Imatinib ameliorates fibrosis in uraemic cardiac disease in BALB/c without improving cardiac function

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Abstract

Background. Cardiovascular disease is one of the major causes of mortality and morbidity in patients with end-stage renal disease (ESRD). It is characterized by multiple left ventricular abnormalities, referred to as ‘uraemic cardiomyopathy’. The aim of the study was to investigate uraemic cardiac disease in a mouse model of chronic renal failure induced by subtotal nephrectomy and to evaluate the impact of the tyrosine kinase inhibitor imatinib and its antifibrotic as well as functional properties on the extent of the disease.
Introduction

Cardiovascular disease is one of the major causes of mortality and morbidity in patients with end-stage renal disease (ESRD). It is characterized by multiple left ventricular abnormalities, referred to as ‘uraemic cardiomyopathy’. The aetiology and pathophysiology remain unclear, however, cardiac hypertrophy caused by hypertension-independent accumulation of myocardial interstitial substance is a characteristic finding in uraemic cardiomyopathy [1,2]. This myocardial fibrosis is associated with changes like rhythm abnormalities, providing a link to the high risk of sudden death in the population of patients suffering from ESRD [3].

Main modulators of interstitial fibrosis are platelet-derived growth factors B and D (PDGF-B, PDGF-D), members of the PDGF/vascular endothelial growth factor family [4,5]. Via PDGF receptor β-signalling, they provide paracrine as well as autocrine stimulation for both vascular and connective tissue growth. Studies using transgenic mice, overexpressing the active core domain of PDGF-D in the heart, revealed a stimulated proliferation of cardiac interstitial fibroblasts. This resulted in cardiac fibrosis followed by dilated cardiomopathy and subsequent cardiac failure [4]. The expression of PDGF-D is known to be induced by progressive renal disease [5], and in a subtotal nephrectomy model for chronic renal failure, PDGF-B and PDGF receptor β were markedly increased [6].

Imatinib mesylate (formerly STI-571; Glivec/Gleevec) is a 2-phenylaminopyrimidine compound designed to specifically interact with the adenosine triphosphate (ATP)-binding site of protein tyrosine kinases (PTKs), which is highly active in chronic myeloid leukaemia and gastrointestinal stromal tumours [7]. Furthermore, imatinib potently inhibits the kinase activity of PDGF receptor tyrosine kinases [8]. It also reduced interstitial fluid pressure in an experimental colonic carcinoma model by blocking PDGF-mediated effects on stromal tissue [8].

It was the aim of this study to investigate structural and functional cardiac parameters in a model of chronic renal failure induced by subtotal nephrectomy in mice. Based on the hypothesis that cardiac fibrosis plays a key role in uraemic cardiomyopathy, we evaluated the effects of the tyrosin kinase inhibitor imatinib on the extent of the disease.

Materials and methods

Animals

Two-month-old male BALB/c mice were housed under a 12-hour light/dark cycle. Food and water were supplied ad libitum. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal protocol was reviewed and approved by a governmental animal care and research committee. Surgery was performed under general anaesthesia with ketamine (150 mg/kg i.p.) and xylazine (15 mg/kg i.p.). Briefly, after bilateral dorsal, longitudinal incisions, 5/6-nephrectomized mice (n = 46) were generated by the ablation of two-thirds of the left kidney and removal of the right kidney with preservation of the adrenal glands as previously described [9]. Nephrectomized animals were either left untreated (n = 26) or treated with imatinib (n = 23, 10 mg/kg/day p.o., kindly provided by Novartis Pharma GmbH) dissolved in drinking water which was weekly adjusted with regard to the individual drinking water consumption. Control animals were subjected to sham operation (n = 27) for 45 min and were either left untreated (n = 19) or received imatinib (n = 8, 10 mg/kg/day p.o.). Mice were placed into metabolic cages for urine collection at 8, 16 and 24 weeks, and blood and urine nitrogen and creatinine concentrations were determined. Thereafter, mice were monitored and sacrificed, and kidneys and hearts were harvested for morphological analysis or stored at −80°C for further evaluation.

Mean arterial blood pressure and echocardiography

After sedation with 2% isoflurane, echocardiographic measurements were performed at 16 and 24 weeks of age as previously described [10,11]. Standard views were obtained in 2D as well as M-mode transthoracic echocardiography with a 15-MHz transducer (Hewlett Packard) on a Sonos 5500 (Hewlett-Packard) echocardiograph. The end-diastolic volume (EDV) and end-systolic volume (ESV) were calculated from left ventricular (LVA) area and left ventricular (LVL) length measurements at 8(LVAd)²/3πLVdLd and 8(LVAs)²/3πLVsLs, respectively (where d represents diastole, and s represents systole). The ejection fraction was defined as 100(EDV-ESV)/EDV Fractional shortening (FS) was calculated from left-ventricular diameter in diastole (LVDd) and systole (LVDs) as follows: (LVDd−LVDs)/LVDd. Then mean arterial blood pressure (MAP) was measured invasively in the carotid artery by a pressure sensor (Finapres, Nijmegen, The Netherlands). The animal protocol was reviewed and approved by a governmental animal care and research committee. Surgery was performed under general anaesthesia with ketamine (150 mg/kg i.p.) and xylazine (15 mg/kg i.p.) and consecutively monitored after stabilization of blood pressure for 15 min (KRONLAB Chromatography and Laboratory technology, 0.28 MT 0.61 Labomedic Medical rope).

Histological evaluation

Paraffin-embedded specimens were cut into 4-μm-thick sections for haematoxylin and cosin (HE) and Sirius red staining. Perivascular and interstitial endomyocardial fibrosis and cardiomyocyte size were assessed as previously described [11].
Malondialdehyde concentration in cardiac tissue

Malondialdehyde (MDA) was assessed by a modified method based on the approach of Hong et al. to determine lipid peroxidation and thus oxidative damage [10]. The level of MDA in homogenized cardiac tissue was determined after reaction with thiobarbituric acid (TBA) with an added alkaline hydrolysis step. After heating and centrifugation, MDA amount was detected/measured by high-pressure liquid chromatography (HPLC) and detected by electrochemical detection (ChromSystems, Instruments and Chemicals GmbH, Munich, Germany). A standard curve was constructed with tetraethoxypropane for quantification (Sigma, Munich, Germany). The between-run variation was 3.5%.

Urinary catecholamine excretion and myocardial β-adrenoceptor density

Urinary catecholamines were determined to assess sympathetic activity. Urinary catecholamines, norepinephrine, epinephrine, and dopamine were measured by HPLC from 24-hour urine collected within a container filled with 30 μl 25% HCl using standard technique. Briefly, samples were diluted and titrated to a pH of 7 using 2N NaOH. Samples were assessed by HPLC and detected by electrochemical detection (Chromsystems, Instruments and Chemicals GmbH, Munich, Germany).

Myocardial β-adrenoceptor (βAR) density was estimated using the (−)[125I]-iodocyanopindolol (ICYP)-binding method as described by Brodde et al. [11]. Homogenized myocardial tissue, left and right ventricle separately, was incubated with six concentrations of ICYP ranging from 10 to 200 pM at 37°C for 90 min in a total volume of 250 μl for determination of β2-adrenoceptor density. The radioactivity was determined in a gamma counter (Gamma 4000; Beckman Instruments, Inc. Fullerton, CA). The βAR density is given in femtomole per milligram protein.

Mesenteric resistance vessels

Two segments of second-generation branch resistance vessels (2 mm in length, internal diameter <150 μm) were dissected and mounted in isometric Mulvan–Halpern small vessel myographs (Danish Myo Technology A/S, Aarhus, Denmark) while maintained in Krebs–Henseleit buffer bubbled with 5% CO2 and 95% O2 at pH 7.4 [12]. The length tension for each vessel was assessed, and baseline tension–internal circumference relationship was determined for each vessel [13]. Contraction response of mesenteric resistance vessels was evaluated with cumulatively increasing concentrations of phenylephrine (10^−9 to 10^−5 M) [14]. Endothelium-dependent and endothelium-independent relaxation was determined in phenylephrine pre-contracted vessels (3 × 10^−6 M) with cumulatively increasing concentrations of carbamoyl (10^−9 to 10^−5 M, endothelium-dependent) and isoprenaline (10^−5 to 10^−3 M). All solutions were freshly prepared before each experiment. The agents were purchased from Merck, Darmstadt, Germany (M) and AppliChem, Darmstadt, Germany (A) and prepared similarly in distilled water and diluted to the final bath concentration with Krebs–Henseleit buffer [sodium chloride (A) 119 mM, sodium bicarbonate (A) 25 mM, potassium chloride (A) 4.7 mM, calcium chloride (A) 2.5 mM, magnesium sulfate (M) 1.17 mM, glucose (M) 5.5 mM, KH2PO4 (M) 1.18 mM and EDTA (M) 0.027 mM]. Carbachol, norepinephrine, isoprenaline, phenylephrine and sodium nitroprusside were purchased from Sigma, Munich, Germany.

Statistics

Data are presented as mean ± SD. Parametric data were compared using ANOVA, and a Student’s t-test as post hoc test. P < 0.05 was accepted as statistically significant. Concentration–response curves were fitted for the 5/6 mice receiving IMA as compared to 5/6 mice without IMA. The 5/6 nephrectomized mice tended to have a higher MAP at 16 and 24 weeks compared to sham-operated untreated controls (Table 2). Also, the 5/6 nephrectomized mice treated with imatinib had a significantly lower MAP at 16 and 24 weeks compared to 5/6 nephrectomized controls (placebo) (Table 2). The MAP differences between the imatinib-treated group and the sham-operated untreated mice were only at 16 weeks significant. Wet lung weight (WLW) was measured to assess whether volume overload contributes to higher MAP in 5/6 nephrectomized mice. However, WLW was comparable in all groups, and no significant differences were found (Table 2).

Results

Renal function after 5/6 nephrectomy

Blood urea nitrogen (BUN) levels were significantly elevated following subtotal nephrectomy compared to sham operation. Imatinib treatment after subtotal nephrectomy reduced neither creatinine nor BUN levels (Table 1). Significantly, increased urine volumes as well as decreased urine creatinine and urea concentrations were observed after subtotal nephrectomy as compared to sham animals (data not shown). Imatinib treatment after subtotal nephrectomy did not reduce urine volume, urine creatinine or urea. Subtotal nephrectomy did not induce proteinuria at any time point (Table 1). The body weight (BW) did not differ significantly at any time point (Table 1).

MAP is lower in 5/6 mice receiving IMA as compared to 5/6 mice without IMA

The 5/6 nephrectomized mice tended to have a higher MAP at 16 and 24 weeks compared to sham-operated untreated controls (Table 2). Also, the 5/6 nephrectomized mice treated with imatinib had a significantly lower MAP at 16 and 24 weeks compared to 5/6 nephrectomized controls (placebo) (Table 2). The MAP differences between the imatinib-treated group and the sham-operated untreated mice were only at 16 weeks significant. Wet lung weight (WLW) was measured to assess whether volume overload contributes to higher MAP in 5/6 nephrectomized mice. However, WLW was comparable in all groups, and no significant differences were found (Table 2).

Table 1. MAP, weight measures and renal parameters

<table>
<thead>
<tr>
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<th>Sham</th>
<th>5/6 nephrectomy</th>
<th>5/6 nephrectomy + imatinib</th>
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<tr>
<td>Body weight (g)</td>
<td>24 weeks</td>
<td>10.6 ± 0.3</td>
<td>11.8 ± 0.9 P = 0.066*</td>
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<tr>
<td></td>
<td>16 weeks</td>
<td>10.1 ± 0.3</td>
<td>11.4 ± 0.7 P = 0.085*</td>
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<td></td>
<td>24 weeks</td>
<td>7.2 ± 1</td>
<td>7.0 ± 1</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>8 weeks</td>
<td>32.3 ± 2.2</td>
<td>72.3 ± 5.7 P &lt; 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>16 weeks</td>
<td>34.9 ± 2.2</td>
<td>68.2 ± 3.8 P &lt; 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>24 weeks</td>
<td>33.3 ± 1.8</td>
<td>59.5 ± 3.0 P &lt; 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wet lung weight (mg/g of body weight)</td>
<td>8 weeks</td>
<td>9.5 ± 7.2</td>
<td>11.5 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>16 weeks</td>
<td>2412 ± 43</td>
<td>3222 ± 100 P &lt; 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>24 weeks</td>
<td>2573 ± 0.24</td>
<td>3679 ± 72 P &lt; 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup> P < 0.05 vs. sham.
Cardiomyocyte size ($\mu$m)

<table>
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<tr>
<th></th>
<th>Sham 5/6 nephrectomy</th>
<th>5/6 nephrectomy + imatinib</th>
</tr>
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<tbody>
<tr>
<td>Heart weight (mg/ mm tibia length) 24 weeks</td>
<td>7.4 ± 0.4</td>
<td>6.9 ± 0.4</td>
</tr>
<tr>
<td>Echocardiography End-diastolic volume (mm$^3$)</td>
<td>101 ± 26</td>
<td>71 ± 8</td>
</tr>
<tr>
<td>Cardiomyocyte size ($\mu$m$^2$)</td>
<td>54 ± 16</td>
<td>52 ± 8</td>
</tr>
<tr>
<td>Cardiac function, catecholamine excretion and myocardial $\beta$-receptor density</td>
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<tr>
<th>Urinary catecholamines (ng/24 hours)</th>
<th>Dopamine</th>
<th>Noradrenaline</th>
<th>Adrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham 5/6 nephrectomy</td>
<td>380 ± 174</td>
<td>416 ± 135</td>
<td>52.6 ± 11.0</td>
</tr>
<tr>
<td>5/6 nephrectomy + imatinib</td>
<td>446 ± 44</td>
<td>426 ± 90</td>
<td>52.6 ± 11.0</td>
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</table>

Cardiofibrosis is ameliorated by IMA in 5/6 mice

At 24 weeks, interstitial collagen deposition in the myocardium was significantly elevated after subtotal nephrectomy as compared to sham operation (Figure 2). Imatinib treatment after subtotal nephrectomy was associated with significantly less collagen deposition (Figure 2). Collagen distribution did not differ significantly between sham-operated mice without treatment and subtotal nephrectomized imatinib-treated mice ($P = 0.15$; Figures 2A–D). The collagen deposition in sham-operated mice receiving imatinib was comparable to untreated sham-operated mice. Stenotic coronary lesions were not demonstrable in any of the samples. Cardiomyocyte size was not significantly different between the groups (Table 2).

Renal histology after 5/6 nephrectomy

The mean glomerular surface area (as a measure of glomerular hypertrophy) of mice subjected to subtotal nephrectomy was significantly higher at all time points as compared to sham-operated untreated mice (Table 1). Imatinib treatment of mice subjected to subtotal nephrectomy resulted in a significantly decreased mean glomerular surface area as compared to mice subjected to subtotal nephrectomy (Table 1).

Cardiac function is decreased in 5/6 mice and not preserved by IMA treatment

The heart weight (HW) did not differ significantly at any time point (Table 2). End-diastolic volume was non-significantly decreased in both nephrectomy groups as compared to sham-operated untreated mice (Table 2, Figure 1A). End-systolic volumes were similar between groups (Table 2). The ejection fraction (EF, Figure 1B) was significantly lower in subtotal nephrectomized mice as compared to sham-operated untreated controls after 24 weeks ($P < 0.01$). Imatinib treatment in subtotal nephrectomized mice did not improve EF as compared to untreated subtotal nephrectomized mice. Treatment with imatinib led to the tendency of worsening functional parameters. Next, it was investigated if an altered density of myocardial $\beta$-adrenoceptors contributes to decreased cardiac function in uremic mice. However, $\beta$AR density estimated by the $\left(-\right)^{[125I]}$iodoanopindolol-binding method revealed no significant differences between treated or untreated 5/6 mice and sham-operated untreated animals (Table 1).

Oxidative stress was assessed by determining lipid peroxidation. Thus, MDA concentration was measured in cardiac tissue and was shown to be significantly elevated following subtotal nephrectomy as compared to sham operation. Imatinib treatment after subtotal nephrectomy did not significantly reduce cardiac MDA concentration (Figure 3).

Mesenteric resistance vessels in 5/6 mice are impaired in endothelial-dependent relaxation

Finally, functional aspects of the vasculature in uremic mice were studied. Subtotal nephrectomy impaired neither phenylephrine-induced vasoconstriction nor isoprenaline-induced relaxation. Imatinib treatment in 5/6 or sham-operated mice had no further effect on these parameters. In contrast, subtotal nephrectomy decreased the extent of relaxation after application of carbachol in mice (Figure 4A). The extent of relaxation after application of carbachol in mice treated with 5/6 nephrectomy showed no significant difference compared to mice treated with 5/6 nephrectomy + imatinib (Figure 4B). Thus, imatinib treatment did not restore impaired carbachol-mediated relaxation in subtotal nephrectomy. In contrast, imatinib even led to decreased relaxation in sham-operated mice (Figure 4C). However, imatinib treatment of 5/6 mice did not further augment the disturbed vessel relaxation observed after 5/6 nephrectomy (Figure 4B).
Discussion

Uraemia induced by subtotal nephrectomy in BALB/c mice increases blood pressure, oxidative damage and impaired ejection fraction. Imatinib ameliorates myocardial fibrosis and decreases arterial blood pressure but impaired cardiac function, oxidative cardiac damage and depressed resistance vessel dilatation persist.

So far, studies of uraemic cardiac disease in the remnant kidney model have mainly been performed in rats [15]. The rat model has some different characteristics as compared to the mouse model [16]. In accordance with other studies in the mouse remnant kidney model, proteinuria is only detectable in certain strains [16]. The BALB/c mice used in this study did not develop proteinuria although glomerular hypertrophy, but not glomerulosclerosis, was diagnosed. Furthermore, progressive chronic renal failure after subtotal ablation was not observed in our model. However, endothelial dysfunction observed in our mice model after subtotal nephrectomy is also commonly noted in various rat remnant kidney models [17,18].

In renal insufficiency, sympathetic activation plays a crucial role for both progression of renal failure and the high rate of cardiovascular events in patients with chronic kidney disease. The contribution of sympathetic neural mechanisms to the development of hypertension and the progression of heart failure are well established [19]. Our model of uraemia after subtotal nephrectomy reflects the picture of sympathetic activation, including elevated blood pressure and cardiac dysfunction [19]. Moreover, this
Fig. 2. Cardiac collagen distribution in sham, 5/6 nephrectomy and 5/6 nephrectomy + imatinib (A). Interstitial collagen deposition was enhanced after 5/6 nephrectomy. Imatinib significantly reduced collagen deposition after 5/6 nephrectomy. (B-D) show representative Sirius red stainings in sham operated (B), 5/6 nephrectomy (C) and 5/6 nephrectomy + imatinib (D).

Fig. 3. MDA concentration (micrometre per milligram heart tissue) in mouse heart homogenates at 8, 16 and 24 weeks after sham operation, 5/6 nephrectomy and 5/6 nephrectomy + imatinib.
model demonstrates enhanced oxidative damage which is a typical characteristic of renal insufficiency [19]. As a consequence of both aspects, cardiac fibrosis was increased, and endothelial relaxation was impaired.

PDGF has been evidenced to mediate tyrosine kinase-dependent fibrotic processes. Moreover, crosstalk with catecholamines [20] and the induction of oxidative stress [21] have been demonstrated. Imatinib potently inhibits the kinase activity of PDGF receptor tyrosine kinases [8]. As such, it has been evidenced as anti-fibrotic agent in several diseases [8]. However, the action of imatinib has not been investigated in context with its catecholamines and oxidative damage. Therefore, we introduced imatinib into our model of renal insufficiency.

As expected, imatinib reduced cardiac fibrosis in our model of renal insufficiency [22]. However, the effects remained not restricted to its anti-fibrotic potential. Interestingly, imatinib reduced, after subtotal nephrectomy, the adrenaline concentration without modifying cardiac β-adrenergic receptor density and also reduced the arterial blood pressure. This implies a reduced sensitivity for catecholamines in renal-insufficient mice after imatinib treatment. The mechanism by which imatinib reduces adrenaline levels remains elusive. Crosstalk between tyrosin kinases and adrenoreceptors has been reported [23]. Moreover, there is evidence from the literature that tyrosine kinases have a role in catecholamine secretion; thus, imatinib might simply interfere with the release of catecholamines [24]. In line with the common notion that catecholamines are important in blood pressure control, lower catecholamine levels might be responsible for the reduced arterial blood pressure. Additionally, one might speculate that imatinib lowers arterial blood pressure by interfering with angiotensin-II signalling as angiotensin II mediates hypertension in chronic renal failure [19]. Indeed, there is crosstalk between angiotensin II and imatinib-sensitive receptor tyrosine kinases as reported by Mehta et al. [25]. However, this remains notional. Nevertheless, lower blood pressure also had beneficial effects on end organs as the mean glomerular surface area of the kidney (as a measure of glomerular hypertrophy) was reduced in nephrectomized mice treated with imatinib as compared to untreated 5/6 mice. Glomerular hypertrophy is usually thought to be a consequence of systemic and glomerular hypertension and follows renal mass reduction [26]. Additionally, the cardiac anti-fibrotic action of imatinib after subtotal nephrectomy can also be explained indirectly by the blood pressure low-

Fig. 4. Mulvany–Halpern myographic experiments with mesenteric resistance vessels. Relaxation of smooth muscles (percentage) subsequent to application of carbachol 8 weeks after. (A) Sham operation and 5/6 nephrectomy; (B) 5/6 nephrectomy and 5/6 nephrectomy + imatinib; (C) sham operation and sham operation + imatinib. Points represent the mean ± SEM. The extent of relaxation after application of carbachol in mice treated with 5/6 nephrectomy was significantly decreased compared to mice treated with sham operation alone (log EC50 -6.9 ± 0.06 vs. log EC50 -7.3 ± 0.05, P = 0.016). The extent of relaxation after application of carbachol in mice treated with 5/6 nephrectomy as compared to untreated 5/6 mice. Glomerular hypertrophy is usually thought to be a consequence of systemic and glomerular hypertension and follows renal mass reduction [26]. Additionally, the cardiac anti-fibrotic action of imatinib after subtotal nephrectomy can also be explained indirectly by the blood pressure low-

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ering even though a direct anti-fibrotic effect should also be considered.

In contrast, the renal insufficiency-induced oxidative damage remained unaffected by imatinib treatment. Oxidative stress is a major cause for endothelial dysfunction [27]. Therefore, we investigated endothelial-dependent relaxation. We could demonstrate that imatinib impaired endothelial function in sham-operated mice but did not ameliorate or augment endothelial function after subtotal nephrectomy. This suggests that imatinib has no beneficial effect on endothelial dysfunction. Furthermore, the accumulated oxidative damage may play an important role in this vascular defect and outdoes the blood pressure-lowering effect in this aspect.

As far as the cardiac function is concerned, imatinib had no protective effect after subtotal nephrectomy, although the blood pressure and cardiac fibrosis were significantly reduced. This led us to consider that these aspects are dissociated from uraemic cardiac disease. Our results are in line with data demonstrating that antihypertensive treatment with hydralazine or nifedipine did not protect function [28]. If antihypertensive drugs that are known to reduce oxidative stress are chosen, cardiac protection is reached [29,30]. Therefore, we further suggest that oxidative stress is an important trigger for the reduced cardiac function in our model and that other mechanisms such as other therapeutic target.

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**Conflict of interest statement.** None declared.

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