

# Dyslipoproteinemia in an inbred rat strain with spontaneous chronic progressive nephrotic syndrome

P. Tarugi,\* S. Nicolini,\* L. Albertazzi,\* S. Calandra,\* P. Salvati,† C. Ferti,† and C. Patrono\*\*

Istituto di Patologia Generale,\* Università di Modena, Modena, Italy; Farmitalia Carlo Erba,† Istituto Ricerche, Nerviano (Milano) Italy; and Farmacologia,\*\* Università degli Studi G. D'Annunzio, Chieti, Italy

**Abstract** Rats of the Milan Normotensive Strain (MNS) develop a dyslipoproteinemia that is associated with a spontaneous, age-dependent and slowly progressive nephropathy characterized by proteinuria and hypoalbuminemia (nephrotic syndrome). We assumed that the MNS strain might be a suitable model for studying the features of nephrotic dyslipoproteinemia and its relationship with proteinuria, hypoalbuminemia, and hepatic apolipoprotein production. Plasma lipoproteins were investigated in MNS rats at various ages (4–48 weeks) and in another rat strain (Milan Hypertensive Strain, MHS), genetically related to MNS but free of nephropathy, that was used as control. In MNS rats, abnormal proteinuria was detectable at 20 weeks and increased 2-fold up to 34 weeks with no reduction of plasma albumin (compensated stage). During this stage we found increased levels of plasma cholesterol (+34%), high density lipoprotein-1 (HDL<sub>1</sub>) (+73%), and HDL<sub>2</sub> (+31%) that were positively correlated with proteinuria but not with plasma albumin. The later stage (34–48 weeks) (nephrotic stage) was characterized by a further increase of proteinuria, moderate hypoalbuminemia (–25%), a 2-fold increase of plasma cholesterol, triacylglycerols, low density lipoprotein (LDL), and HDL<sub>1</sub>, and a 1.2-fold increase of HDL<sub>2</sub>. In this stage the levels of LDL, HDL<sub>1</sub>, and HDL<sub>2</sub> were positively correlated with proteinuria and negatively correlated with plasma albumin. The most striking change in apolipoproteins was a progressive increase of the relative content of apoA-I in HDL (in 48-week-old MNS rats the A-I/E ratio was 3-fold that found in MHS rats) that was associated with a similar increase of plasma apoA-I. None of these lipoprotein changes were observed in age-matched MHS rats. At the end of the compensated stage, the hepatic levels of A-I, B, A-II, and albumin mRNA were 5.3-, 3.5-, 1.3-, and 2.0-fold, respectively, those found in age-matched MHS rats. During the nephrotic stage, albumin mRNA continued to increase, whereas A-I, B, and A-II mRNAs decreased toward the levels found in age-matched MHS rats. Thus, nephrotic dyslipoproteinemia in MNS rats starts to develop in the compensated stage before the onset of hypoalbuminemia, is characterized by an early elevation of HDL<sub>1</sub> + HDL<sub>2</sub>, and is associated with an increased content of hepatic mRNAs of some apolipoproteins, especially apoA-I. The slow progression of nephrotic syndrome with the long-standing proteinuria and no reduction in plasma albumin renders the MNS strain the most suitable animal model for the study of the effect of proteinuria

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**Supplementary key words** proteinuria • plasma albumin • plasma lipoproteins • apolipoprotein gene expression

The nephrotic syndrome is classically defined as a clinical entity of diverse etiology that is characterized by the triad: proteinuria, hypoalbuminemia, and edema (1). These manifestations are usually associated with a complex disorder of plasma lipoproteins (nephrotic dyslipoproteinemia) (2–8). A variable elevation of apoB-containing lipoproteins (VLDL, IDL, and LDL) and a normal or a decreased level of HDL are frequently reported in human nephrotic syndrome (2–8). It is still uncertain whether this lipoprotein disorder is caused by an increased production, an altered catabolism of some lipoprotein species, or both (9–13).

Dyslipoproteinemia is also the hallmark of the experimental nephrotic syndrome in the rat (14–18). In the rat acute or chronic forms of nephrotic syndrome develop after the administration of drugs such as adriamycin (AD) or puromycin aminonucleoside (PAN). The acute form is characterized by a massive proteinuria, a severe hypoalbuminemia, and a rapid elevation of all lipoprotein classes (14, 15). It has been reported that an increased production of lipoproteins by the liver plays a major role in this lipoprotein disorder (19–25) even though defects in

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; MNS, Milan Normotensive Strain; MHS, Milan Hypertensive Strain; PAN, puromycin aminonucleoside.

catabolism of certain lipoproteins may also occur, especially in the most severe forms of nephrotic syndrome (26). Unfortunately, the rapid progression and the severity of drug-induced nephrotic syndrome make it difficult to properly investigate the relationship between proteinuria, hypoalbuminemia, and the changes in the concentration, composition, and synthetic rate of specific lipoprotein classes (15, 16). These studies might be conducted in rats with less severe symptoms and a slow progression of kidney lesions as observed in chronic nephrotic syndrome induced by repeated intradermal injections of PAN (27, 28). However, under these circumstances one cannot rule out the possible side effect of the drug on tissue lipid metabolism (19).

The availability of animal strains that spontaneously develop a congenital form of slowly progressive nephrotic syndrome would be the most suitable model for the study of the pathogenesis of nephrotic dyslipoproteinemia. These features are present in the Milan Normotensive Strain (MNS), a rat strain that was developed from Wistar rats by inbreeding initiated in 1965 (29). MNS rats develop age-dependent, slowly progressive glomerular lesions that are associated with proteinuria, hyperlipidemia and, in the long run, with hypoalbuminemia and reduced renal function (30).

In the present study we used the MNS strain to define: *a*) the features of the dyslipoproteinemia during the development of nephrotic syndrome; *b*) the relationship between proteinuria and dyslipoproteinemia; and *c*) the effect of proteinuria on the level of albumin and some apolipoprotein mRNAs in the liver.

## MATERIALS AND METHODS

### Animals

Male MHS and MNS rats of the F60 generation were used. MNS and MHS rat strains are genetically related as they were both developed from common ancestor Wistar rats by two-way selection and inbreeding initiated in 1965 (29). MNS rats develop an age-related focal glomerulosclerosis whereas MHS rats do not (29). Despite the presence of glomerular lesions, MNS rats do not develop hypertension (thus they were designated normotensive) (29, 31). Despite the presence of hypertension, MHS rats do not develop glomerular lesions (29). Thus MHS rats were used as control animals.

Newborn MNS rats were divided into eight groups (eight animals each) and were killed at 4, 20, 24, 26, 28, 34, 44, and 48 weeks of age. Newborn MHS rats were divided into four groups (eight animals each) and were killed at 24, 28, 34, and 48 weeks of age. Animals received a standard chow diet (Altromin MTA Rieper Vandois, Italy) containing 23% protein, 5.5% fat, and 59% carbohydrates, and had free access to food and water through-

out the experiment. Rats were placed in metabolic cages and after an adaptation period of 3 days, 24-h urine samples were collected. The animals were fasted overnight and were anesthetized with Na-Pentobarbital (50 mg/kg) and blood was collected from the abdominal aorta. Na<sub>2</sub>EDTA was used as anticoagulant. Livers were removed, weighed, washed in cold buffered saline, immediately frozen in liquid nitrogen, and then stored at -80°C until used for RNA extraction.

### Plasma lipids and lipoproteins

Total plasma cholesterol and triacylglycerols were measured by using automated enzymatic methods (Ames Division, Miles, England). Plasma lipoproteins were separated by two procedures.

*Continuous density gradient ultracentrifugation.* Before the separation of plasma lipoproteins, equal aliquots of plasma taken from animals from each age group were pooled. Lipoproteins were isolated from the pooled plasma by density gradient ultracentrifugation in an SW 41 Beckman rotor (32). After centrifugation, aliquots of 500  $\mu$ l (fraction 1) or 400  $\mu$ l (fractions 2–20) were collected and their protein concentration was measured (33).

*Sequential density ultracentrifugation.* Plasmas of six animals of some age groups (20, 34, 44, and 48 weeks) were subjected to sequential ultracentrifugation to separate the following lipoprotein fractions: VLDL ( $d < 1.006$  g/ml), LDL (1.006–1.050 g/ml); HDL<sub>1</sub> (1.050–1.080 g/ml); and HDL<sub>2</sub> (1.080–1.210 g/ml) (17). Aliquots (100–400  $\mu$ g of protein) of each lipoprotein class were extracted with chloroform-methanol 2:1 (v/v) (34). Lipid classes were separated by thin-layer chromatography and measured colorimetrically (15).

### Apolipoprotein analysis

Aliquots (30–50  $\mu$ g of protein) of lipoprotein fractions isolated by ultracentrifugation were precipitated in 10% trichloroacetic acid (TCA); TCA-precipitates were extracted with ethanol-diethyl ether 3:2 (v/v), dissolved in 2% SDS, 3.5% 2-mercaptoethanol, 0.05 M Tris-HCl (pH 7) and heated at 95°C for 3 min. This material was applied to an SDS-polyacrylamide gel slab. A linear polyacrylamide gradient (5–20%) was used as a running gel (35). Gels were stained with Coomassie Blue R-250. The content of some apolipoproteins was estimated from the 605-nm absorbance of the Coomassie Blue stain eluted from the gel with 25% pyridine in 1% acetic acid (36).

### Urinary proteins

Urine was centrifuged at 800 g for 10 min to remove cells and particulate materials and dialyzed in 0.154 M NaCl and 0.01 M Na<sub>2</sub>EDTA for 48 h before the assay of protein content (33). Aliquots of urine (25–50  $\mu$ g of protein) were precipitated in 10% trichloroacetic acid (TCA), dissolved in 2% SDS, 3.5% 2-mercaptoethanol, 0.05 M

Tris-HCl (pH 7), heated at 95°C for 3 min, and separated by SDS-polyacrylamide gradient (5–20%) gel electrophoresis (35).

### Immunochemical analysis

Plasma concentrations of apoA-I and albumin were measured by electroimmunoassay as previously described (17). Isolation and purification of albumin and apoA-I from rat plasma were performed as described in a previous report (17). Specific polyclonal antibodies were raised in New Zealand white rabbits (17).

### Analysis of liver RNA

Total cellular RNA was extracted from the liver of individual animals (four animals for each age group) by the guanidine-HCl method as previously described (37). Northern blot analysis of albumin, A-I, A-II, E, and B apolipoprotein mRNAs was carried out using total liver RNA (15 µg) (37). Equal aliquots (20 µg) of liver RNA from each animal per group were pooled before the quantification of specific mRNAs by slot-blot hybridization. For slot-blot analysis, serial dilutions of RNA (starting from 2–4 µg/slot) were alkaline-denatured and blotted onto Zeta-Probe membranes (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. Each slot-blot assay included as control one slot containing 4 µg of yeast tRNA that did not hybridize to the cDNA probes used in this study. Yeast tRNA was added to balance the amount of liver RNA applied to each slot. Conditions of pre-hybridization and hybridization were as previously described (37). <sup>32</sup>P-labeling of the cDNA probes for rat apolipoproteins was performed using the Multiprime Kit (Amersham, England); labeling of the cDNA probes for rat albumin and human β-actin was performed by nick-translation (38). The specific activity of <sup>32</sup>P-labeled

cDNA probes ranged from 1.5 to 2.5 × 10<sup>8</sup> cpm/µg of DNA. The cDNA probes for rat apolipoproteins (A-I, A-II, E, and B) were kindly provided by Dr. L. Chan (Baylor College of Medicine, Houston, TX). cDNA probe for rat albumin was the clone pRSA 57 containing the whole coding region for rat plasma albumin (39). cDNA probe for human β-actin was clone pHF A-1 encoding the entire coding region of β-actin protein (40). Membranes were exposed to Hyperfilm MP (Amersham) for autoradiography at –80°C and stored wet to allow stripping and re-hybridization. Quantitative analysis of autoradiograms was performed using an LKB 2202 Ultrascan Laser densitometer. Each lane was scanned twice and the relative band intensities were determined by averaging the integrated intensities. Multiple autoradiographic exposures of each membrane were scanned and compared to ascertain that all densitometric data reported were measured within the linear range of the film. Slot-blot hybridization curves were calculated by linear regression analysis.

### Statistical analysis

Values are given as the mean ± standard deviation of the mean (SD). Statistical comparison between the groups was made using the Student's *t*-test. Correlation coefficients were obtained by linear regression analysis. A value for *P* < 0.05 was considered to be statistically significant.

## RESULTS

### Physiological parameters of MNS rats during the development of proteinuria

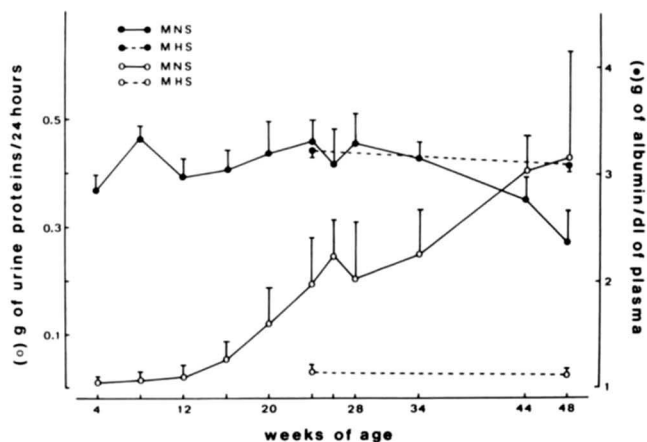
The changes of some physiological parameters of MNS rats over a period of 4–48 weeks of age are shown in **Table 1** and **Fig. 1**. Proteinuria increased slowly during the first

TABLE 1. Physiological parameters in MNS rats

Age	Body Weight	Liver Weight	LW/BW <sup>a</sup>	Kidney Weight
<i>wk</i>	<i>g</i>	<i>g</i>	<i>%</i>	<i>g</i>
MNS				
4	62.7 ± 10.7	1.7 ± 0.4	2.7 ± 0.2	0.64 ± 0.1
20	3.76 ± 18.0	8.9 ± 0.7	2.3 ± 0.2	2.41 ± 0.2
24	403.2 ± 27.0	9.8 ± 1.4	2.4 ± 0.2	2.69 ± 0.3
26	417.7 ± 34.9	10.7 ± 1.0	2.5 ± 0.1	2.78 ± 0.2
28	413.7 ± 20.3	9.8 ± 1.2	2.4 ± 0.2	2.74 ± 0.3
34	425.5 ± 24.5	11.2 ± 1.1	2.6 ± 0.1	2.86 ± 0.2
44	436.0 ± 26.0	11.4 ± 1.0	2.6 ± 0.2	3.25 ± 0.3
48	435.6 ± 23.9	11.5 ± 1.2	2.6 ± 0.2	2.63 ± 0.2
MHS				
48	517.0 ± 27.3	10.8 ± 0.9	2.09 ± 0.1	2.29 ± 0.1
MNS (48 wk) vs. MHS (48 wk)	<i>P</i> < 0.001	NS	<i>P</i> < 0.001	<i>P</i> < 0.001

Values are given as mean ± standard deviation; statistical analysis using Student's *t*-test.

<sup>a</sup>Liver weight/100 g of body weight.



**Fig. 1.** Time-course changes of proteinuria and plasma albumin during the development of nephrotic syndrome in MNS rats. Each value represents the mean  $\pm$  standard deviation from eight animals per group. Statistical analysis using Student's *t*-test. Plasma albumin: MNS (24 wk) vs. MHS (24 wk) NS; MNS (48 wk) vs. MHS (48 wk)  $P < 0.005$ ; MNS (20 wk) vs. MNS (34 wk) NS; MNS (34 wk) vs. MNS (44 wk)  $P < 0.025$ ; MNS (34 wk) vs. MNS (48 wk)  $P < 0.001$ ; MNS (44 wk) vs. MNS (48 wk)  $P < 0.05$ .

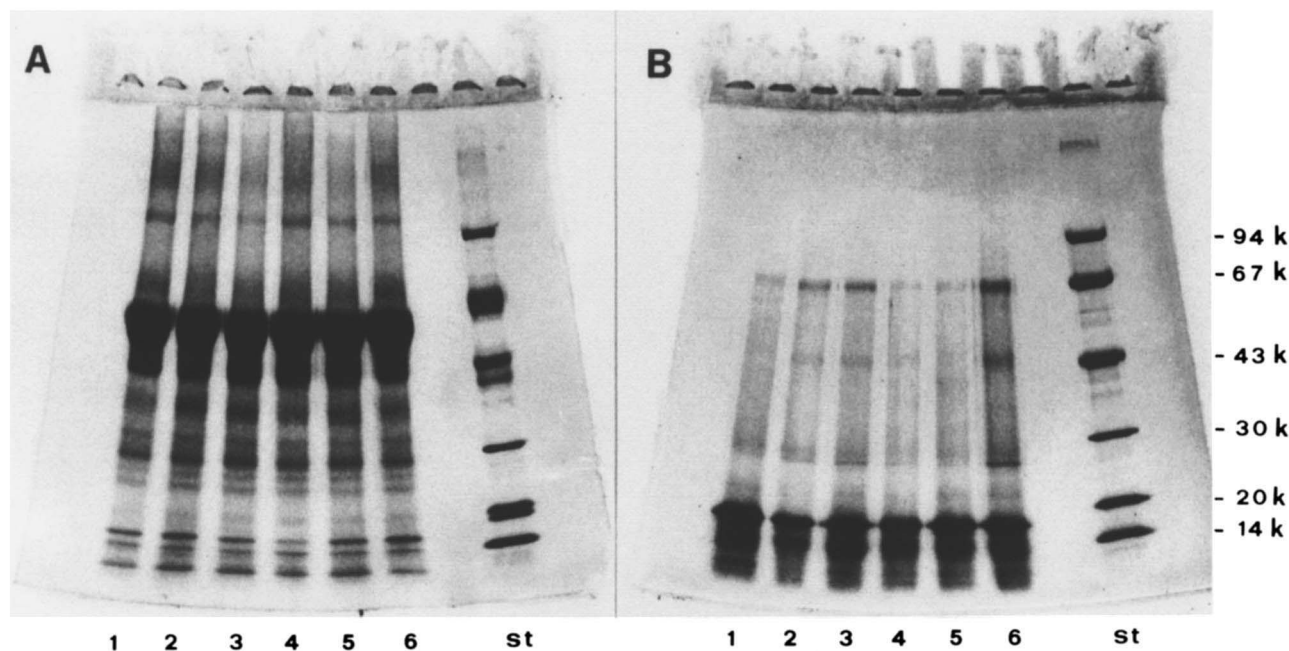
16 weeks of age but increased dramatically afterwards. At 48 weeks it was 18-fold more than that found in the age-matched MHS rats ( $0.45 \pm 0.20$  vs.  $0.025 \pm 0.005$  g/24 h,  $P < 0.001$ ) (Fig. 1). The level of proteinuria observed at 20 weeks of age in MNS rats ( $0.12 \pm 0.063$  g/24 h, 4-fold above the value found at 20–24 weeks of age in MHS rats,  $0.03 \pm 0.005$  g/24 h) was chosen as an arbitrary cut-off value for establishing the presence of an abnormal

glomerular permeability. In all age groups of MNS rats albumin was found to be the main protein lost in the urine as illustrated in Fig. 2.

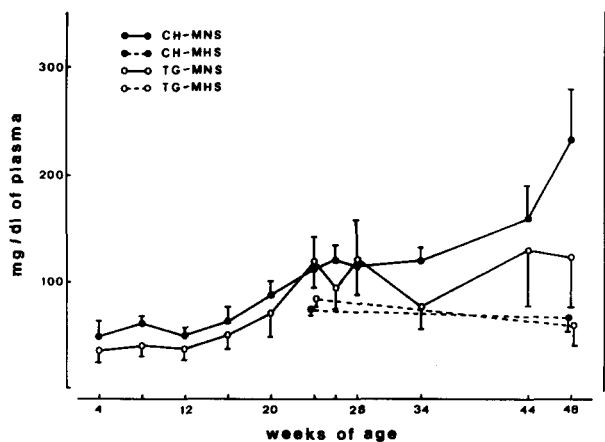
Despite the loss of large amounts of albumin in the urine, the level of plasma albumin remained constant up to 34 weeks of age but decreased at 44 weeks when proteinuria reached the value of 0.4 g/24 h (Fig. 1). At 48 weeks of age, plasma albumin of MNS rats was 75% of that found in the age-matched MHS rats. We designated as “compensated stage” the period ranging from 20 to 34 weeks (characterized by proteinuria only) and as “overt nephrotic stage” the subsequent period (characterized by severe proteinuria and hypoalbuminemia). In the compensated stage no correlation was found between proteinuria and the level of plasma albumin ( $r = -0.265$ ,  $n = 26$ , NS). By contrast, in the overt nephrotic stage there was a negative correlation between these parameters ( $r = -0.692$ ,  $n = 17$ ,  $P < 0.002$ ).

### Plasma lipids

In MNS rats the level of total plasma cholesterol increased between 12 and 24 weeks, remained stable up to 34 weeks, and increased sharply afterwards. At 48 weeks the level of plasma cholesterol in MNS rats was 3.5-fold that found in the age-matched MHS rats (Fig. 3). In MNS rats the level of plasma triacylglycerols paralleled that of total cholesterol up to 24 weeks (35% higher than the level found in the age-matched MHS rats) and slowly increased afterwards. At 48 weeks the level of plasma triacylglycerols in MNS rats was 2-fold that found in the age-matched MHS rats (Fig. 3).



**Fig. 2.** SDS-polyacrylamide gradient (5–20%) gel electrophoresis of urine proteins in 48-week-old MNS (A) and MHS (B) rats. Lanes 1–6 show the urine proteins of six animals per group. The migration of molecular weight standards is shown on the right.



**Figure 3.** Time-course changes of plasma cholesterol and triacylglycerols during the development of nephrotic syndrome in MNS rats. Each value represents the mean  $\pm$  standard deviation from eight animals per group. Statistical analysis using Student's *t*-test.

	CH	TG
MNS (24 wk) vs. MHS (24 wk)	$P < 0.001$	$< 0.0025$
MNS (48 wk) vs. MHS (48 wk)	$P < 0.001$	$< 0.01$
MNS (20 wk) vs. MNS (34 wk)	$P < 0.001$	NS
MNS (34 wk) vs. MNS (44 wk)	$P < 0.0025$	$< 0.01$
MNS (34 wk) vs. MNS (48 wk)	$P < 0.001$	$< 0.025$
MNS (44 wk) vs. MNS (48 wk)	$P < 0.0025$	NS

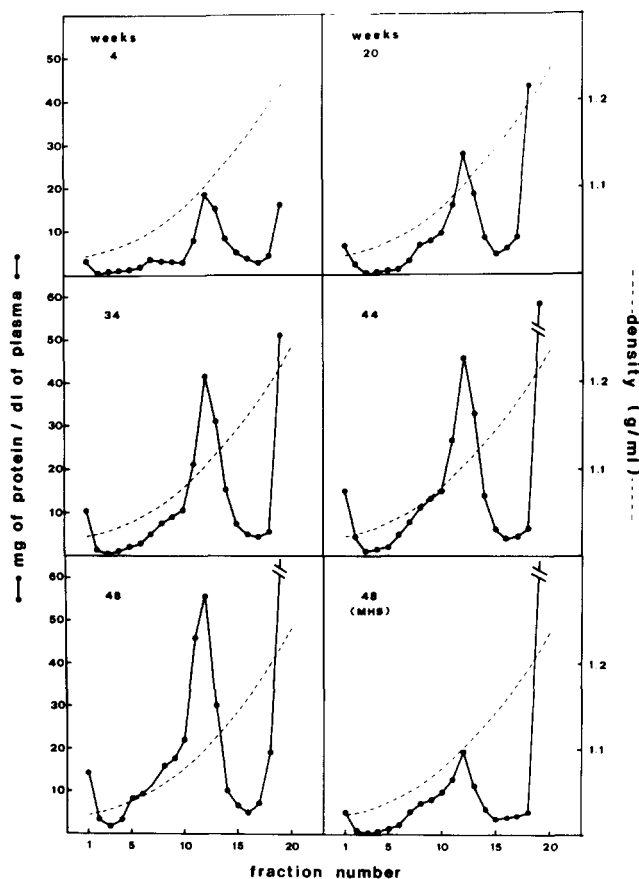
In the compensated stage a positive correlation was found between proteinuria and both plasma cholesterol ( $r = 0.856$ ,  $n = 26$ ,  $P < 0.001$ ) and triacylglycerols ( $r = 0.414$ ,  $n = 26$ ,  $P < 0.05$ ). These correlations were also present in the overt nephrotic stage ( $r = 0.867$ ,  $n = 17$ ,  $P < 0.001$  for cholesterol and  $r = 0.666$ ,  $n = 17$ ,  $P < 0.01$  for triacylglycerols). No correlation was found between the plasma levels of albumin and total cholesterol ( $r = -0.041$ ,  $n = 26$ , NS) in the compensated stage, whereas a highly significant correlation was observed in the overt nephrotic stage ( $r = -0.794$ ,  $n = 17$ ,  $P < 0.001$ ).

### Plasma lipoproteins

The density profile of plasma lipoproteins observed in MNS rats at 4 weeks of age was characterized by a major peak encompassing the density interval of HDL and two minor peaks in the VLDL and LDL density interval, respectively (Fig. 4). At 8 and 12 weeks of age the density profiles were superimposable to that found at 4 weeks (data not shown). At 16–20 weeks of age, the area of HDL peak was 30% larger than that found at 4 weeks (Fig. 4). At 24, 26, and 28 (data not shown) and at 34, 44, and 48 weeks of age (Fig. 4) the density profiles observed in MNS rats were characterized by: *a*) a progressive elevation of the HDL peak ( $d$  1.075–1.150 g/ml) (fractions 10–15 of the density gradient); *b*) an enlargement of the shoulder preceding the HDL peak ( $d$  1.040–1.065 g/ml) (fractions 6–9 of the density gradient) and including LDL and light

HDL; and *c*) a less pronounced increase of the VLDL + IDL peak ( $d < 1.040$  g/ml) (fractions 1–3 of the density gradient). In 48-week-old MNS rats the area of HDL peak was approximately 3-fold larger than that found in the age-matched MHS rats (Fig. 4).

In order to quantify the lipoprotein changes shown in Fig. 4, plasma lipoproteins were isolated by sequential ultracentrifugation (Table 2). At the end of the compensated stage (34 weeks of age) the plasma levels of LDL, HDL<sub>1</sub>, and HDL<sub>2</sub> were 129%, 171%, and 131%, respectively, of those observed at 20 weeks of age. At 48 weeks we observed a further increase in LDL (+89%), HDL<sub>1</sub> (+83%), and HDL<sub>2</sub> (+25%) compared to the values observed at the end of the compensated stage. In 48-week-old MNS rats the levels of all plasma lipoproteins were 2- to 3-fold those found in the age-matched MHS rats. During the compensated stage the level of HDL<sub>2</sub> was positively correlated with proteinuria ( $r = 0.734$ ,  $n = 14$ ,  $P < 0.005$ ) but not with plasma albumin. During the nephrotic stage the levels of VLDL, LDL, HDL<sub>1</sub>, and



**Fig. 4.** Density profile of plasma lipoproteins during the development of nephrotic syndrome in MNS rats. Plasma lipoproteins were separated by density gradient ultracentrifugation. Pools of plasmas taken from eight MNS rats of 4, 20, 34, 44, and 48 weeks of age and eight MHS rats of 48 weeks of age were used. VLDL + IDL, fractions 1–3; LDL, fractions 4–6; HDL, fractions 7–15.

TABLE 2. Concentration of plasma lipoproteins

Rats	Age	VLDL (1.006) <sup>a</sup>	LDL (1.006-1.050)	HDL <sub>1</sub> (1.050-1.080)	HDL <sub>2</sub> (1.080-1.210)
<i>mg lipoprotein-protein/dl plasma</i>					
MNS	20	13.2 ± 3.7	9.9 ± 1.6	16.5 ± 5.3	93.4 ± 12.0
	34	15.2 ± 3.4	12.9 ± 1.9	28.5 ± 3.9	122.1 ± 14.9
	44	19.7 ± 3.9	15.3 ± 3.2	40.7 ± 3.4	138.7 ± 20.1
	48	18.6 ± 7.2	24.3 ± 5.9	52.2 ± 7.5	155.0 ± 26.3
MHS	48	7.6 ± 2.9	8.1 ± 2.7	22.5 ± 9.2	50.3 ± 9.8
MNS (48 wk) vs. MHS (48 wk)		<i>P</i> < 0.01	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.0001
MNS (20 wk) vs. MNS (34 wk)		NS	<i>P</i> < 0.01	<i>P</i> < 0.0025	<i>P</i> < 0.0025
MNS (34 wk) vs. MNS (48 wk)		NS	<i>P</i> < 0.0025	<i>P</i> < 0.001	<i>P</i> < 0.025

Values are given as mean ± standard deviation; statistical analysis using Student's *t*-test.  
<sup>a</sup>Lipoprotein density (g/ml) shown in parentheses.

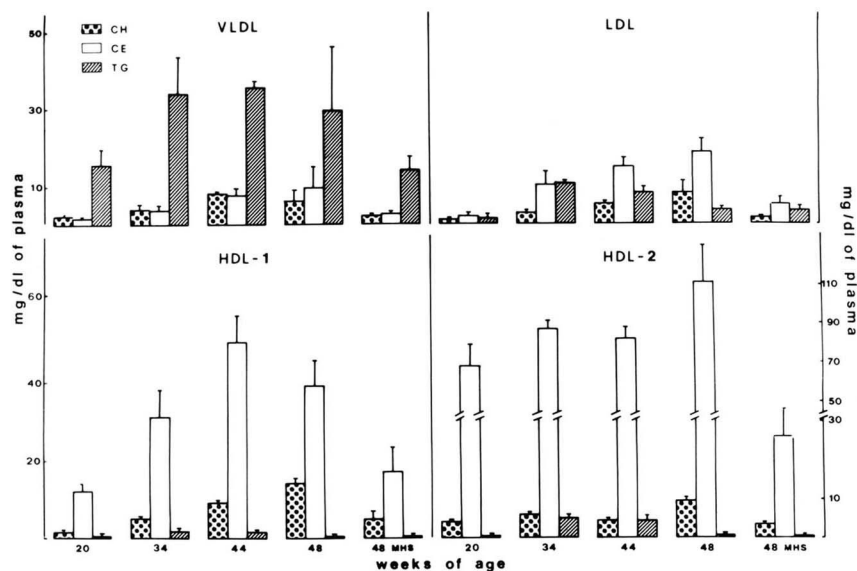


Figure 5. Lipoprotein-lipids during the development of nephrotic syndrome in MNS rats. Plasma lipoproteins were separated by sequential ultracentrifugation from six MNS rats of 20, 34, 44, and 48 weeks of age and from six 48-week-old MHS rats. VLDL, *d* < 1.006 g/ml; LDL, *d* 1.006-1.050 g/ml; HDL<sub>1</sub>, *d* 1.050-1.080 g/ml; and HDL<sub>2</sub>, *d* 1.080-1.210 g/ml. Statistical analysis using Student's *t*-test.

	VLDL	LDL	HDL <sub>1</sub>	HDL <sub>2</sub>
MNS (20 wk) vs. MNS (34 wk)	CH	<i>P</i> < 0.025	0.0025	0.0005
	CE	<i>P</i> < 0.01	0.0025	0.005
	TG	<i>P</i> < 0.005	0.0005	0.0025
MNS (34 wk) vs. MNS (48 wk)	CH	<i>P</i> , NS	0.0025	0.0005
	CE	<i>P</i> < 0.025	0.01	0.0005
	TG	<i>P</i> , NS	0.0005	0.0005
MNS (48 wk) vs. MHS (48 wk)	CH	<i>P</i> < 0.025	0.0025	0.0005
	CE	<i>P</i> < 0.01	0.0005	0.0005
	TG	<i>P</i> < 0.05	NS	NS

HDL<sub>2</sub> were positively correlated with proteinuria ( $r = 0.634$ ,  $P < 0.02$ ;  $r = 0.741$ ,  $P < 0.005$ ;  $r = 0.808$ ,  $P < 0.0005$ ;  $r = 0.766$ ,  $P < 0.0005$ ,  $n = 15$ ). The levels of LDL, HDL<sub>1</sub>, and HDL<sub>2</sub> were negatively correlated with the level of plasma albumin ( $r = -0.729$ ,  $P < 0.005$ ;  $r = -0.669$ ,  $P < 0.005$ ;  $r = -0.684$ ,  $P < 0.005$ ,  $n = 15$ ).

The cholesterol and triacylglycerol contents of the plasma lipoproteins were also determined (Fig. 5). At the end of the compensated stage we observed the following changes with respect to the levels observed at 20 weeks of age: *a*) an increase of triacylglycerol, cholesterol and cholesteryl esters of VLDL and LDL; and *b*) an increase of cholesterol and cholesteryl esters of HDL<sub>1</sub> and HDL<sub>2</sub>. At 48 weeks of age we observed the following changes compared to the levels observed at 34 weeks: *a*) an elevation of VLDL-cholesteryl esters; *b*) an increase of cholesterol and cholesteryl esters of LDL, HDL<sub>1</sub>, and HDL<sub>2</sub>; *c*) a reduction of triacylglycerol of LDL, HDL<sub>1</sub>, and HDL<sub>2</sub>.

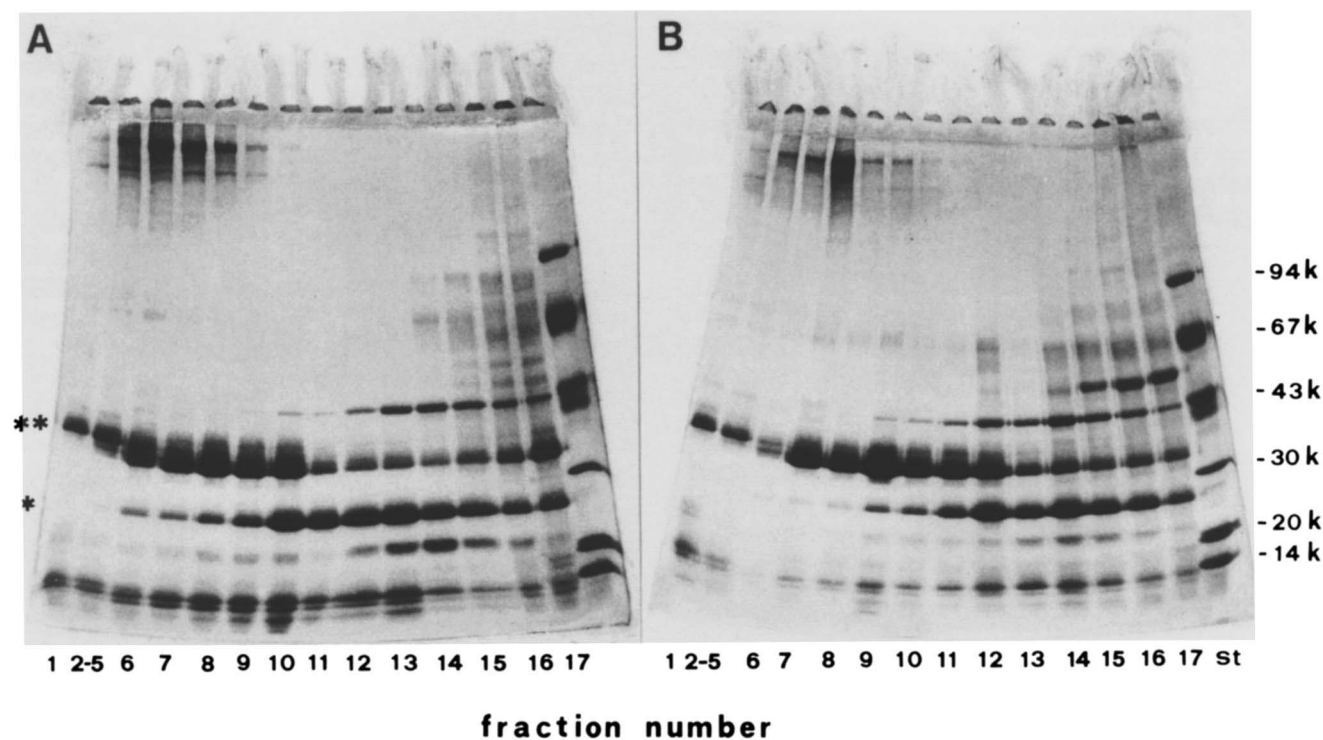
#### Apolipoprotein composition of plasma lipoproteins

At all ages the apolipoprotein patterns found in MNS and MHS rats were qualitatively similar (data not shown). During the development of nephrotic syndrome the apoA-I/apoE ratio in HDL<sub>1</sub> and HDL<sub>2</sub> of MNS rats increased progressively. At 48 weeks of age the apoA-

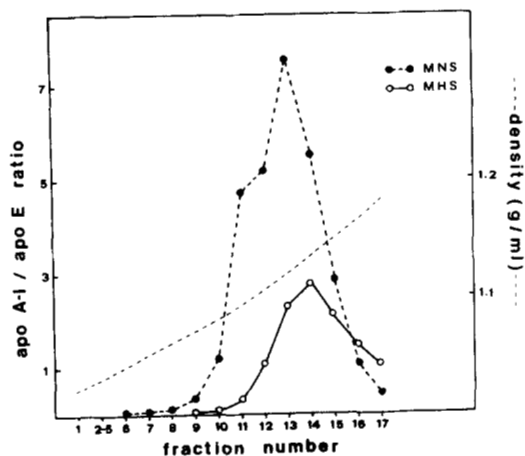
I/apoE ratio in lipoproteins of MNS rats was approximately 3-fold higher than the corresponding value of age-matched MHS rats (Fig. 6 and Fig. 7). This finding was consistent with the changes of plasma apoA-I, whose level in MNS rats increased approximately 3-fold from 20 to 48 weeks (Table 3).

#### Albumin and apolipoprotein mRNAs in the liver

To investigate whether in MNS rats the normal or the only moderately decreased level of plasma albumin (in the presence of a massive loss of albumin in urine) and the development of dyslipoproteinemia were accompanied by an enhanced hepatic production of albumin and lipoproteins (19–25), the levels of mRNA for albumin and some apolipoproteins were measured. Linear plots of slot-blot hybridizations of albumin, apoA-I, apoA-II and apoB mRNAs at selected stages of development of nephrotic syndrome are shown in Fig. 8. The regression coefficients (slopes) of these linear plots were used to determine the concentrations of the various mRNAs. To ensure that variations in mRNA were not due to differences in RNA content, all slot-blots were re-analyzed with human  $\beta$ -actin cDNA. There were minimal variations in  $\beta$ -actin mRNA levels during aging or the development of nephrotic syndrome (data not shown). Fig. 9 shows the variations of albumin and apolipoprotein mRNAs in both MNS and



**Fig. 6.** SDS-polyacrylamide gradient (5–20%) gel electrophoresis of apolipoproteins of 48-week-old MNS (A) and MHS (B) rats. Thirty to 50  $\mu$ g of lipoprotein-protein isolated by density gradient ultracentrifugation was applied to each lane. The numbers below each lane indicate the lipoprotein fractions of the density gradient shown in Fig. 4. The migration of molecular weight standards is shown on the right; \*, apoA-I (mol wt 28,000); \*\*, apoE (mol wt 34,000).



**Fig. 7.** ApoA-I/apoE ratio in plasma lipoproteins of 48-week-old MNS and MHS rats. Apolipoproteins of lipoprotein density fractions (Fig. 4) were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue R-250 (Fig. 6). The bands corresponding to apoA-I and apoE were cut and the stain was eluted from the gel with 25% pyridine in 1% acetic acid. The absorbance of Coomassie Blue stain was measured at 605 nm.

MHS rats relative to the levels observed in MNS rats at 4 weeks of age (100%). In MNS rats the level of: *i*) albumin mRNA steadily increased from 20 weeks onward reaching the highest level at 48 weeks (2-fold the level found at 4 weeks of age and 3.6-fold the level found in 48-week-old MHS rats) (Figs. 8, and 9-panel A); *ii*) apoA-I mRNA increased strikingly, reaching the highest level at 34 weeks (2.6-fold the level found at 4 weeks and 5.5-fold the level found in 34-week-old MHS rats). At 48 weeks apoA-I mRNA in MNS rats was similar to that found at 4 weeks and in the age-matched MHS rats (Figs. 8 and 9-panel B); *iii*) apoB mRNA increased, reaching the highest level at 28 weeks (1.7-fold the level found at 4 weeks and 2.6-fold the level found in the age-matched MHS rats); it decreased at the later stages so that by 48 weeks it reached a level similar to that found in age-matched MHS rats (Figs. 8 and 9-panel C); *iv*) apoA-II mRNA increased at 20 weeks, remained constant up to 44 weeks (4-fold the level found at 4 weeks but 1.5-fold the level found in 28- and 34-week-old MHS rats) and decreased at 48 weeks (Figs. 8 and 9-panel D); *v*) apoE mRNA showed no major variation during the whole time period, nor was it different with respect to the level found in MHS rats (data not shown).

## DISCUSSION

The first aim of the present study was the characterization of a disorder of plasma lipoproteins that occurs in an inbred strain of rats designated Milan Normotensive Strain (MNS). In MNS rats dyslipoproteinemia develops in association with a kidney disease characterized by age-

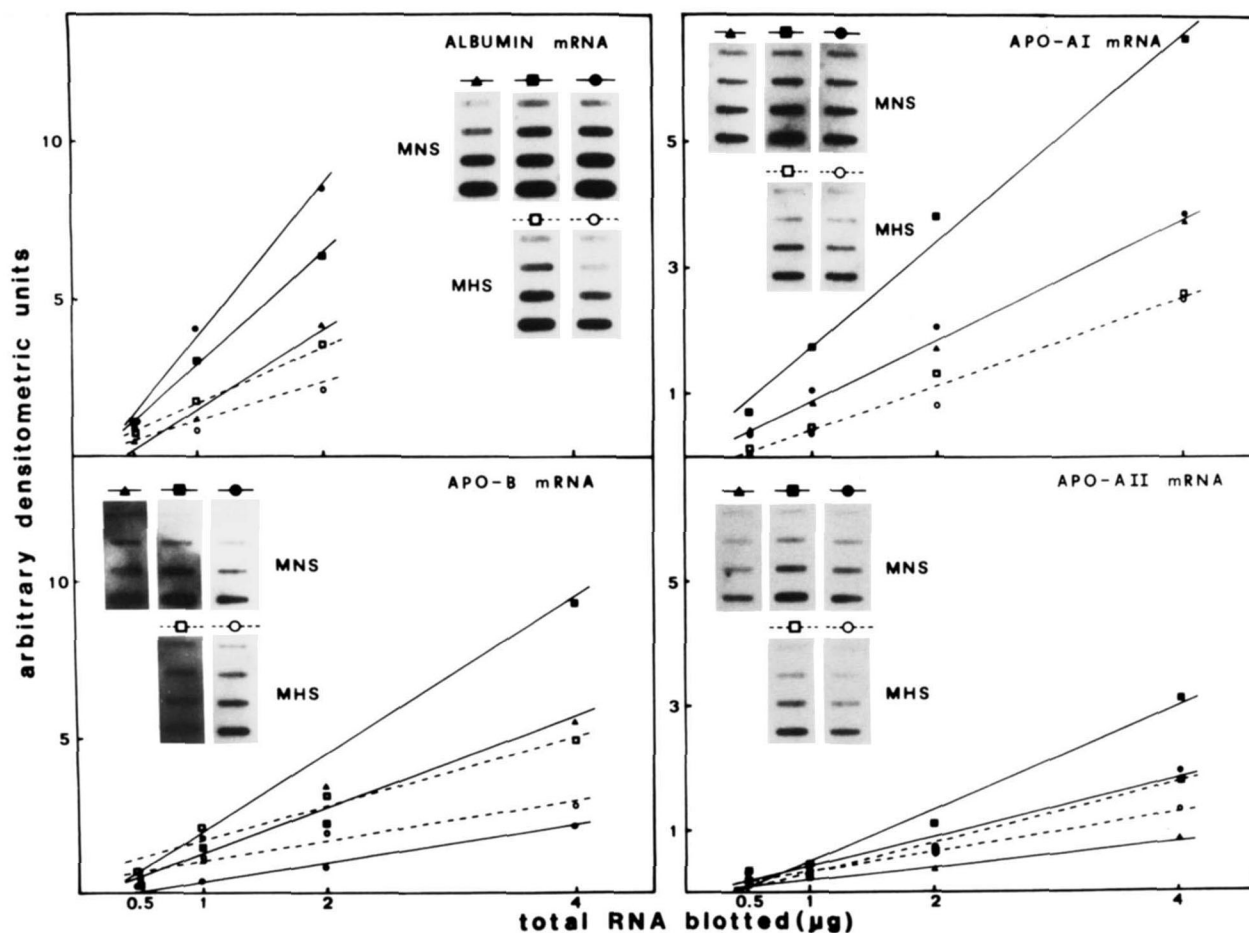
related glomerular lesions, proteinuria, and hypoalbuminemia (nephrotic syndrome) (29-31). In order to discriminate the effect of aging on plasma lipoproteins from that associated with the kidney disease, rats from another inbred strain (Milan Hypertensive Strain, MHS) genetically related to MNS (29-31) were used as controls. In MNS rats the daily loss of protein in urine progressively increased with age from 0.001 g at 4 weeks to 0.45 g at 48 weeks whereas in adult MHS rats (24-48 weeks of age) proteinuria ranged from 0.025 to 0.033 g. We chose the level of proteinuria at 20 weeks (Fig. 1) as an arbitrary value to set the initiation of the "abnormal" glomerular permeability. Nephrotic syndrome in MNS rats is characterized initially by a moderate-severe proteinuria without hypoalbuminemia (compensated stage) and later by a severe proteinuria and a moderate hypoalbuminemia (overt nephrotic stage).

In MNS rats dyslipoproteinemia is clearly detectable at the beginning of the compensated stage (20 weeks) and becomes progressively more severe as proteinuria increases and hypoalbuminemia develops. The earliest and most remarkable feature of this dyslipoproteinemia is the elevation of high density lipoproteins (HDL<sub>1</sub> + HDL<sub>2</sub>) whose level at 48 weeks of age is 3-fold that observed in the age-matched MHS rats. High density lipoproteins that accumulate in plasma of nephrotic MNS rats are enriched in apoA-I and cholesteryl esters. That is not surprising since we and others have previously reported that in drug-induced acute nephrotic syndrome there is an accumulation in plasma of large and "light" HDL particles (d 1.050-1.090 g/ml) that are rich in apoA-I and, unlike those in MNS rats, almost devoid of apoA-IV and apoE (14, 16, 17, 41, 42). The mechanism underlying the altered apolipoprotein composition of HDL might reflect several abnormalities such as: 1) an enhanced catabolism or a reduced production of apoA-IV and apoE; 2) a defect in the transfer of apoA-IV and apoE from triacylglycerol-rich lipoproteins to HDL (43) due to an impairment of chylomicrons and VLDL lipolysis (43-45); and 3) an in-

**TABLE 3.** Variations of apolipoprotein A-I concentration in plasma of MNS rats during the development of nephrotic syndrome

Rats	Age	Apolipoprotein A-I	
		mg/dl	%
MNS	4	51.4 ± 6.9	100
	20	64.7 ± 8.7	126
	24	87.6 ± 11.9	170
	26	79.1 ± 10.2	154
	28	82.2 ± 13.6	160
	34	81.4 ± 9.6	158
	44	140.7 ± 13.2	274
	48	160.9 ± 20.0	313
MHS	24	72.3 ± 6.2	140
	48	68.1 ± 7.3	132





**Fig. 8.** Slot-blot hybridization analysis of total liver RNA isolated from MNS and MHS rats during the development of nephrotic syndrome. Increasing amounts of RNA (0.5–1–2–4  $\mu$ g) isolated from rat liver as described in Methods were blotted onto Zeta-probe filters. The filters were hybridized with  $^{32}$ P-labeled rat albumin, apoA-I, apoB, and apoA-II cDNA probes. Filters were exposed to X-ray films. Autoradiograms (insets) were scanned and the results were plotted. Data are shown for some representative time points during the development of nephrotic syndrome: at 4 ( $\blacktriangle$ ), 28 ( $\blacksquare$ ), and 48 ( $\bullet$ ) weeks of age for MNS rats and at 28 ( $\square$ ) and 48 ( $\circ$ ) weeks of age for MHS rats.

creased production of apoA-I by liver and/or intestine (20, 25, 26). The first mechanism seems unlikely since in rats with various types of drug-induced nephrotic syndrome: a) there is no evidence that HDL containing predominantly apoE or apoA-IV are more rapidly removed from the plasma compartment (46) or are preferentially lost in urine with respect to HDL containing predominantly apoA-I; b) isolated and perfused liver of nephrotic rats secretes more apoE than does control liver (25). The second mechanism, which was described in the severe hypertriglycerolemia present in PAN-induced nephrotic syndrome (43), can be ruled out in nephrotic MNS rats in which plasma triacylglycerol level was 1/5–1/7 of that present in severe PAN-induced nephrotic syndrome (43–45). It is most likely, therefore, that the increased content of apoA-I in HDL of MNS rats reflects an overproduction of apoA-I by liver and/or intestine not compensated for by a parallel increase in the rate of catabolism of apoA-I-containing HDL (41).

The second aim of the present study was to clarify the relationship between the level of plasma lipoproteins and those of plasma albumin and proteinuria during the development of nephrotic syndrome. We found that during the compensated stage the level of plasma lipids (especially cholesterol) and HDL<sub>2</sub> was positively correlated with proteinuria but showed no correlation with the level of plasma albumin. In the overt nephrotic stage the level of plasma lipids, LDL, HDL<sub>1</sub>, and HDL<sub>2</sub> was positively correlated with proteinuria and inversely correlated with plasma albumin.

The third aim of the present study was to investigate whether the development of dyslipoproteinemia in MNS rats was associated with changes in the expression of some apolipoprotein genes in the liver, since much evidence supports the increased hepatic production of lipoproteins as the major contributor to the elevation of plasma lipoproteins in experimental nephrotic syndrome (20–26). We performed a time-course study of the levels of hepatic

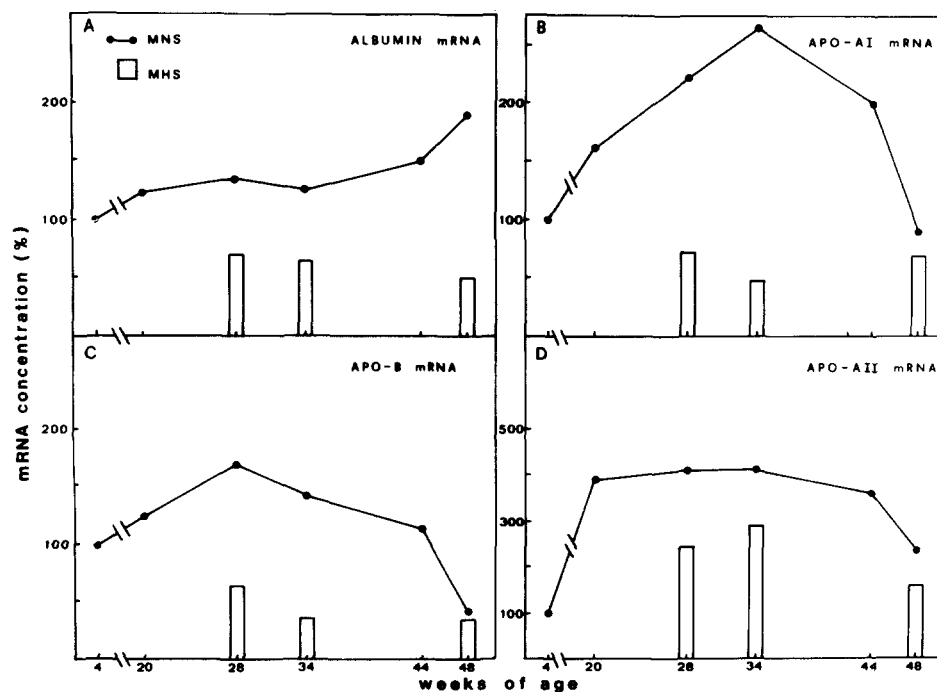


Fig. 9. Relative concentrations of albumin and apolipoprotein mRNAs during the development of nephrotic syndrome. Concentration of mRNA for albumin (A), apoA-I (B), apoB (C), and apoA-II (D) were determined from the slopes of the linear plots of slot-blot experiments (Fig. 8). For each probe mRNA concentration was given relative to the concentration of specific mRNA in the liver of 4-week-old rats (100%).

mRNA for B, A-I, E, A-II apolipoproteins and albumin. Our results can be summarized as follows: *i*) apoE mRNA does not change over the whole period of observation; *ii*) apoB mRNA shows a moderate but a transient increase in the middle of the compensated stage; *iii*) apoA-I mRNA increases during the compensated stage but returns to basal level in the overt nephrotic stage; *iv*) apoA-II mRNA level, which shows age-dependent changes (it increases from 4 to 20 weeks of age in both MNS and MHS rats), increases during the compensated stage; and *v*) albumin mRNA steadily increases during the whole period of observation. Among the apolipoprotein mRNAs, apoA-I mRNA showed the most striking increase; at the end of the compensated stage the hepatic apoA-I mRNA level in MNS rats was several-fold that observed either at 4 weeks or in the age-matched MHS rats. An increased level of hepatic apoA-I mRNA had been previously documented in our laboratory in rats with acute PAN-induced nephrotic syndrome (37). This observation was recently confirmed and extended by Marshall et al. (42) who measured the levels of apoB, A-I, A-II, and albumin mRNA in rats with PAN-induced acute nephrotic syndrome. They found that in pre-nephrotic animals (investigated 4 days after PAN injection and before the onset of proteinuria), hepatic mRNA levels for albumin, A-II, B, and E apolipoproteins were all elevated between 1.5- and 2.2-fold, whereas apoA-I

mRNA level was not increased. In nephrotic animals (with heavy proteinuria and severe hypoalbuminemia) the levels of mRNA for apoA-II, apoB, and apoE did not increase further, whereas albumin and apoA-I mRNA increased 3.8- and 6.1-fold, respectively. Although our results in MNS rats are not strictly comparable with those obtained by Marshall et al. (42) (in terms of severity of dyslipoproteinemia, rate of progression of nephrotic syndrome, and the possible effect of puromycin aminonucleoside on mRNA metabolism) these studies show that: *a*) a variable increase of the level of mRNAs for some apolipoproteins occurs in the liver early in the development of nephrotic syndrome; and *b*) the most striking increase involves the mRNA for apoA-I.

The early elevation of hepatic mRNA for apoA-I observed in MNS rats during the compensated stage may account for an increased production of apoA-I-rich lipoproteins by the liver and, in turn, for the increased level of apoA-I-rich HDL in plasma (see above). This simple explanation, however, may not be sufficient to account for the situation observed in overt nephrotic stage where apoA-I mRNA tends to return to basal level, whereas plasma apoA-I and HDL continue to increase. There are several possible explanations for this discrepancy: *a*) the rate of catabolism of HDL (as well as that of other plasma proteins) might be reduced as a compensatory adaptation to heavy proteinuria; *b*) the rate of translation of apoA-I

mRNA might be increased; and *c*) increased production of apoA-I might occur in other tissues (intestine, kidney, etc.). Further studies are needed to clarify this point.

Our observations that during the compensated stage: *a*) the hepatic level of mRNA for apoA-I, apoB, and apoA-II increased; *b*) the level of plasma lipids and HDL<sub>2</sub> was correlated with proteinuria but not with plasma albumin; and *c*) the elevation of some plasma lipoproteins (HDL<sub>1</sub> + HDL<sub>2</sub>) preceded the onset of hypoalbuminemia, raise new questions concerning the role of proteinuria on the pathogenesis of dyslipoproteinemia in MNS rats and in other forms of drug-induced nephrotic syndrome in the rat. It is generally accepted that the reduction of plasma oncotic pressure and/or viscosity resulting from the excessive loss of plasma proteins in urine provides the stimulus for the hepatic compensatory synthesis of albumin and other liver-derived plasma proteins (including lipoproteins) (47). This mechanism seems rather unlikely in MNS rats, at least during the compensated stage, since dyslipoproteinemia develops in the presence of a normal concentration of albumin that is difficult to reconcile with a reduction of plasma oncotic pressure and/or viscosity. This mechanism, however, may be operating in the stage of overt nephrotic syndrome when hypoalbuminemia develops. Our observations provide further support for the hypothesis that, in nephrotic syndrome, hyperlipidemia is not linked to serum albumin concentration but results at least in part from proteinuria (48). In this context one can assume that the development of glomerular lesions (of which proteinuria is a classical marker) leads, in some way, to the loss of some yet unidentified factor(s) that regulates the hepatic synthesis of plasma proteins or to the production by the kidney of some factors capable of stimulating hepatic synthesis of export proteins and lipids.

In view of our findings, MNS rat strain appears to be a new and unique model for the study of the pathogenesis of the dyslipoproteinemia associated with kidney disease (nephrotic syndrome) that offers several advantages over the animal models of drug-induced nephrotic syndrome used so far. First, MNS rats appear to have been selected for a spontaneous, genetically determined kidney disease. For this reason one might expect that glomerular lesions, proteinuria, lipoprotein disorder, etc. show less inter-individual variations than those observed in acute or chronic nephrotic syndrome induced by drugs. Second, the slow progression of the kidney lesions and, especially, the long-standing proteinuria without hypoalbuminemia allow the study of the effect of proteinuria on protein and lipid metabolism independently of hypoalbuminemia. Third, MNS strain would be a suitable model for the study of the role of kidney diseases in the changes of plasma lipoproteins related to age in the rat, since it is well recognized that a chronic nephropathy (heralded by proteinuria and characterized by glomerular alterations

similar to those observed in MNS rats) is a common disease in aged (2- to 3-year-old) laboratory rats (49).

In conclusion, in MNS rats dyslipoproteinemia develops in association with a mild and slowly progressive nephrotic syndrome; it develops before the onset of hypoalbuminemia and is characterized by an early elevation of plasma HDL<sub>1</sub> and HDL<sub>2</sub> enriched in apoA-I that is associated with an increased content of apoA-I mRNA in the liver. These observations support the contention that the accumulation of apoA-I-rich HDL in plasma is one of the most specific features of nephrotic dyslipoproteinemia in the rat (17). ■■

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