Nephroprotective effect of the HMG-CoA-reductase inhibitor cerivastatin in a mouse model of progressive renal fibrosis in Alport syndrome

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Abstract

Background. Alport syndrome is caused by mutations in genes encoding for the α3, α4 or α5 chain of type IV collagen leading to excessive production of fibrotic tissue and end-stage renal failure. HMG-CoA-reductase-inhibitors exhibit pleiotropic effects by which they modulate the production of connective tissue. The aim of this study was to examine the anti-fibrotic effect of the HMG-CoA-reductase-inhibitor, cerivastatin, in COL4A3 knockout mice, an animal model of Alport syndrome with progressive renal fibrosis.

Methods. Forty homozygous COL4A3 knockout mice received cerivastatin, starting 28 or 49 days after birth. Mice were sacrificed at day 52 or 66 after birth. Immunohistochemistry against laminin and fibronectin was performed. Inflammatory cell infiltration was determined by F4/80- and CD3-staining. Myofibroblasts were identified by an α-smooth muscle actin staining. Expression of the profibrotic cytokines, TGF-β1 and CTGF, were determined by immunoblot.

Results. The lifespan of treated COL4A3 knockout mice was increased by 28% compared with untreated animals (71±6 vs 91±9 days, P<0.01). Early cerivastatin treatment reduced cholesterol levels (113±13 vs 141±19 mmol/l in untreated animals, P<0.05) and serum urea (164±235 mmol/l, day 66, P<0.05). Treatment also decreased proteinuria (5.5 vs 12 g/l at day 66, P<0.05). Deposition of laminin and fibronectin, expression of TGF-β and CTGF was reduced. Infiltration of T-cells and macrophages as well as myofibroblasts appeared to be reduced in kidneys from cerivastatin-treated mice.

Conclusion. Cerivastatin prolongs the lifespan of COL4A3 knockout mice, reduces proteinuria and delays uraemia. These effects are associated with decreased renal fibrosis and a reduction of inflammatory cell infiltration.

Keywords: alport syndrome; extracellular matrix; renal fibrosis; statins; type IV collagen

Introduction

Kidney fibrosis, associated with renal failure, is known to be the most common final stage of progressive renal disease. Alport syndrome (AS) is a hereditary disorder associated with progressive renal failure and kidney fibrosis. This disease is characterized by mutations in genes encoding for either the α3, α4 or α5 chain of type IV collagen, leading to the absence of all three chains forming the triple helical collagen monomers [1,2]. Thus, the glomerular basement membrane (GBM) in AS consists of only the α1 and α2 chains of type IV collagen, making this altered basement membrane more susceptible to endoproteolysis [3]. In the course of the disease, focal thickening and splitting of the basement membrane occurs, associated with haematuria and proteinuria and later progressive renal fibrosis leads to loss of renal function.

Several animal models of AS have been established, one of which is a COL4A3 knockout mouse model developed by Cosgrove et al. [4]. This model allows examination of a large number of animals exhibiting a phenotype similar to human AS. To date, there is no specific therapy for AS and the only treatment at present is dialysis and renal transplantation. To evaluate the effect of a now established therapy in renal disease, COL4A3 knockouts have been treated with an ACE-inhibitor and AT1–antagonist; these
achieved a prolongation of lifespan in the mouse model, paralleled by reduction of proteinuria and renal fibrosis [5,6].

Inhibitors of HMG-CoA-reductase (statins) are potent inhibitors of cholesterol synthesis. However, effects beyond lipid reduction have become increasingly recognized. Statins improve endothelial dysfunction and reduce thrombogenicity, while in the kidney, statins inhibit proliferation of mesangial cells and mesangial matrix deposition [7,8]. Statins intervene in the regulation of apoptosis and modify monocyte infiltration and production of the profibrotic cytokine TGF-β1 [9,10]. Zoja et al. [11] found that renal injury in passive Heymann nephritis could be ameliorated by an ACE-inhibitor and AT1-antagonist. Further adding a statin to that treatment significantly improved proteinuria, inflammatory and fibrotic processes. Abbate and Remuzzi [12] argue that these numerous beneficial effects of statins in different experimental settings make them an interesting target for evaluation of their nephroprotective potential in the COL4A3 knockout model of renal fibrosis.

Methods

Mice

Heterozygous COL4A3 knockout mice (Jackson Immunoresearch Laboratories, Westgrove, PA, USA) were crossbred on a SvJ/129 background. Treatment protocols for the mice were previously approved by local German authorities and supervised by veterinarians. The genotype of the mice was determined by PCR as described previously [5]. To exclude artefacts, PCR was repeated in all homozygous mice. In addition, tail biopsies and PCR were repeated in all homozygous animals living longer than 84 days. No animals died from infection or obvious side effects. Col4A3 –/– mice were either sacrificed after 32, 42, 52 or 66 days, eight wild-type mice were sacrificed at 52 days of age, four wild-type mice at 66 days of age. A subgroup of mice in each group was used to monitor lifespan (no treatment group n = 20; early treatment group n = 8; late treatment group, n = 8).

Administration of cerivastatin

Cerivastatin (Bayer, Leverkusen, Germany) was mixed to standard western diet (20 mg/kg body weight following recommendations by the manufacturer). A total of 76 COL4A3 –/– animals were divided into different treatment groups:

(i) untreated animals (n = 36)
(ii) early cerivastatin treatment, starting at day 28 (n = 24)
(iii) late cerivastatin treatment, starting at day 49 (n = 16).

Statistics

Data were analysed by log rank statistic (survival analysis) and one-way analysis of variance (ANOVA). All results are given as mean ± SD.

Urinary protein analysis, serum cholesterol and urea

To obtain urine samples, mice were placed in metabolic cages. Proteinuria was measured using a gradient polyacrylamide gel, stained with Coomassie Blue and analysed by densitometry, a semiquantitative method described previously [5]. Serum for measurements of urea and cholesterol were taken, using heparin-covered capillaries on a Hitachi 917 automatic analyser (Boehringer Mannheim, Germany).

Light and electron microscopy

Mice were sacrificed, transcardially perfused with a solution of paraformaldehyde and glutaraldehyde and kidneys were immersion-fixed as described previously [5]. Semithin and thin sections were taken on a Reichert Ultratcut UCT ultramicrotome. A Zeiss Axioshot and a Zeiss EM 902 microscope (Göttingen, Germany) were used for histological documentation.

Immunohistochemistry

For paraffin embedding, fresh kidneys were fixed in 4% paraformaldehyde in 0.1 mol/l phosphate-buffered saline (PBS) and vacuum-embedded in a Shandon Citadel 1000 (Pittsburg, PA, USA). Sections of 4-μm were cut on a Micron HM355S (Walldorf, Germany). Sections were rehydrated in graded alcohol, treated with 1% Trypsin for 7 min and blocked with 5% bovine serum albumin (BSA) at room temperature. The primary antibodies were allowed to incubate overnight at 4°C (rabbit-anti-mouse EHS-laminin and rabbit-anti-mouse fibronectin, 1:1000, gift from M. Paulsson, Cologne, Germany). Goat anti-rabbit Cy3 (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) served as secondary antibody. POX-conjugated anti-α-smooth muscle actin (α-SMA) (DAKO, Hamburg, Germany) was used to stain activated fibroblasts. Staining of CD3-positive T-cells and F4/80-positive monocytes/macrophages (rat anti-CD3, 1:100, clone CD3-12, Serotec, Raleigh, NC and rat anti-F4/80, 1:25, clone A3-1, Serotec, Raleigh, NC) was performed on paraffin-embedded material as previously described [13]. Sections were analysed on a Zeiss Axioshot microscope (Göttingen, Germany). Immunohistochemistry was repeated three times.

Immunoblotting

Three to four kidneys from different animals were pooled to minimize individual differences. Protein was extracted in a Tris-buffered saline (TBS) solution, using protease inhibitors phenylmethylsulfonyl-fluoride (PMSF) and n-ethylmaleimide (NEM). Aliquots with 30 μg protein as shown by BCA protein assay were dissolved in sodium dodecy/sulphate (SDS)-sample buffer, separated by electrophoresis on a 4–12% NuPage Novex Bis-Tris Gel (Invitrogen, Carlsbad, USA), transferred to a polyvinyliden-fluoride-membrane, and blocked with 5% BSA and milk powder. Mouse anti-TGFβ1 (1:100, R&D Systems, Minneapolis, USA) and rabbit anti-CTGF (1:5000, Abcam Limited, Cambridge, UK) were incubated for 60 min. The membrane was then incubated with the secondary antibody conjugated with horseradish potash (HRP) (Dako, Hamburg, Germany). The blot was developed using
chemoluminescence. Protein expression was analysed by densitometry using a gel-pro analyser software (Media Cybernetics, USA). Immunoblots were repeated three times.

**Results**

**Treatment with cerivastatin extends lifetime of COL4A3 knockout mice**

The lifespan of untreated COL4A3 /− /− animals was 71.3 days (mean; SD ±5.8). Homozygous knockout mice to which cerivastatin was given pre-emptively from day 28 on (early treatment) lived 91.3 days (±9.4) which was an increase of 28.0% over untreated COL4A3 /− /− mice (P < 0.01). Mice treated with cerivastatin from day 49 on (late treatment) lived 90.5 days (±6.2), an increase of 26.9% over untreated COL4A3 /− /− mice (P < 0.01). Differences of the increased lifespan between the two treatment groups were not significant (Figure 1).

**Cerivastatin reduces cholesterol levels and proteinuria and delays uraemia**

Cholesterol levels were elevated in COL4A3 knockouts when compared with wild-type mice, 52 days of age (131.1 ± 15.7 vs 96.8 ± 12.1 mmol/l, P < 0.05). Treatment with cerivastatin reduced cholesterol levels in the knockouts almost to untreated wild-type level (99.6 ± 17.2 mmol/l, P < 0.05). However it should be noted that wild-type mice treated with cerivastatin had a slight reduction in cholesterol levels (90.9 ± 10.7 mmol/l). At the age of 66 days, cholesterol in untreated knockouts was 140.8 ± 19.3 mmol/l, cholesterol in the knockouts treated with cerivastatin was reduced to 112.8 ± 12.7 mmol/l, P < 0.05 (Figure 2A). Cholesterol in untreated and treated wild-type animals had also increased (105.5 ± 14.4 and 103.5 ± 16.0 mmol/l, respectively).

Proteinuria developed at the age of 42 days in untreated knockout mice and increased from 1 g/l to 4.5 g/l at the age of 52 days, mirroring the beginning of morphological changes in the kidneys of COL4A3 /− /− mice. By 66 days of age, just before death, proteinuria was 12 g/l in untreated animals. Early as well as late treatment with cerivastatin had an anti-proteinuric effect (Figure 2B). In the animals treated pre-emptively, urinary protein was 2.5 g/l at 52 days (vs 4.5 g/l in untreated animals, P < 0.05) and 5.5 g/l at 66 days of age (vs 12 g/l in untreated animals, P < 0.05). In animals receiving late treatment, an anti-proteinuric effect could be seen by day 66 (7 g/l vs 12 g/l in untreated animals, P < 0.05). The difference between early and late treatment was not significant.

Blood urea began to rise after 52 days in COL4A3 /− /− mice. However at this age, urea had increased only mildly when compared with wild-type animals. No significant differences could be seen between the two treatment groups.
treated and untreated animals (52.5 ± 1.6 mg/dl in untreated COL4A3 knockouts, 50.8 ± 8.4 mg/dl in the early treatment group; 39.6 ± 4.9 mg/dl in wild-type mice). By 66 days of age, blood urea had increased to 235.4 ± 21.8 mg/dl in the untreated animals. Mice treated with cerivastatin had significantly lower serum urea concentrations (163.6 ± 45.7 mg/dl in the early treatment group, 151 ± 37 mg/dl in the late treatment group, \( P < 0.05 \)). No significant differences could be seen between early and late treatment (Figure 2C).

**Deposition of extracellular matrix molecules is reduced in mice treated with cerivastatin**

In 52-day-old knockout animals, light microscopy showed glomerular hypercellularity and thickening of the Bowman’s capsule as morphological correlates with the beginning of proteinuria and disturbed renal function. By 66 days, severe glomerulosclerosis was seen in most of the glomeruli. Fibrosis and tubular atrophy dominated in the interstitium. These morphological changes were less pronounced in mice treated with cerivastatin, as intact glomerular architecture and less interstitial fibrosis could be seen (Figure 3).

At day 66, glomeruli of 12 different kidney sections of three different animals in each group were evaluated for glomerulosclerosis by a blinded observer. Glomerulosclerosis was defined as loss of more than 50% of glomerular lumen due to extracellular matrix accumulation. One out of 80 (1.3%) glomeruli of healthy controls showed sclerosis, whereas 76 out of 92 (83.0%) did in untreated COL4A3 −/− knockouts. In contrast, only 26 out of 82 (31.7%) of glomeruli in the early treatment group and 35 out of 87 (40.2%) of glomeruli in the late treatment group showed sclerosis (\( P < 0.05 \) early/late treatment vs untreated). Tubulointerstitial fibrosis was evaluated in a similar matter by grading 12 different kidney sections of three different animals per experimental group (total of 36 sections) into zero, 1+ and 2+ accumulation of extracellular matrix by a blinded observer. Healthy controls showed no accumulation of extracellular matrix in any section; untreated COL4A3 −/− showed an average matrix score of 1.78. Early cerivastatin treatment reduced the matrix score to 1.02, in the late treatment group, the matrix score was 1.11 (\( P < 0.05 \) early/late treatment vs untreated).

Electron microscopy showed characteristic thickening and splitting of the GBM in COL4A3 knockouts...
irrespective of treatment. There was complete effacement of podocyte foot processes at day 66 in untreated animals (Figure 3), these changes were less severe in treated animals.

These findings were confirmed in immunofluorescence analysis (Figure 4). Fibronectin staining was used as an indicator of fibrosis. Staining was markedly less pronounced in sections from animals treated with cerivastatin, not very dissimilar to wild-type animals which showed only scant fibronectin. However, kidney sections from untreated COL4A3−/− mice showed a distinct fibronectin staining within the glomerulus and in the periglomerular matrix. Laminin was stained with a polyclonal antibody reacting with the three subunits of laminin 1, the α1, β1 and γ1 chain. Again, untreated COL4A3−/− mice showed marked laminin staining within the glomeruli, in the periglomerular region and interstitially, whereas wild-type animals displayed only a fine staining of the glomerular and tubular basement membranes. Compared with untreated COL4A3−/− mice, the periglomerular and interstitial signal was markedly reduced upon statin treatment (Figure 4).

Reduction of the profibrotic cytokines TGF-β1 and CTGF by cerivastatin

To elucidate the pathways by which cerivastatin acts on the deposition of matrix molecules and renal fibrosis, the profibrotic cytokine TGF-β1 and its downstream mediator CTGF were analysed by western blot. At the age of 66 days, TGF-β1 and CTGF were up-regulated in untreated knockouts compared with wild-type littermates. TGF-β1 signal was reduced to 35.7% (integrated optical density (IOD) 3.43) at day 66 in the knockouts receiving early cerivastatin treatment compared with untreated knockouts (IOD 9.61, \( P < 0.05 \)). CTGF was reduced to 56.1% (IOD 3.12) compared to untreated knockouts (IOD 5.56, \( P < 0.05 \)). Reduction of the profibrotic cytokines was not as pronounced when treatment is initiated at day 49; late treatment reduced TGF-β1 and CTGF expression to 78.6% (IOD 7.56) and 80.6% (IOD 4.48) (differences not significant), respectively (Figure 5).

Cerivastatin reduces the accumulation of macrophages, inflammatory cells and activated fibroblasts

To evaluate the role of inflammatory cell infiltration, macrophages were stained with an antibody to the F4/80 antigen, which is specifically expressed by macrophages.

Figure 6 shows accumulation of macrophages in the interstitium of 66-day-old homozygous COL4A3−/− mice. In kidneys of mice treated with cerivastatin the accumulation of macrophages was reduced, though there was still an increase in macrophage accumulation compared with wild-type animals.

Interstitial T-cell infiltration was evident in untreated COL4A3 knockouts by the age of 52 days, as shown by immunohistochemical staining against CD3. T-cells were seldom observed in wild-type as well as in pre-emptively treated animals. At the age of 66 days, interstitial T-cells were not as numerous as in kidneys of 52-day-old animals (Figure 6). However, no striking difference in T-cell infiltration between treated and untreated knockouts was noted, consistent with the observation that infiltration of inflammatory cells is one of the first steps in the pathogenesis of fibrosis. Interstitial myofibroblasts play a crucial role in the pathogenesis of fibrosis, as they contribute to matrix deposition into the tubulointerstitial space. Therefore, staining for α-SMA as a marker of activated fibroblasts was performed. The α-SMA-positive cells were abundant in the periglomerular region and in the interstitium of untreated 66-day-old COL4A3 knockouts. The number of activated cells appeared markedly reduced in animals treated with cerivastatin.

Fig. 4. Deposition of the extracellular matrix molecules, laminin and fibronectin, at 66 days of age. Upper row: immunohistochemical staining for laminin 1; (A) wild-type; (B) COL4A3−/−, early cerivastatin treatment; (C) untreated COL4A3−/−. The lower row shows immunohistochemistry for fibronectin; (D) wild-type; (E) COL4A3−/− early cerivastatin treatment; (F) untreated COL4A3−/−. Original magnification: 1:100.

Fig. 5. Western blot analysis of TGF-β1 and CTGF. Lanes 1–4 show TGF-β1 and CTGF expression in kidneys of 66-day-old animals: (1) COL4A3−/− late cerivastatin treatment, (2) untreated COL4A3−/−, (3) COL4A3−/− early cerivastatin treatment, (4) untreated wild-type. Lanes 5–7 represent kidneys at day 52 of life: (5) untreated COL4A3−/−, (6) COL4A3−/− early cerivastatin treatment, (7) untreated wild-type. IOD stands for integrated optical density.
as Figure 6 shows. There was only scarce staining of α-SMA-positive cells in wild-type animals.

Discussion

Statins are inhibitors of HMG-CoA-reductase and are potent drugs in the treatment of hypercholesterolaemia. However, apart from their cholesterol-lowering action, numerous pleiotropic effects have also been identified. It has been shown that statins exert anti-proliferative, anti-inflammatory and anti-fibrotic effects on a wide variety of tissues. There is growing evidence from in vitro and in vivo experiments suggesting beneficial effects of statins in progressive renal disease. Lovastatin inhibits proliferation of mesangial cells in cell culture and attenuates the expression of proinflammatory mediators [10]. Pravastatin and simvastatin diminish the accumulation of extracellular matrix molecules by mesangial cells [8]. Statins ameliorate renal injury in many models of renal disease such as 5/6 nephrectomy Sprague Dawley rats [14] and obese Zucker rats [15].

In this study, we examined the effects of cerivastatin on the development of renal disease in the COL4A3 knockout mouse model of human Alport syndrome.
The fully synthetic cerivastatin was chosen because of its lipophilicity. In vitro experiments suggest that hydrophilic statins such as pravastatin may be devoid of anti-proliferative effects, presumably due to their poor cell membrane permeability [16]. However, it is not yet clear whether lipophilicity is an important factor for the exhibition of pleiotropic effects.

The lifespan of COL4A3 knockout mice treated with cerivastatin was increased by 28% compared with untreated knockouts. Consistent with this, we found that serum urea at 66 days of life was significantly decreased in knockouts treated with cerivastatin. Light microscopy showed that fibrotic changes in the kidney were also much improved by cerivastatin treatment. Immunohistochemistry revealed a significantly marked increase in laminin and fibronectin staining during the course of disease; this was greatly reduced upon cerivastatin treatment. Cerivastatin also reduced proteinuria in COL4A3 /– mice. Interestingly, cerivastatin did not affect the structure of the GBM, which showed characteristic thickening and splitting. However, effacement of podocyte foot processes improved by cerivastatin treatment. As cerivastatin treatment had no major effect on restructuring the pathological GBM yet reduced fibrosis and proteinuria, we speculated that statins could interfere in the secondary events leading to fibrosis in Alport syndrome. Two important mechanisms have been identified in the COL4A3 model. TGF-β1 contributes to the focal thickening of the GBM and renal fibrosis. Antagonizing the effects of TGF-β1 ameliorates focal thickening of the GBM, but has no effect on podocyte foot process effacement in COL4A3 knockouts [17]. Cosgrove et al. [17] hypothesized that α1β1-integrin, the most abundant integrin on the surface of mesangial cells, may be responsible for foot process effacement by mediating the deposition of non-native laminin isoforms in the GBM. These isoforms, namely laminin 2 and 4, might mask binding of the podocytes to their preferred ligand, laminin 11, leading to loss of focal adhesion and finally to foot process effacement [17]. This group also demonstrated that inhibition of the TGF-β1 pathway by a TGF-β1 antibody attenuated interstitial fibrosis but did not extend lifetime, whereas α1-integrin/COL4A3 double knockouts lived about 70% longer and showed less GBM damage [17]. The inflammatory reaction preceding fibrosis might be important in this context.

In our study, the expression of TGFβ1 and its downstream mediator CTGF was markedly reduced. The expression of profibrotic cytokines was significantly reduced in the early treatment group compared with the late treatment group. Infiltration of CD3-positive T-cells, macrophages and activated fibroblasts was attenuated by cerivastatin treatment. Though one would expect that treatment initiation before onset of nephron damage should be more efficient, no difference was seen between the early and late treatment group. Only TGF-β1 was significantly reduced in the early treatment group compared with the late treatment group. However, early TGF-β1 reduction had no influence on lifespan, uraemia and proteinuria. Data of the Cholesterol and Recurrent Events (CARE) Trial suggest that treatment with pravastatin may delay loss of renal function in patients with chronic renal insufficiency, but only in an advanced stage of the disease [18]. The reasons for this phenomenon remain unknown. In the present study, the combination of several ‘nephroprotective’ effects of early and late cerivastatin treatment might have resulted in preserved renal architecture and prolonged lifespan: anti-proteinuric (Figure 2), anti-fibrotic (Figures 3–5) and anti-inflammatory effects (Figure 6).

How statins exert their pleiotropic effects in the kidney is not yet entirely clear. Animal studies have shown that hyperlipidaemia itself contributes to renal injury and progressive scarring, perhaps by mimicking some of the atherosclerotic mechanistic pathways. Most Alport patients develop nephrotic syndrome during adolescence including hypercholesterinaemia (unpublished data). This hypercholesterinaemia results from extensive protein loss and not from an inadequate food intake. Similar to other nephrotic renal diseases in children it is unclear if this hypercholesterinaemia on its own contributes to the severity of disease and need to be treated or not. Dietary hypercholesterolaemia leads to deposition of lipids in the glomerulus and tubulointerstitium and induces interstitial inflammation and fibrosis in rats. It has been shown that additional presence of glomerular disease aggravates nephron damage in this case [19]. In vitro experiments examining the potential mechanism of lipid-induced renal damage revealed a crucial role for low density lipoprotein, LDL. LDL activates mesangial cells and in turn becomes oxidized to cytotoxic oxLDL. LDL leads to increased production of matrix molecules and chemoattractant proteins [20]. Lipid reduction prevents glomerular injury in 5/6 nephrectomized rats [14]. However, a large body of evidence suggests that statins exert beneficial effects independent of their lipid lowering properties. Inhibition of HMG-CoA-reductase not only impairs cholesterol synthesis, but also the production of intermediates derived from the same metabolic pathway. Besides cholesterol, the mevalonate pathway leads to the formation of non-sterol isoprenoids that are involved in the lipid modification of a number of intracellular proteins, e.g. small G-proteins and proteins from the Rho, Ras, Rab and Rap family. This process, named isoprenylation, is necessary for their binding to the cell membrane. Thus, statins regulate the cellular signalling pathways involved in many processes within the cell [20]. Some of the statins’ pleiotropic effects are reversed by adding downstream metabolites in the cholesterol cascade such as mevalonate, geranylgeranyl- or farnesyl-pyrophosphate [7].

In conclusion, our data show that cerivastatin reduces proteinuria and delays uraemia and hence prolongs the lifespan of COL4A3 knockout mice. These effects are associated with decreased renal fibrosis and a reduction of inflammatory cell
infiltration. There was no significant difference in the course of disease between beginning treatment either early or late. The molecular mechanisms underlying the beneficial effects of cerivastatin in the COL4A3 knockout model still need further investigation. Both, reduction of cholesterol levels as well as direct anti-fibrotic and anti-inflammatory effects may contribute to the beneficial effect of cerivastatin observed in this model of fibrotic renal disease. However, our data support a new treatment option for children with Alport syndrome and, possibly, other fibrotic renal diseases. Further experiments should address, if the combination of statins with ACE-inhibitors could provide additive nephroprotective effects in Alport patients.

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