muscle phenotypic modulation is an early event in aortic aneurysms, *J. Thorac. Cardiovasc Surg.* 138 (6) (2009) 1392–1399.
- [5] A. Zalewski, Y. Shi, A.G. Johnson, Diverse origin of intimal cells: smooth muscle cells, myofibroblasts, fibroblasts, and beyond? *Circ. Res.* 91 (8) (2002) 652–655.
- [6] G.K. Owens, M.S. Kumar, B.R. Wamhoff, Molecular regulation of vascular smooth muscle cell differentiation in development and disease, *Physiol. Rev.* 84 (3) (2004) 767–801.
- [7] J.B. Knox, G.K. Sukhova, A.D. Whittemore, P. Libby, Evidence for altered balance between matrix metalloproteinases and their inhibitors in human aortic diseases, *Circulation* 95 (1) (1997) 205–212.
- [8] A.L. Dewar, K.V. Doherty, T.P. Hughes, A.B. Lyons, Imatinib inhibits the functional capacity of cultured human monocytes, *Immunol. Cell Biol.* 83 (1) (2005) 48–56.
- [9] P. Boucher, M. Gotthardt, W.P. Li, R.G. Anderson, J. Herz, LRP: role in vascular wall integrity and protection from atherosclerosis, *Science* 300 (5617) (2003) 329–332.
- [10] M.M. Hoeper, R.J. Barst, R.C. Bourge, et al., Imatinib mesylate as add-on therapy for pulmonary arterial hypertension: results of the randomized

- IMPRES study, *Circulation* 127 (10) (2013) 1128–1138.
- [11] F. Grimminger, R.T. Schermuly, H.A. Ghofrani, Targeting non-malignant disorders with tyrosine kinase inhibitors, *Nat. Rev. Drug Discov.* 9 (12) (2010) 956–970.
 - [12] M. Lassila, T.J. Allen, Z. Cao, et al., Imatinib attenuates diabetes-associated atherosclerosis, *Arterioscler. Thromb. Vasc. Biol.* 24 (5) (2004) 935–942.
 - [13] R. Seggewiss, K. Lore, E. Greiner, et al., Imatinib inhibits T-cell receptor-mediated T-cell proliferation and activation in a dose-dependent manner, *Blood* 105 (6) (2005) 2473–2479.
 - [14] A. Juurikivi, C. Sandler, K.A. Lindstedt, et al., Inhibition of c-kit tyrosine kinase by imatinib mesylate induces apoptosis in mast cells in rheumatoid synovia: a potential approach to the treatment of arthritis, *Ann. Rheum. Dis.* 64 (8) (2005) 1126–1131.
 - [15] J. Sun, G.K. Sukhova, M. Yang, et al., Mast cells modulate the pathogenesis of elastase-induced abdominal aortic aneurysms in mice, *J. Clin. Invest.* 117 (11) (2007) 3359–3368.
 - [16] K. Shimizu, R.N. Mitchell, P. Libby, Inflammation and cellular immune responses in abdominal aortic aneurysms, *Arterioscler. Thromb. Vasc. Biol.* 26 (5) (2006) 987–994.
 - [17] A. Daugherty, L. Cassis, Chronic angiotensin II infusion promotes atherogenesis in low density lipoprotein receptor $-/-$ mice, *Ann. N. Y. Acad. Sci.* 892 (1999) 108–118.
 - [18] E. Vorkapic, A.M. Lundberg, M.I. Mayranpaa, P. Eriksson, D. Wagsater, TRIF adaptor signaling is important in abdominal aortic aneurysm formation, *Atherosclerosis* 241 (2) (2015) 561–568.
 - [19] S.S. Rensen, P.A. Doevendans, G.J. van Eys, Regulation and characteristics of vascular smooth muscle cell phenotypic diversity, *Neth Heart J.* 15 (3) (2007) 100–108.
 - [20] G.D. Demetri, M. von Mehren, C.D. Blanke, et al., Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors, *N. Engl. J. Med.* 347 (7) (2002) 472–480.
 - [21] T.I. Mughal, A. Schrieber, Principal long-term adverse effects of imatinib in patients with chronic myeloid leukemia in chronic phase, *Biologics Targets Ther.* 4 (2010) 315–323.
 - [22] E. Atallah, J.B. Durand, H. Kantarjian, J. Cortes, Congestive heart failure is a rare event in patients receiving imatinib therapy, *Blood* 110 (4) (2007) 1233–1237.
 - [23] A.L. Ribeiro, M.S. Marcolino, H.N. Bittencourt, et al., An evaluation of the cardiotoxicity of imatinib mesylate, *Leukemia Res.* 32 (12) (2008) 1809–1814.
 - [24] K. Bartolovic, S. Balabanov, U. Hartmann, et al., Inhibitory effect of imatinib on normal progenitor cells in vitro, *Blood* 103 (2) (2004) 523–529.
 - [25] P.L. Schwartzberg, A.M. Stall, J.D. Hardin, et al., Mice homozygous for the *abl*^{m1} mutation show poor viability and depletion of selected B and T cell populations, *Cell* 65 (7) (1991) 1165–1175.
 - [26] M.I. Mayranpaa, J.A. Trosien, V. Fontaine, et al., Mast cells associate with neovessels in the media and adventitia of abdominal aortic aneurysms, *J. Vasc. Surg.* 50 (2) (2009) 388–395 discussion, 395–386.
 - [27] L. Jiao, Z. Xu, F. Xu, S. Zhang, K. Wu, Vascular smooth muscle cell remodelling in elastase-induced aortic aneurysm, *Acta Cardiol.* 65 (5) (2010) 499–506.
 - [28] S.T. Hollenbeck, K. Sakakibara, P.L. Faries, B. Workhu, B. Liu, K.C. Kent, Stem cell factor and c-kit are expressed by and may affect vascular SMCs through an autocrine pathway, *J. Surg. Res.* 120 (2) (2004) 288–294.
 - [29] N. Grewal, A.C. Gittenberger-de Groot, M.C. DeRuiter, et al., Bicuspid aortic valve: phosphorylation of c-Kit and downstream targets are prognostic for future aortopathy, *Eur. J. Cardiothorac. Surg.* 46 (5) (2014) 831–839.
 - [30] L. Fredriksson, H. Li, U. Eriksson, The PDGF family: four gene products form five dimeric isoforms, *Cytokine Growth Factor Rev.* 15 (4) (2004) 197–204.
 - [31] B. Liu, J.N. Zhang, P.Y. Pu, Expressions of PDGF-B and collagen type III in the remodeling of experimental saccular aneurysm in rats, *Neurol. Res.* 30 (6) (2008) 632–638.
 - [32] S. Kanazawa, T. Miyake, T. Kakinuma, K. Tanemoto, T. Tsunoda, K. Kikuchi, The expression of platelet-derived growth factor and connective tissue growth factor in different types of abdominal aortic aneurysms, *J. Cardiovasc. Surg. (Torino)* 46 (3) (2005) 271–278.
 - [33] D. Wagsater, C. Zhu, H.M. Bjorck, P. Eriksson, Effects of PDGF-C and PDGF-D on monocyte migration and MMP-2 and MMP-9 expression, *Atherosclerosis* 202 (2) (2009) 415–423.
 - [34] H. Karvinen, J. Rutanen, O. Leppanen, et al., PDGF-C and -D and their receptors PDGFR- α and PDGFR- β in atherosclerotic human arteries, *Eur. J. Clin. Invest.* 39 (4) (2009) 320–327.
 - [35] R. Sarzani, G. Arnaldi, A.V. Chobanian, Hypertension-induced changes of platelet-derived growth factor receptor expression in rat aorta and heart, *Hypertension* 17 (6 Pt 2) (1991) 888–895.