Effect of uremia on HDL composition, vascular inflammation, and atherosclerosis in wild-type mice

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Chronic renal failure increases the risk of cardiovascular disease (11, 12). Although often encountered in uremic patients, hypercholesterolemia, hypertension, and smoking cannot explain the increased risk (9). However, less firmly established risk factors such as increased plasma concentrations of triglyceride-rich apolipoprotein B-containing lipoproteins (1, 18) (with normal total plasma cholesterol concentrations) and inflammation (18) may play a role. In addition, the maturation of HDL seemingly is a defect implying that uremia could impair the otherwise cardioprotective effect of HDL (31).

Moderate uremia (induced by 5/6 nephrectomy) leads to markedly increased atherosclerosis in hypercholesterolemic apolipoprotein E (apoE)-deficient mice (3–7, 15, 20). This effect cannot be ascribed to changes in the total plasma cholesterol level (3). Nevertheless, in apoE-deficient mice uremia confers increases in the expression of inflammatory genes in the arterial wall (5, 6), suggesting that inflammation could play a role. Interestingly, systemic inflammation has been suggested to convert HDL from a protective to a harmful lipoprotein with proatherogenic effects (30). This effect has been suggested to be related to the increase in serum amyloid A (SAA) plasma concentrations that occurs during inflammation (22, 30). SAA is an amphiphilic protein that can incorporate in HDL (10, 14). SAA-enriched HDL is believed to have proatherogenic effects, and SAA has been detected in atherosclerotic lesions where it colocalizes with apoA-I (25). It is unknown, however, whether SAA-enriched HDL may be formed during uremia and how it may affect development of atherosclerosis. The apoE-deficient mouse model may not be suited to address this issue, since their plasma lipoprotein composition is dominated by a vast accumulation of atherogenic apoB48-containing lipoproteins in plasma (34).

Mice normally have very low plasma levels of apoB-containing VLDL and LDL, with the major portion of the plasma cholesterol being in HDL. Nevertheless, the inbred C57BL/6J mouse strain is susceptible to development of fatty-streak-like atherosclerotic lesions when fed a plasma cholesterol-raising cholic acid-containing diet (24, 27), whereas lesion formation occurs but is minimal when the mice are fed a Western-type diet enriched with cholesterol and fat (29). Interestingly, dietary cholic acid raises plasma SAA concentrations (26), although the importance of this effect in relation to atherosclerosis is unclear (17, 28). Two previous studies have examined atherosclerosis in 5/6 nephrectomized C57BL/6J mice with moderate uremia. One study showed that uremic mice indeed can develop oil-red-O-stained lesions when fed a cholic acid-free, cholesterol- and fat-enriched diet (29). A more recent study noted that three of eight uremic wild-type mice had aortic lesions 12 wk after 5/6 nephrectomy when fed a low-cholesterol, low-fat diet (7).

In this study, we sought to examine to what extent moderate uremia might affect plasma lipoproteins, vascular inflammation, and development of atherosclerosis in C57BL/6J mice. For this purpose, we examined plasma lipids and lipoprotein profiles, SAA, hepatic expression of genes involved in synthesis of apoB- and apoA-I containing lipoproteins, and aortic expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) mRNA, as well as nitrotyrosine staining and lesion formation in aortas of uremic and control mice fed a cholesterol- and fat-enriched diet and in mice fed a cholic acid-containing diet.

MATERIALS AND METHODS

Animals. C57BL/6J wild-type mice (Taconic M&B Laboratory Animals and Services for Research, Ry, Denmark) were housed in a...
temperature-controlled facility (21–23°C) with a 12:12-h light-dark cycle. The mice were allowed free access to food and water. The experiments were performed according to the principles stated in the Danish law on animal experiments and were approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark. Before induction of uremia, the mice were fed a standard mouse chow (Altromin 1314, Altromin, Lage, Germany). After induction of uremia or sham operation, mice were switched to a high-cholesterol/high-fat diet (C13002, Research Diets, New Brunswick, NJ) with or without sodium cholic acid (0.5% wt/wt) produced by mixing Purina mouse chow 5015 (75 g); casein, 80 mesh (75 g); dextrose (25 g); maltodextrin (42 g); sucrose (16.25 g); cellulose BW200 (12.5 g); cocoa butter (75 g); mineral mix S10001 (8.75 g); vitamin mix V10001 (2.5 g); choline bitartrate (1.25 g); and cholesterol USP (12.5 g).

Moderate uremia was induced by a two-step surgical procedure (3). Briefly, the upper and lower poles of the right kidney were resected in 10-wk-old mice. Two weeks later, the left kidney was removed. Control mice underwent sham operations at both 10 and 12 wk of age. Anesthesia was achieved with a mixture (ratio 3:0.2:4) of fentanyl (0.050 mg/kg), midazolam (5.0 mg/ml), and droperidol (2.5 mg/ml, 20 µg/g body wt sc), and buprenorphine 0.3 mg/ml (0.1 g/g body wt sc twice daily for 3 days) was used as an analgesic after the surgical procedures. In mice on the C57BL/6J background, 5/6 NX does affect blood pressure and as such does not inflict insufficient adrenal function (3). NX and sham-operated mice were allocated to a cholesterol- and fat-enriched diet either with or without cholic acid. The NX mice fed the cholic acid-containing diet did not thrive and were killed before the end of the study. The cause of the poor compliance to the cholic acid-containing diet is unknown. At the time of death, several of the mice had grossly enlarged livers, gallstones, and elevated plasma liver enzyme concentrations.

### Plasma biochemistry

Blood from the retroorbital venous plexus was collected in heparinized microtubes (Capjet, Terumo Medical, Elkton, MD) and centrifuged at 2,000 g for 10 min at 4°C. Plasma was stored at −80°C. Plasma sodium, potassium, chloride, creatinine, total calcium, phosphate, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, albumin, and bilirubin concentrations were measured with an Modular P Hitachi Automatic Analyzer (Roche, Hvidovre, Denmark) using reagents from Roche. Plasma urea was measured with a Modular P Hitachi Automatic Analyzer (Roche, Hvidovre, Denmark) using reagents from Roche. Plasma cholesterol, triglycerides, and phospholipids were measured by an enzymatic colorimetric method (Wako Chemicals, Neuss, Germany) for semiquantitative analyses of plasma apoA-I, we loaded 0.16 µl plasma/fane. Analyses of plasma dilutions with loadings of 0.0625, 0.125, and 0.25 µl showed proportionality between band intensities and amounts of loaded apoA-I.

### mRNA quantification

After homogenization of mouse liver biopsies with a Tissuelyser (Qiagen Retsch, Hann, Germany), total RNA was isolated with TRIzol (Invitrogen). Aortic RNA was made as previously described (5). RNA concentrations were assessed from the absorbance at 260 nm, and RNA integrity was ensured with RNA Nano LabChip (Agilent Technologies, Narum, Denmark). cDNA was made from 1 µg of total RNA with M-MULV reverse transcriptase (20 U, Roche) and random hexamer primers in 10-µl reactions at 37°C for 60 min. Real-time PCR with a LightCycler (Roche) was used to determine the mRNA expression of ICAM-1, VCAM-1, hypoxanthine phosphoribosyl transferase (HPRT), β-actin, microsomal triglyceride transfer protein (MTP), apoB, apoA-I, and GAPDH. The forward and reverse primers were 5′-GGGGCCTGTCGTCTGTT-3′ (apoA-I-51) and 5′-ACGGTTGAACCCAGAGTGTC-3′ (apoA-I-31), respectively, for apoA-I and 5′-TGCGCGCTCATCT-TACGCTTT-3′ (m-HPRT-31) and 5′-AACGTGGCTGTGAAAGGA-3′ (m-HPRT-51), respectively, for HPRT. Other primers have been described previously (2, 6, 21, 23). Each reaction mixture (20 µl) contained cDNA synthesized from 20 ng total RNA, 2.0–3.5 mM MgCl2, 10 pmol of each primer, 2 µl of LightCycler Faststart DNA Master SYBR Green 1 mix (Roche), and PCR-grade water. Standard curves were made by serial dilutions of pools of mouse liver or aortic mRNA. All mRNA were quantified in duplicate in separate runs. Agarose gel electrophoresis and DNA sequencing confirmed the specificity of the individual PCR reactions.

In the livers, the crude expression of β-actin was lower in NX mice and cholic acid-fed mice than in sham-operated control (data not shown), whereas the crude expression of GAPDH was similar in the three groups. Hence GAPDH expression was used to normalize hepatic gene expression. In aortas, the expression of β-actin, GAPDH, and HPRT was similar in NX and sham-operated mice, and the average expression of the three genes was used to normalize aortic gene expression. Importantly, the overall results and conclusions were similar whether the results were analyzed with or without normalization with housekeeping gene expression.

### Evaluation of aortic pathology and quantification of atherosclerosis

To study aortic atherosclerosis, mice were anesthetized and the aortic aortas were collected. The aortic root and aortic arch were sectioned and fixed in 10% buffered formaldehyde for 48 h, then dehydrated, cleared, and embedded in methacylate. The aortic arch and aortic root were sectioned in the transverse plane with a low-speed saw (Isomet 2000, Buehler). The sections were examined at a magnification of ×200 with a Zeiss Axioskop microscope fitted with a color video camera (Nikon, Tokyo, Japan) and analyzed with Image Pro-Plus software (Media Cybernetics). The total area of atherosclerotic plaques was calculated by following the periphery of the lesions. In aortas, the expression of aortic gene expression. In aortas, the expression of aortic gene expression. In aortas, the expression of aortic gene expression. In aortas, the expression of aortic gene expression.
vol, pH 7.0, Sigma-Aldrich, Vallensbak Strand, Denmark) and reembedded in OCT compound. Serial 10-μm-thick sections of the aortic sinus were cut in a cryostat. With the appearance of the first aortic valve, sections were collected on SuperFrost slides (Menzel-Glaser) precoated with 1% gelatin (Sigma). When all three valves were visible, 15 sections were collected on 3 slides with 5 sections on each. Two of the slides with sections of the best quality were stained with oil red O (Sigma) and hematoxylin (Sigma). Digital images were collected from six random sections at ×4.0 magnification using a Nikon eclipse TE300 light microscope, a Nikon DXM 1200 digital camera, and the software control manager ACT-1. The oil red O-stained areas in the intima and inner media were measured with Leica IM50 Image Manager, version 4.0.

Table 1. Effect of NX and a cholic acid-containing diet on plasma biochemical parameters related to liver function

<table>
<thead>
<tr>
<th></th>
<th>NX (n = 7)</th>
<th>Cholic Acid (n = 7)</th>
<th>Sham (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Creatinine, μmol/l</strong></td>
<td>28±3.5†</td>
<td>12±0.7</td>
<td>10±0.5</td>
</tr>
<tr>
<td>Total calcium, mmol/l</td>
<td>2.59±0.06‡</td>
<td>2.45±0.05†</td>
<td>2.26±0.03</td>
</tr>
<tr>
<td>Phosphate, mmol/l</td>
<td>2.18±0.31</td>
<td>1.73±0.08*</td>
<td>2.13±0.10</td>
</tr>
<tr>
<td>Ca × phosphate, mmol²</td>
<td>5.73±0.98</td>
<td>4.24±0.20</td>
<td>4.83±0.26</td>
</tr>
<tr>
<td>Sodium, mmol/l</td>
<td>159±3.2</td>
<td>160±5.1</td>
<td>155±2.2</td>
</tr>
<tr>
<td>Potassium, mmol/l</td>
<td>6.98±0.31‡</td>
<td>5.86±0.51</td>
<td>5.18±0.15</td>
</tr>
<tr>
<td>Chloride, mmol/l</td>
<td>109±2.1</td>
<td>107±4.9</td>
<td>108±1.3</td>
</tr>
<tr>
<td>Body weight, % baseline</td>
<td>120±5.5‡</td>
<td>115±4.4</td>
<td>160±6</td>
</tr>
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</table>

Values are means ± SE 26 wk after NX or sham operation. †P < 0.05, ‡P < 0.01, and §P < 0.001 compared with sham mice.

Fig. 1. Effect of % nephrectomy (NX) and a cholic acid-containing diet on plasma urea in C57BL/6J mice. NX mice (n = 7); sham mice (n = 11); cholic acid-fed mice (n = 7). Values are means ± SE. ***P < 0.01 and ***P < 0.001 compared with sham mice.

Fig. 2. Effect of NX and a cholic acid-containing diet on plasma markers of kidney and liver function. Plasma urea was increased 2.5- to 4.5-fold by NX and was unaffected by the cholic acid-containing diet (Fig. 1). Also, the plasma potassium, creatinine, and calcium concentrations were increased in sections dehydrated through a series of alcohol solutions, and a coverglass was mounted with Pertex (HistorLab Products, Göteborg, Sweden). Sections incubated with rabbit immunoglobulins (Negative Control Rabbit Immunoglobulin Fraction, Normal, Dako) in the same immunoglobulin concentration as the primary antibodies were included as negative controls.

Statistical analyses. Statistical calculations were performed using GraphPad Prism, version 4.03 for Windows. Results were analyzed by Student’s unpaired t-tests with Welch’s correction when appropriate except for plasma SAA values, where the Mann-Whitney U-test was used. Data are presented as means ± SE, with n indicating the number of mice studied.

RESULTS

Effect of NX and a cholic acid-containing diet on plasma markers of kidney and liver function. Plasma urea was increased 2.5- to 4.5-fold by NX and was unaffected by the cholic acid-containing diet (Fig. 1). Also, the plasma potassium, creatinine, and calcium concentrations were increased in Table 2. Effect of NX and a cholic acid-containing diet on plasma biochemical parameters related to liver function

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<tr>
<td><strong>Alanine aminotransferase, U/l</strong></td>
<td>27±5†</td>
<td>362±61†</td>
<td>68±11</td>
</tr>
<tr>
<td><strong>Alkaline phosphatase, U/l</strong></td>
<td>145±9‡</td>
<td>183±24†</td>
<td>81±6</td>
</tr>
<tr>
<td><strong>Lactate dehydrogenase, U/l</strong></td>
<td>423±88</td>
<td>885±67‡</td>
<td>232±15</td>
</tr>
<tr>
<td><strong>Albumin, g/l</strong></td>
<td>30±1*</td>
<td>30±2</td>
<td>33±1</td>
</tr>
<tr>
<td><strong>Bilirubin, μg/l</strong></td>
<td>4.8±0.4</td>
<td>16.2±7.6</td>
<td>4.1±0.1</td>
</tr>
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Values are means ± SE 26 wk after NX or sham operation. *P < 0.05, †P < 0.01, and ‡P < 0.001 vs. sham.
the NX compared with the sham group, whereas the cholic acid-containing diet increased plasma calcium and decreased plasma phosphate (Table 1). NX did not affect plasma sodium and chloride concentrations (Table 1). Both NX and the cholic acid-fed mice gained less weight than the sham-operated controls (Table 1). Plasma alkaline phosphatase and lactate dehydrogenase were both increased 1.8-fold, whereas plasma alanine aminotransferase and albumin were decreased by 60 and 10%, respectively, in the NX group compared with the sham group (Table 2). The cholic acid-containing diet increased plasma alkaline phosphatase, lactate dehydrogenase, and alanine aminotransferase 2.3-, 3.8-, and 5.3-fold, respectively, compared with the sham group, but did not affect plasma bilirubin or albumin (Table 2).

Effect of NX on HDL composition and serum amyloid A. To analyze the impact of NX on HDL lipid composition, we isolated HDL from NX and sham mice by sequential ultracentrifugation. The relative cholesterol content of HDL was significantly reduced in NX compared with sham mice, whereas the triglyceride content was increased and the phospholipid and total protein contents were unaffected (Table 3). The average

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<td>Triglyceride, mmol/l</td>
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<td>Cholesterol, mmol/l</td>
<td>0.84±0.06 (15.0±0.8*)</td>
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<td>Protein, mg/l</td>
<td>1.10±0.07 (51.1±2.2)</td>
<td>1.03±0.07 (50.7±2.71)</td>
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Values are means ± SE. Three pools of HDL each from 3–8 mice were isolated from plasma from NX or sham mice with sequential ultracentrifugation. Data are plasma HDL lipid and protein concentrations assuming that the recovery of HDL during purification by ultracentrifugation was 100%. Numbers in parentheses are percentages of total HDL mass. Phospholipid, triglyceride, and cholesterol masses were calculated using molecular masses of 773.2, 885.0, and 386.6 g/mol, respectively. *P ≤ 0.05 vs. sham.

Effect of NX and a cholic acid-containing diet on plasma lipoproteins and hepatic expression of MTP and apoB mRNA. NX did not affect the total plasma cholesterol concentration, whereas it was increased by the cholic acid-containing diet (Fig. 2A). On gel filtration analyses of pooled plasma from 4, 13, 19, and 26 wk after NX, there was a consistent, albeit small, elevation of VLDL/LDL cholesterol and a corresponding decrease in HDL cholesterol in the NX group compared with the sham group (Fig. 2B). The cholic acid-containing diet caused a massive increase in VLDL/LDL cholesterol (Fig. 2B).

The hepatic production of VLDL is affected by the mRNA expression of both MTP and apoB (2, 23). The expression of liver MTP mRNA was reduced both by NX and by the cholic acid-containing diet (Fig. 3A), whereas the expression of apoB mRNA was decreased by the cholic acid-containing diet but not by NX (Fig. 3B).

Effect of NX on HDL composition and serum amyloid A. To analyze the impact of NX on HDL lipid composition, we isolated HDL from NX and sham mice by sequential ultracentrifugation. The relative cholesterol content of HDL was significantly reduced in NX compared with sham mice, whereas the triglyceride content was increased and the phospholipid and total protein contents were unaffected (Table 3). The average

![Image](https://via.placeholder.com/150)

Table 3. Lipid composition of HDL from NX and sham mice

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plasma apoA-I concentration and hepatic apoA-I mRNA expression did not differ in a statistically significant manner between NX and sham mice (data not shown).

The mean plasma SAA concentration increased markedly in the NX compared with the sham-operated mice (Fig. 4A). The cholic acid-containing diet also increased plasma SAA but to a lesser extent than NX (Fig. 4A). On gel filtration and Western blotting analyses of plasma from NX mice, SAA and apoA-I were associated with HDL-sized lipoproteins (Fig. 4B). It was striking that the magnitude of the increase in plasma SAA concentrations above the average value in sham-operated mice varied greatly between NX mice (−0- to 53-fold). The cause of this variation is unknown. The mice were kept in a clean facility with frequent testing excluding infections, none of the mice had signs of wound infection after surgery and the rise in SAA was not associated with the extent of uremia (judged from plasma urea concentrations). To examine whether an increase in plasma SAA was associated with a decrease in plasma apoA-I, we compared the plasma apoA-I concentration in individual NX mice with the plasma SAA concentration. NX mice with high-plasma SAA had low apoA-I concentrations (Fig. 4C). However, the hepatic apoA-I mRNA expression was not decreased in parallel with the increase in SAA (Fig. 4C).

**Effect of NX on aortic expression of adhesion molecules.** Vascular inflammation is a hallmark of the accelerated atherosclerosis occurring in hypercholesterolemic, uremic, apoE-deficient mice (5, 6). To examine whether moderate uremia

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**Fig. 5.** Effect of NX on aortic expression of ICAM-1 and VCAM-1 mRNA and plasma sICAM-1 and sVCAM-1 in C57BL/6J mice. A: aortic expression of ICAM-1 and VCAM-1 mRNA [normalized with hypoxanthine phosphoribosyl transferase (HPRT), β-actin, and GAPDH as described in MATERIALS AND METHODS] 12 wk after NX. The aortas were harvested from NX and sham-operated control mice used in a previous study (5). Filled bars, NX mice (n = 18); open bars, sham mice (n = 11). B: plasma sICAM-1 and sVCAM-1 concentrations 26 wk after NX. Filled bars, NX mice (n = 7); hatched bars, cholic acid-fed mice (n = 6); open bars, sham mice (n = 11). Values are means ± SE. Brackets and P values show results of 2-group comparisons.

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**Fig. 6.** Effect of NX and a cholic acid-containing diet on aortic atherosclerosis and nitrotyrosine staining in C57BL/6J mice. A: area of oil red O-stained lesions 26 wk after NX. Filled bars, NX mice (n = 7); hatched bars, cholic acid-fed mice (n = 6); open bars, sham mice (n = 11). Values are means ± SE. Brackets and P values show results of 2-group comparisons. B: immunohistochemical nitrotyrosine staining (brown) and negative control staining (insets) of aortas from a C57BL/6J NX mouse (B), a C57BL/6J sham-operated control mouse (C), and an apoE-deficient NX mouse with atherosclerosis (D; the aorta was harvested 36 wk after NX and also used in a another study) (4). Note the uniform staining of nitrotyrosine in the media of NX and sham C57BL/6J mice as opposed to the strong nitrotyrosine staining of the foam cell intimal lesion in the apoE-deficient NX mouse.
Expression is decreased in uremic rats (32), the hepatic apoA-I concentrations. Despite that the hepatic apoA-I gene expression was not reduced in the NX mice. This suggests that chronic inflammation in mice with moderate uremia confers a decrease in plasma apoA-I, which is not necessarily due to lowering of the hepatic apoA-I gene expression. HDL from uremic mice also contained more triglycerides and less cholesterol per microgram protein than control HDL. These changes in HDL lipid composition are similar to those described after induction of acute inflammation with an injection of croton oil in rabbits or with LPS in baboons (8). Nevertheless, in mice LPS-induced acute inflammation does not change the lipid composition of HDL (despite increasing plasma SAA), suggesting that, at least in mice, chronic inflammation associated with uremia affects HDL differently than acute inflammation perhaps due to reduced hepatic lipase activity (16) and/or plasma LCAT activity (33). Interestingly, stable isotope turnover studies support the notion that triglyceride-enrichment of HDL increases its catabolic rate (19).

NX dramatically accelerates the development of atherosclerosis in the aortas of apoE-deficient mice, which have elevated plasma VLDL/LDL cholesterol (3). In apoE-deficient mice, NX also increases apoE expression of the genes encoding ICAM-1 and VCAM-1 as well as plasma concentrations of sICAM-1 and sVCAM-1 (4, 6). The increase in ICAM-1 expression (and perhaps also VCAM-1) precedes lesion formation in NX apoE-deficient mice, suggesting that vascular inflammation may be an important precipitating factor in uremic atherosclerosis. In the present study, neither the aortic expression of VCAM-1 and ICAM-1 mRNA nor the plasma concentrations of sICAM-1 and sVCAM-1 were increased in NX wild-type mice. This supports the idea that vascular inflammation in uremic mouse to a large extent is dependent on concomitant hypercholesterolemia. Similarly, when aortic oxidative stress was examined with nitrotyrosine staining, we saw no difference between NX and sham-operated control mice. Of note, in NX apoE-deficient mouse aortas nitrotyrosine staining was predominantly within the intimal lesions. These results further suggest that the proatherogenic effect of uremia in mouse arteries relies on hypercholesterolemia. Accordingly, there was no difference in atherosclerotic lesion areas in the aortic arch between wild-type NX and sham-operated control mice, and the average lesion area in NX mice was less than in the cholic-acid fed mice. Thus the small increase in VLDL/LDL cholesterol and the proinflammatory changes in HDL composition were insufficient to induce atherosclerosis in NX C57BL/6J mice. Notably, this result does not exclude that formation of proinflammatory HDL contributes to accelerated lesion formation in the setting of more markedly elevated VLDL/LDL cholesterol concentrations such as in apoE-deficient mice and possibly humans.

**ACKNOWLEDGMENTS**

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REFERENCES


