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1 **Abstract**

2 **Background**

3 Chronic interstitial fibrosis is the primary barrier against the long-term survival of transplanted
4 kidneys. Extending the lifespan of allografts is vital for ensuring the long-term health of patients
5 undergoing kidney transplants. However, few targets and their clinical applications have been
6 identified. Moreover, whether dyslipidemia facilitates fibrosis in renal allograft remains unclear.

7 **Methods**

8 Blood samples were collected from patients who underwent kidney transplantation. Correlation
9 analyses were conducted between the Banff score and body mass index, ¹ and serum levels of
10 triacylglycerol, total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein
11 cholesterol. A rat model of renal transplantation was treated with the lipid-lowering drug, fenofibrate,
12 and kidney fibrosis levels were determined by histochemical staining. Targeted metabolomic
13 detection was conducted in blood samples from patients who underwent kidney transplantation and
14 were divided into fibrotic and non-fibrotic groups. Rats undergoing renal transplantation were fed
15 either an ¹⁴ n-3 or n-6 polyunsaturated fatty acid (PUFA)-enriched diet. Immunohistochemical ¹⁹ and
16 Masson's trichrome staining were used to determine the degree of fibrosis.

17 **Results**

18 Hyperlipidemia was associated with fibrosis development. Treatment with fenofibrate contributed
19 to improve fibrosis in a rat model of renal transplantation. Moreover, n-3 polyunsaturated fatty acids
20 (PUFAs) from fibrotic group showed significant downregulation compared to patients without
21 fibrotic renal allografts, and n-3 PUFAs-enriched diet contributed to delayed fibrosis in a rat model
22 of renal transplantation.

23 Conclusions

24 This study suggests that hyperlipidemia facilitates fibrosis of renal allografts. Importantly, a new
25 therapeutic approach was provided that may delay chronic interstitial fibrosis in transplanted
26 kidneys by augmenting the n-3 PUFA content in the diet.

27 Background

28 When alternative therapies have been exhausted,¹⁵ kidney transplantation is the sole solution for end-
29 stage renal disease. Owing to a dearth of available donor kidneys, extending the lifespan of
30 transplanted kidneys has become necessary. Nevertheless, sustaining renal allografts over the long
31 term presents considerable difficulties. Chronic allograft nephropathy was redefined as⁴ interstitial
32 fibrosis and tubular atrophy[1], and represents a major factor affecting⁴ the long-term survival of
33 renal allografts[2]. Renal allograft fibrosis is a multifactorial disease characterized by inflammation,
34 T-cell-mediated rejection, immunosuppressive drug toxicity, and the epithelial-mesenchymal
35 transition, all of which are closely associated with the development of fibrosis[3-5]. However, few
36 targets and clinical applications to this end have been discovered. Moreover, whether dyslipidemia
37 facilitates fibrosis in renal allograft remains unclear.

38 Polyunsaturated fatty acid (PUFA) are fatty acids with more than two unsaturated bonds. Linoleic
39 acid (LA) and α -linolenic acid (α -LA) are essential fatty acids. They function as precursors for the
40 biosynthesis of PUFAs by adding unsaturated bonds and elongating the fatty acid chain. PUFAs are
41 classified based on the distance between the unsaturated bonds and the first methyl group. PUFAs
42 are defined as n-3 PUFAs when the unsaturated bond is situated¹⁶ at the third carbon atom and as n-
43 6 PUFAs when the unsaturated²² bond is situated at the sixth carbon atom[6]. PUFAs participate in
44 several cellular processes and can affect disease progression[7]. PUFAs in the blood of patients with

45 liver cirrhosis were decreased compared to that in the control group, and supplementary essential
46 fatty acids and long-chain PUFAs contributed to improvement of the cirrhosis[8]. In addition, n-3
47 PUFAs are risk predictors of heart failure[9]. A recent study demonstrated a positive correlation
48 between elevated n-3 PUFA levels in the bloodstream and improved survival rates for renal
49 allografts[10]. However, whether the addition of n-3 PUFAs improves fibrosis in renal allografts
50 remains unclear.

51 One study revealed that hyperlipidemia significantly contributes to the development of fibrotic
52 lesions in renal allografts. Treatment with lipid-lowering drug fenofibrate contributed towards
53 improving fibrosis¹⁰ in a model of rat kidney transplantation. Analysis of blood sample collected from
54 renal transplant recipients showed that the reduction of n-3 PUFAs was associated with fibrosis
55 progression in renal allografts. An n-3 PUFA enriched diet attenuated fibrosis in a rat renal transplant
56 model. Based on the results from blood samples of renal transplant recipients and rat models of
57 renal transplantation, a low fat and high-proportioned PUFA diet may be beneficial to the extended
58 upkeep of renal allografts.

59 **Materials and methods**

60 **Blood specimens**

61 Patients were recruited from the First Hospital of Jilin University, China. All clinical records were
62 obtained with the approval of the Ethics Committee. Clinical records were divided into three groups;
63 69 cases with ci0, 63 cases with ci1, and 19 cases with ci2/3. Targeted lipidomic detection used
64 blood samples from seven patients with ci0, six patients with ci1, and one patient with ci2. There
65 was a partial overlap in the above samples for different projects.

66 **Rat kidney transplantation model**

67 Sprague-Dawley rats (180-200g) were used as donors and receptors. Prior to operation, anesthesia
68 was administered to the mice using isoflurane. For the donor, heparinized saline (at a concentration
69 of 125 U/mL) was injected, and the blood supply was halted using styptic clips. The left renal vein
70 was removed before perfusion. The left renal vein, ureter, and artery were ligated after perfusion
71 was complete. Heparinized saline (0~4°C) was used as a preserving fluid for the separated left
72 kidney. For the recipient, the left kidney, renal artery, renal vein, and ureter were separated and cut
73 off. The renal artery, vein, and ureter were then anastomosed between the donor and recipient before
74 the blood supply was restored. Bladder anastomoses were created to avoid ureteral obstruction,
75 which may lead to unsuccessful ureter anastomosis. For postoperative management, 5 mL saline
76 and ketoprofen (5 mg/kg) were injected into the abdominal cavity before the abdomen was closed.
77 After renal transplantation, penicillin was administered for 3 days and cyclosporin A for 1 week.
78 The animals were included in the study if they underwent successful renal transplantation, defined
79 by improved blood supply of renal veins and arteries.

80 Immunofluorescence staining

81 The sections were dewaxed and washed with water conventionally. Thermal antigen retrieval with
82 citrate for 5 min at 95°C was performed. 5% BSA was used to block the sections for 30 min before
83 being incubated with the primary antibodies, including antibodies against acyl-CoA oxidase 1
84 (ACOX1) (DF12046, Affinity Biosciences, Ohio, USA) and α -smooth muscle actin (α -SMA)
85 (ab7817, Abcam, Cambridge, UK), in PBS containing 1% BSA, and the sections were incubated
86 overnight at 4°C. The sections were washed thrice before being incubated with the different
87 fluorescent dye-conjugated secondary antibodies for 1 h. After washing the sections three times
88 with PBS, Lotus Tetragonolobus Lectin (LTL) (FL-1321-2, Vector, California, US) was used for

89 incubation for 30 min. The sections were then washed thrice before the cell nuclei were stained with
90 DAPI for 10 min. After washing them again thrice, the sections were sealed with antifade mounting
91 medium (P0128, Beyotime, Shanghai, China). The sections with stained tissues were mounted onto
92 a confocal microscope and images were captured.

93 Hematoxylin & eosin.

94 ²⁰ Sections were dewaxed twice with xylene for 30 min, then dipped into a concentration gradient of
95 ethyl alcohol ²⁴ for 5 min. They were then washed with distilled water and subjected to 5 min of
96 hematoxylin staining, differentiated with acid alcohol, blued with ammonia water, and subjected to
97 a 10 min eosin staining procedure. The sections were dehydrated with a gradient of 70%, 80%, 95%
98 I, 95% II, 95% III, 100% I, and 100% II ethyl alcohol. The samples were immersed twice in xylene
99 for a duration of 10 min before they were sealed with neutral resins.

100 Masson

101 The sections underwent a range of procedures, including dewaxing, staining with hematoxylin and
102 ponceau-acid fuchsin solution, washing with 2% glacial acetic acid, differentiation with 1%
103 phosphomolybdic acid, staining with aniline blue, dehydrating, and sealed with neutral resin.

104 Targeted lipidomics

105 A standard solution was prepared. Fatty acid calibrators at concentrations of 10–40000 ng/mL were
106 prepared by mixing the stock solutions of individual fatty acids with a fatty acid-free matrix. The
107 samples were resuspended and homogenized in liquid nitrogen with 300 μ L of a mixture of
108 isopropanol and acetonitrile (1:1) containing internal standards, which were mixed. The samples
109 were subsequently underwent centrifugation at 12,000g for a duration of 10 min. The 2 μ L of
110 supernatant was extracted and injected into the LC-MS/MS system. The fatty acids were quantified

111 using a UHPLC-MS/MS system. The concentration series of the standard solutions were detected
112 using LC-MS. The ⁷ratio of standard to internal standard concentrations was determined using the x-
113 axis, whereas the y-axis, denoting the ⁷peak area of the standard to internal standard, was designated
114 as the ordinate to assess the linearity of the standard solution. Each metabolite was allowed to reach
115 a correlation coefficient (r) > 0.99. The limit of quantification was determined using the signal-to-
116 noise ratio method.

117 Immunohistochemistry (IHC)

118 The sections were dewaxed and washed with water conventionally. Thermal antigen retrieval with
119 citrate for 5 min at 95°C was performed. An ultra-sensitive TM SP IHC kit procured from MXB
120 biotechnologies (KIT-7710, Fujian, China) was used for IHC detection. Tissues then underwent the
121 following treatment steps: incubation with Reagent I for 15 min, washed thrice with PBS for 5 min,
122 and incubation with Reagent II for 15 min. The tissues were then incubated with dilute specific
123 antibodies, including Fibrillin 1 1:200 (AF0429, Affinity Biosciences, Ohio, USA), MMP7 1:4000
124 (AF0218, Affinity Biosciences, Ohio, USA), and collagen IV 1:400 (AF0510, Affinity Biosciences,
125 Ohio, USA), at 4 °C overnight. The next day, the tissues were subjected to ¹¹three washes with PBS
126 and were ¹¹then incubated with Reagent III. Subsequently, the tissues were ¹¹washed again with PBS,
127 ¹¹incubated with Reagent IV, and then washed three more times with PBS. Subsequently, 3,3-
128 diaminobenzidine (DAB) staining was performed until tissues were stained with brown or red, then
129 terminated with water. In this step, one antibody performed same dyeing time. Cell nuclei were
130 visualized by staining the sections with hematoxylin. To retain the sample, the sections were
131 dehydrated and preserved using neutral resin.

132 ⁸Total cholesterol (TC) and triacylglycerol (TG) detection in rat blood

133 Serum was collected from rats with renal transplantation.⁸ TC (A111-1-1) and TG (A110-1-1)
134 detection kits were obtained from³ Nanjing Jiancheng Bioengineering Institute, located in Nanjing,
135 China. Serum (2.5μL) or standard substance (TG 2.39mmol/L, TC 6.15mmol/L) was maximixed
136 with working solution in 96 pore plate and subsequently⁶ incubated at 37°C for 10 min. Absorbance
137 was measured at a wavelength of 500 nm. TC and TG concentrations were counted using the
138 subsequent equations:⁵ TG (mmol/L) = (A_{sample}-A_{blank}/A_{standard}-A_{blank})x 2.39 mmol/L;⁵ TC
139 (mmol/L) = (A_{sample}-A_{blank}/A_{standard}-A_{blank}) x 6.15 mmol/L.

140 Statistical analysis

141 GraphPad Prism 9, developed by GraphPad Software in California (USA), was utilized to perform
142 statistical analyses in this study. The data have been provided as means ± standard errors of the
143 means.² Statistical significance was determined using Student's *t*-tests, where **P* < 0.05, ***P* < 0.01,
144 ****P* < 0.001, and *****P* < 0.0001 signify statistical significance.

145 Results

146 **Disordered lipid metabolism in blood¹⁰ is associated with interstitial fibrosis in renal allografts**

147 Blood samples were collected during various post-transplantation periods from 151 patients who
148 underwent kidney transplantation. To confirm whether obesity was associated with fibrosis in
149 transplanted kidneys, the relationship between fibrosis and body mass index (BMI) was analyzed;
150 level of fibrosis was measured by ci-scores ranging from 0 to 3 to determine the progress of fibrosis
151 according to the Banff score. BMI did not correlate with the ci-score (Fig. 1A). Early causes or acute
152 reactions, such as ischemia reperfusion and acute rejection, also result in injury-induced fibrosis.
153 Thus, to exclude acute injury-induced fibrosis in transplanted kidneys, cases that had undergone
154 transplantation more than 5 years prior were analyzed. The percentage of overweight patients

(BMI>25) displayed an increasing trend along with the progression of fibrosis (Fig. 1B). The results revealed that the amount of TG in patients with ci-scores of 2 and 3 was significantly higher than that in patients with ci-scores of 0 or 1 (Fig. 1C). Further analysis revealed that cases with percentages of high TG (>1.75 mmol/L) accounted for 52.00% of ci0 cases, 67.57% of ci1 cases, and 85.71% of ci 2/3 cases (Fig. 1D). In addition, TC levels were higher in ci 2/3 patients than in ci 0 and ci 1 patients (Fig. 1E). Cases with percentage of high TC (>5.2 mmol/L) accounted for 64.29% which was far higher than ci 0 and ci 1 cases (Fig. 1F). A correlation analyses of BMI, TG, TC, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) with several other indices of the Banff score were further conducted, including total interstitial inflammation (ti), tubulitis (t), glomerulitis (g), C4d deposition, vascular fibrous intimal thickening (cv), and arteriolar hyaline thickening (ah), in cases where transplantation was performed more than 5 years prior. The results showed that the proportion of patients with high TG and LDL-C levels progressively increased alongside cv and ah progression (Table 1). These findings indicate that abnormal lipid levels in blood are correlated with fibrotic lesions.

Fenofibrate attenuates fibrosis in a rat model of renal transplantation

Fenofibrate activates peroxisome proliferator-activated receptor alpha (PPAR α), which has a critical function in promoting fatty acid oxidation[11]. This drug is used for the treatment of hypertriglyceridemia and mixed dyslipidemia[12]. As renal transplant patients with serious fibrosis display increased blood TG and TC levels, rat models of renal transplantation were treated with fenofibrate for four weeks (Fig. 2A). Fenofibrate treatment increased the expression of CD36 and ACOX1 in liver which were responsible for the uptake and oxidation of lipid (Fig. 2B, C). TG and TC levels in blood were decreased after fenofibrate treatment (Fig. 2D), indicating that fenofibrate

177 treatment produced the expected effects. Following treatment with fenofibrate, transplanted kidneys
178 displayed a ruddier color and softer grains than those in the untreated group (Fig. 2E).
179 Immunofluorescence (IF) detection showed that fenofibrate also upregulated ACOX1 expression in
180 kidney, and decreased α -SMA expression (Fig. 2F), indicating that it may decrease lipid level in
181 renal allograft. Hematoxylin-eosin staining showed that fenofibrate treatment improved kidney
182 injury in the rat models of renal transplantation (Fig. 2G), while Masson's and Sirius red staining
183 showed that fenofibrate treatment inhibited the development of fibrosis (Fig2. G, H). Taken together,
184 the lipid-lowering drug, fenofibrate, delayed the progression of fibrosis in renal allografts.

185 **The n-3 PUFA level is decreased in serum from renal transplantation recipients with fibrosis**

186 To ascertain the association between the serum lipid composition and fibrosis progression, blood
187 samples were collected from patients who underwent kidney transplantation more than five years
188 prior, including seven patients with ci-score 0 (Group A), six cases with ci-score 1, and one case
189 with ci-score 2 (Group B) (Fig. 3A). ¹There was no statistically significant difference in total TG
190 levels between the two groups (Fig. 3B). Blood samples were also analyzed using targeted
191 metabolomics to determine the free fatty acid content. The results showed that saturated fatty acids
192 (SFAs) were unchanged between groups A and B (Fig. 3 C). However, the majority of PUFAs
193 decreased in the blood of patients with allograft fibrosis (Fig. 3D, E). It is noteworthy that n-3
194 PUFAs showed more significant decrease than n-6 PUFAs, especially the essential fatty acid α -LA.
195 In summary, serum obtained from fibrotic renal transplantation recipients showed decreased PUFA
196 levels compared to that of non-fibrotic patients, proposing that n-3 PUFAs may affect fibrosis
197 progression in renal allografts.

198 **An n-3 PUFA enriched diet contributes to improved fibrosis in a rat renal transplant model**

199 Targeted metabolomics results showed that LA was not altered in either group; nevertheless, α -LA
200 was significantly decreased in Group B (Fig. 3D, E). This suggests that although both ²¹n-3 and n-6
201 PUFAs are necessary to prevent fibrosis in renal allografts, a lack of n-3 PUFAs may play a more
202 crucial role in facilitating fibrosis. To verify whether the addition of n-3 PUFAs alleviated fibrosis
203 progression in renal allografts in vivo, a rat renal transplantation model was established and the rats
204 were supplied with soybean oil feed (SbOF), a type of edible oil, or linseed oil feed (LOF) (Fig.
205 4A). The total lipid content in both feeds was 7%. SFAs and monounsaturated fatty acids were
206 present in similar proportions. The biggest difference between the feeds was that soybean oil
207 includes more n-6 PUFAs, whereas linseed oil has a higher amount of n-3 PUFAs (Fig. 4B).
208 Compared with normal kidneys (right), transplanted kidneys (left) showed a lower volume (Fig. 4C).
209 In addition, transplanted kidneys in rats fed with SbOF were smaller in volume, whiter in color, and
210 tougher in grains than those treated with LOF (Fig. 4C). The n-3 PUFA diet effectively alleviated
211 the development of fibrosis in renal allografts (Fig. 4D, E) and did not produce observable lesions
212 in other organs (Fig. 4F). The IHC analyses revealed that the n-3 PUFA diet downregulated the
213 expression of fibrosis-related proteins, including collagen IV (Col 4), matrix metalloproteinase 7
214 (MMP7), and fibrillin 1 (FBN1) (Fig. 4G, H). In addition, LOF did not affect the expression of
215 ACOX1 and CD36 in liver (Fig. 4I, J) or the TG and TC levels in blood (Fig. 4H), indicating that
216 n-3 PUFA diet improved fibrosis in renal allograft and the desired effects were not dependent on
217 reducing blood lipid levels. These findings suggest that augmenting the n-3 PUFA content in the
218 diet may delay chronic interstitial fibrosis in renal allografts.

219 Discussion

220 Chronic interstitial fibrosis is a ¹⁷major challenge in patients undergoing kidney transplantation.

221 Preventing the progression of fibrosis and prolonging the survival time of renal allografts are urgent
222 issues. Although multiple underlying mechanisms for renal fibrosis have been elucidated[5], few
223 therapeutic targets have been developed into applications suitable for use in clinical practice.

224 Obesity is a critical factor that accelerates liver fibrosis in the development of non-alcoholic fatty
225 liver disease[13]. However, the contribution of blood fat to the progression of renal allograft fibrosis
226 remains unclear. The results showed that hyperlipidemia may contribute to the progression of
227 fibrosis in renal allografts. Patients who undergo renal transplantation are usually instructed to
228 control their diet, and while fatty acid oxidation is the primary energy supplement for the kidneys,
229 fibrotic renal tissue cannot oxidize enough fatty acids, resulting in lipid accumulation in the kidneys
230 and blood. Fibrotic renal allografts, therefore, may induce hyperlipidemia.

231 Fenofibrate has been extensively used clinically to lower blood lipid levels[14]; however, it also
232 functions at the cellular level. While the results showed that lowering blood lipid with fenofibrate
233 improved fibrosis in a rat model of renal transplantation, the possible effect of the drug on cells
234 cannot be excluded. For example, a recent study has shown that fenofibrate affects the vitality,
235 function, and phenotype of proximal tubular cells[15], macrophages[16], and T cells[17], which are
236 critical factors influencing fibrosis in renal allograft fibrosis. In addition, a previous study reported
237 that fenofibrate improved fibrosis by inhibiting oxidative stress in renal allografts[18].

238 Unquestionably, fenofibrate is a potential agent for delaying fibrosis in renal allografts though its
239 molecular mechanism requires further elucidation.

240 In addition to energy supplement, fatty acids such as arachidonic acid, play a vital role in signal
241 transduction[19]. The results showed that serum PUFA levels were decreased in fibrotic renal
242 transplant patients; however, the causality as to why PUFAs decreased remains unclear. Fibrotic

243 renal tissues consume excess PUFAs, giving rise to implications that n-3 PUFAs being consumed
244 may be leading to rise in some stress status. Consistent with this understanding, n-3 PUFA
245 supplementation provenly improves oxidative stress in mice with chronic kidney disease[20], and
246 patients with intestinal failure[21]. In addition, n-3 PUFA supplementation inhibits endoplasmic
247 reticulum stress[22]. Both oxidative and endoplasmic reticulum stress play critical roles in the
248 fibrosis of renal allografts [23, 24]. Thus, a diet supplemented with n-3 PUFAs may improve fibrosis
249 in renal allografts by suppressing stress.

250 **Strengths and limitations**

251 This study effectively revealed the association between dyslipidemia and fibrosis progression in
252 renal allografts. Changes in blood lipid levels following treatment with lipid-lowering drug
253 fenofibrate and an n-3 PUFAs enriched diet are effective approaches towards maintaining the long-
254 term survival of renal allografts. Fenofibrate is commonly used in clinical medicine and n-3 PUFAs
255 are essential fatty acids for humans, indicating that both these approaches had the potential of
256 clinical transformation. Several limitations also exist in this study. First, abnormal lipid metabolism
257 may affect the function of immune cells and the transformation of epithelial cells. Future research
258 should be focused on the mechanistic area of study. Second, LOF was a complicated component, it
259 will be constructive to specify which n-3 PUFAs play the critical role in suppressing fibrosis in renal
260 allografts.

261 **Conclusion**

262 Taken together, these findings¹ suggest that hyperlipidemia is a risk factor for the advancement of
263 fibrosis in renal allografts. The lipid-lowering drug fenofibrate is a potential therapeutic agent for
264 the treatment of fibrosis. These findings support a new therapeutic approach that may delay chronic

265 interstitial fibrosis in renal allografts by augmenting the dietary n-3 PUFA content. This study
266 indicated that patients who underwent renal transplantation should follow a dietary habit, a low fat
267 but high-proportioned PUFAs diet, which may contribute in delaying the survival time of a renal
268 allograft.