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Lipoprotein Secretion by Isolated Perfused Livers from Streptozotocin-Diabetic Rats

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Summary. In liver perfusion from sucrose-fed, streptozotocin-diabetic rats there was in comparison with normal animals, a decrease in very low density lipoprotein concentration in the perfusion medium (38.6 ± 6.3 versus 64.4 ± 8.4 $\mu g \cdot g$ liver $^{-1}$ $3 h^{-1}$, $p < 0.05$) and an increase in high density lipoprotein concentration (33.5 ± 6.5 versus 14.0 ± 1.9 $\mu g \cdot g$ liver^{-1} $3 h^{-1}$, $p < 0.005$), which was paralleled by enhanced secretion of apoprotein A-I. The triglyceride: protein ratio was lower in very low density lipoprotein from diabetic animals (8.8 versus 13.4). Analysis of the apoprotein composition showed that diabetic very low density lipoprotein lacked arginine-rich protein (apo-E) and apo-C peptides; diabetic high density lipoprotein also lacked arginine-rich protein but contained more A-IV and apo-C-peptides. This may indicate net transfer of C peptides to high density lipoprotein from the degradation of very low density lipoprotein particles. The ratio of ^{3}H-leucine: ^{14}C-glucosamine incorporation was decreased in all diabetic lipoprotein classes suggesting increased glycosylation of apoproteins. These changes in particle composition may influence lipoprotein metabolism in diabetes through their effects on lipoprotein lipase and lecithin cholesterol acyl transferase activity, plasma half-life and tissue binding.

Key words: Sucrose-feeding, streptozotocin diabetes, lipoprotein secretion, apoprotein composition, liver perfusion, incorporation of leucine, glucosamine.

It has previously been found that in experimental diabetes mellitus in the sucrose-fed rat there are increased plasma levels of very low density (VLDL), low density (LDL) and high density (HDL) lipoproteins [1]. Subsequent studies on the half-life of injected HDL showed that the increase in HDL was due to an augmented synthetic rate with a secondary decrease in catabolism [2]. The mechanism for the increased VLDL levels is thought to be due to a defect in removal caused by decreased activity of extrahepatic lipoprotein lipase [3]. Recently the presence of a plasma factor which interferes with the removal of VLDL-triglyceride has been demonstrated in experimental diabetes mellitus [4]. The parallel increase in both VLDL and HDL concentrations is of interest since there is usually an inverse relationship between these lipoproteins [5] and because the HDL particle is partially derived from the metabolism of VLDL [6]. We have previously shown that in the perfused liver from diabetic rats the incorporation of leucine into protein was decreased whilst that of glucosamine was unchanged or even enhanced [7]. We therefore wished to extend this work by examining the incorporation of these labels into the specific lipoproteins in the study of the hyperlipidaemia of experimental diabetes.

We have measured the secretion of lipoproteins by isolated perfused livers in streptozotocin-diabetic rats to assess how much hepatic synthesis determines the levels of circulating lipoproteins without the influence of peripheral catabolism by lipoproteins lipase.

Materials and Methods

Animals and Diet

Male rats of the Hebrew University strain, weighing 250–350 g were fed a semi-purified sucrose rich diet consisting of 20% vitamin-free casein, 60% sucrose, 5% lard, 1% vitamin mixture, 4% salt mixture and 10% cellulose. The rats were fed for 3 weeks before induction of diabetes. Streptozotocin (40 mg/kg body weight) was injected into the tail vein as previously described [1]. Experiments were performed 2–4 days after the streptozotocin injection. In all the diabetic animals blood glucose concentration was over 19 mmol/l.

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Table 1. Lipoprotein protein in perfusates from diabetic and non-diabetic rats after perfusion for 3 h

	VLDL	LDL	HDL	Apoprotein A-I
	(μg/g liver)			
Diabetic rats	38.3 ± 6.3 (10)	6.1 ± 0.8 (10)	33.5 ± 6.5 (6)	13.4 ± 2.4 (5)
Non-diabetic rats	64.4 ± 8.4 (7)	10.1 ± 1.9 (9)	14.0 ± 1.9 (12)	8.6 ± 1.6 (5)
p	< 0.05	NS	< 0.005	NS

Results expressed as mean ± SEM; number of experiments in parentheses; NS = not significant

Liver Perfusion

Isolated liver perfusion was performed as described by Mortimore et al. [8], published in detail [7]. The perfusate consisted of Krebs-Ringer bicarbonate (pH 7.4) containing glucose 4 mg/ml, streptomycin 0.1 mg/ml, penicillin 1000 μ/ml, bovine serum albumin 30 g/l, a mixture of the naturally occurring amino-acids (excluding leucine) 0.1 mmol/l of each, and heparin 1 u/ml. Human red blood cells, washed twice with 0.154 mol/l saline (pH 7.4), were added to the perfusate to achieve a haematocrit of approximately 20%. The liver was washed first with 40 ml Krebs-Ringer bicarbonate solution. After washing, the liver was perfused in a recycling system with a volume of 60–70 ml. Throughout the perfusion, sodium palmitate complexed with serum albumin (4 : 1 molar ratio) was infused into the portal vein at a rate of 0.2 mmol/h, using a constant perfusion pump [9]. Oxygenation with 95% O_2, 5% CO_2, was carried out as described by Hamilton et al. [10] using silastic tubing (Dow-Corning, Michigan). At zero time, 50 μCi of (^{3}H) leucine and 10 μCi of (^{14}C) glucosamine were added to the perfusate immediately after washing. Samples of perfusate were taken for the determination of apoprotein A-I, at zero time, and every 30 min thereafter for 3 h. At the end of the perfusion the perfusate was centrifuged and the supernatant measured and stored at 5 °C after the addition of sodium azide (1 mg/ml) and EDTA (2.1 mmol/l).

Separation of Lipoproteins

Lipoproteins were isolated by the method of Havel et al. [11], using the Ti 50, Ti 60 or SW 41 rotors in the Beckman model L-50 ultracentrifuge at 15 °C. VLDL of d 1.006 and LDL, d 1.006–1.063, were separated by 20 h ultracentrifugation at 200,000 *g*, while HDL of d 1.063–1.21 were spearated after 40 h at the same speed. Densities above 1.006 were adjusted by the addition of KBr solutions. The isolated fractions were separated using a Spinco tube slicer, washed once by resuspending them in their respective density solutions and then repeating the ultracentrifugation. The washed lipoprotein fractions were exhaustively dialysed against water with 0.134 mmol/l EDTA pH 7.0 at 4 °C.

To obtain apolipoproteins free of lipid the isolated fractions were lyophilised and delipidated at 4 °C with a mixture of 3 : 1 absolute ethanol : anhydrous diethyl-ether, as described by Brown et al. [12]. Cold ether was then added to the mixture to adjust it to 1 : 1 ethanol (v/v) [13]. The precipitate was centrifuged at 4 °C at 1000 *g* for 45 min. The delipidated apolipoproteins were then dissolved in 0.2 mol/l *Tris* HCL buffer (pH 8.2) containing 0.006 mol/l sodium dodecyl sulphate.

Chemical Methods

Lipoprotein lipids were extracted in 99% (v/v) isopropanol and triglyceride and cholesterol levels were determined by the Technicon autoanalyser II method [14]. The coefficient of variation for these determinations never exceeded 6%. Lipoprotein protein was measured according to the method of Lowry et al. [15]. Apoprotein A-I levels were estimated by radioimmunoassay [16]. The intra-assay coefficient of variation was 4.5% and the inter-assay coefficient was 6%. Protein radioactivity of the erythrocyte free perfusate and the lipoprotein fractions were determined by the method of Mans and Novelli [17].

Sodium Dodecyl Sulphate (SDS) Polyacrylamide-Gel Electrophoresis

This was performed according to the method of Shapiro et al. [18], as subsequently modified by Maizel [10]. Ten percent polyacrylamide gels were polymerized in tubes and a continuous buffer system consisting of 0.1 mol/l sodium phosphate (pH 7.0) containing 0.1% SDS was used. Electrophoresis was carried out for a 20 h period at 30 V, with the temperature maintained at 15 °C and 20–40 μg of protein being applied to each gel. The gels were stained with 0.2% Coomassie Blue in 50% methanol and 99% acetic acid for 20–24 h. Densitometric scanning of gels was recorded by a Quick Quant II scanner (Helena Laboratories, Beaumont, Texas, USA). With the exception of apoprotein A-I levels, identification of the different apoproteins was based solely on their different mobilities using the nomenclature of Swaney et al. [20].

Materials

L-(4,5-^{3}H)-leucine 60.0 Ci/mmol, D-(1-^{14}C)-glucosamine 59.0 mCi/mmol were purchased from the Radiochemical Centre, Amersham, UK. Streptozotocin was kindly supplied by Dr. W. Dulin of the Upjohn, Kalamazoo, Michigan, USA.

Statistical Analysis

The statistical significance of the difference between the means of the experimental groups was evaluated using Student's t-test (two-tailed).

Results

Lipoproteins Isolated from the Perfusate

Table 1 shows the protein content of the different lipoprotein fractions isolated after three hours liver perfusion. VLDL levels were decreased in the diabetic preparations and HDL levels were increased. There were no significant differences in the levels of LDL or apoprotein A-I (apo-A-I). The secretion of apo-A-I,

however, was higher in the perfusates from the diabetic animals during the whole time-course of the experiment, parallel with the raised HDL levels (Fig. 1). LDL protein levels were approximately 16% of the VLDL protein in each group.

Cholesterol and Triglyceride Content of VLDL (Table 2)

Livers from diabetic animals secreted significantly less cholesterol and triglyceride matching the decrease in VLDL protein. The triglyceride : protein ratio in the VLDL from the diabetic preparations was 8.8 ± 1.3 and in the fraction from control animals 13.4 ± 2.5. The triglyceride : cholesterol ratios were 20.3 ± 6.4 and 15.5 ± 5.1, respectively.

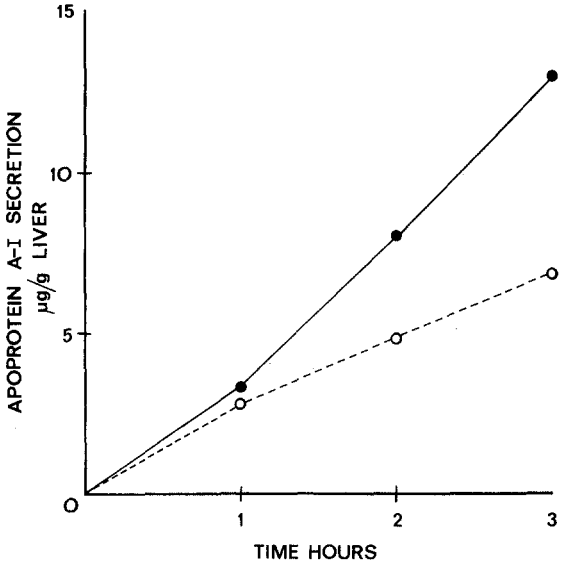

Fig. 1. The secretion of apoprotein-A-I into the perfusate during 3 h of isolated liver perfusion of non-diabetic (O – – – – O) and diabetic (●——●) rats. Apoprotein A-I was determined by radioimmunoassay on perfusate samples taken hourly. Each curve is a representative of two liver perfusions

Table 2. Triglyceride and total cholesterol secretion in VLDL after liver perfusion for 3 h

	Triglyceride (µg/g liver)	Cholesterol (µg/g liver)
Diabetic rats	336 ± 65	17 ± 3
Non-diabetic rats	864 ± 15	56 ± 9
p	< 0.001	< 0.005

Results expressed as mean ± SEM; six experiments in each group

Specific Activities of Radioactive Labels in Different Lipoproteins

The specific activities of ^{3}H-leucine and ^{14}C-glucosamine in the different lipoprotein subclasses are shown in Table 3. ^{3}H-Leucine specific activity was significantly lower in all lipoproteins isolated from diabetic animals. ^{14}C-glucosamine specific activities were similar in both groups. ^{3}H-leucine : ^{14}C-glucosamine ratios (Table 4) were significantly lower in all lipoproteins in the diabetic groups. A comparison was made of the percentage of the proteins which were secreted as lipoproteins (Table 5). The percentage ^{3}H-leucine incorporation into VLDL and LDL was unchanged, while in HDL it was increased but this did not reach statistical significance. The percentage incorporation of ^{14}C-glucosamine was higher in diabetic HDL but similar in VLDL and LDL.

The Results of the Lipoprotein Electrophoresis

Sodium dodecyl sulphate-polyacrylamide disc gel electrophoresis of VLDL is shown in Figure 2, together with the densitometric scan. The gels are representative of at least eight different experiments, all with similar patterns. VLDL from diabetic animals lacked arginine rich protein and apo-C peptides but contained equal if not more apoprotein A-IV. HDL from diabetic preparations also lacked arginine rich protein but contained more apoprotein A-IV and apo-C than the non-diabetic lipoprotein.

Discussion

In the perfusates from streptozotocin-diabetic rats there were significantly decreased amounts of VLDL protein but increased levels of HDL. VLDL-protein secretion in non-diabetic rats was 21.5 $\mu g \cdot g^{-1} h^{-1}$

Table 3. The specific activity of ^{3}H-leucine and ^{14}C-glucosamine in lipoproteins isolated after liver perfusion for 3 h

Density		Non-diabetic rats (cpm/µg protein)	Diabetic rats (cpm/µg protein)	*p*
1.006, 1.063	^{3}H-leucine	722 ± 192	133 ± 43	< 0.05
	^{14}C-glucosamine	90 ± 18	54 ± 4	NS
1.21	^{3}H-leucine	121 ± 27	26 ± 12	< 0.05
	^{14}C-glucosamine	19 ± 2	18 ± 8	NS

Mean ± SEM of four observations

which compares with values of 39 $\mu g \cdot g^{-1}h^{-1}$ found by Marsh [21] in a non-recycling preparation and 58 $\mu g \cdot g^{-1}h^{-1}$ calculated from the results of Ruderman et al. [22] using a haemoglobin-free system. Triglyceride secreted during liver perfusion in fed rats are all in the VLDL fractions [23] and VLDL-triglyceride production of 288 $\mu g \cdot g^{-1}h^{-1}$ (Table 2) compares with the results obtained by these groups of 283 and 290 $\mu g \cdot g^{-1}h^{-1}$ respectively. The reasons for the lower rates of VLDL-protein synthesis are not clear but may be related to differences in the strain of rats and their diet. Sucrose feeding increases the ratio of triglyceride : protein in VLDL [1].

In their studies on perfused livers from alloxan-diabetic rats, Heimberg et al. have also noted decreased trigylceride output and apoprotein synthesis [24, 25] but there has been little work on changes in the different lipoprotein subclasses in experimental diabetes.

The levels of LDL found in the perfusates probably reflected catabolism of VLDL during the course of the experiments. Recycling of labelled VLDL in the perfusion apparatus *without* a liver did not lead to any degradation of the lipoprotein which suggests that any changes observed were due to hepatic synthesis and/or catabolism. Circulating LDL is considered to be formed principally from the metabolic conversion of VLDL [26, 27]. In both the diabetic and non-diabetic preparations, LDL levels were approximetely 16% those of VLDL suggesting that the hepatic fractional catabolism of VLDL was the same.

Since it has yet to be satisfactorily demonstrated that the liver secretes HDL particles, interpretation of the increased levels of this lipoprotein found in the perfusates is not clear. The increased levels of apoprotein A-I suggest enhanced synthesis de novo by the liver which would be in agreement with metabolic studies in the whole animal [2]. Alternatively, the HDL may be derived from increased degradation of VLDL [6], especially in the presence of heparin in the perfusate.

The decreased specific activity of ^{3}H-leucine in VLDL (Table 3) was in agreement with the decreased total levels of this lipoprotein in the perfusates. The situation for HDL is, however, less clear. Whilst the total HDL concentration was elevated, the specific activity of leucine was decreased, even though the amount of HDL secreted as a percentage of the total protein was increased (Table 5). These apparently contradictory observations may be explained by either increased turnover of HDL protein or perhaps by the apoproteins of HDL from diabetic animals containing less leucine.

Whilst lipoprotein synthesis as shown by ^{3}H-leucine incorporation was decreased in all fractions from the diabetic animals there was no change in the ^{14}C-glucosamine incorporation (Table 3). The lowered ratio of ^{3}H-leucine : ^{14}C-glucosamine in lipoprotein from diabetic livers, suggests that there is increased glycosylation of apoproteins in diabetes (Table 4). Enhancement of the incorporation of glucosamine into secretory proteins by the perfused diabetic liver has been described previously [7]. When incorporation of the radioactive labels was expressed as a percentage of the total secreted protein, there was an increase in the ^{14}C-glucosamine content of HDL from diabetic rats. This may reflect increase in apo-C proteins which are rich in carbohydrate residues [28]. We believe that these changes in incorporation are due to the diabetic state and are not secondary to alterations in the pool sizes of leucine and glucosamine. The pool sizes have been measured in hepatocyte preparations and were found to be similar with respect to leucine but slightly increased for glucosamine in the diabetic animals [7].

The composition of newly secreted VLDL is practically the same as that of circulating lipoprotein apart from the content of apo-C proteins which probably play only a minor role in its structural integrity [27, 29, 30]. However, VLDL from the perfusates of diabetic

Table 4. The ratio of ^{3}H-leucine : ^{14}C-glucosamine incorporated into lipoproteins isolated after liver perfusion for 3 h

	Lipoprotein fractions of density 1.006 and 1.063	Lipoprotein fraction of density 1.21
Diabetic rats	2.5 ± 0.9	1.8 ± 0.4
Non-diabetic rats	7.4 ± 0.8	5.6 ± 0.7
p	< 0.02	< 0.01

Mean ± SEM of four observations

Table 5. Percentage of total trichloroacetic acid precipitated material secreted as lipoprotein estimated by ^{3}H-leucine and ^{14}C-glucosamine counts per minute after liver perfusion for 3 h

Density		Non-diabetic rats	Diabetic rats	p
1.006, 1.063	^{3}H-leucine	0.51 ± 0.04	0.37 ± 0.08	NS
	^{14}C-glucosamine	0.27 ± 0.03	0.25 ± 0.07	NS
1.21	^{3}H-leucine	0.071 ± 0.01	0.176 ± 0.05	NS
	^{14}C-glucosamine	0.048 ± 0.001	0.067 ± 0.005	< 0.02

Mean ± SEM four observations

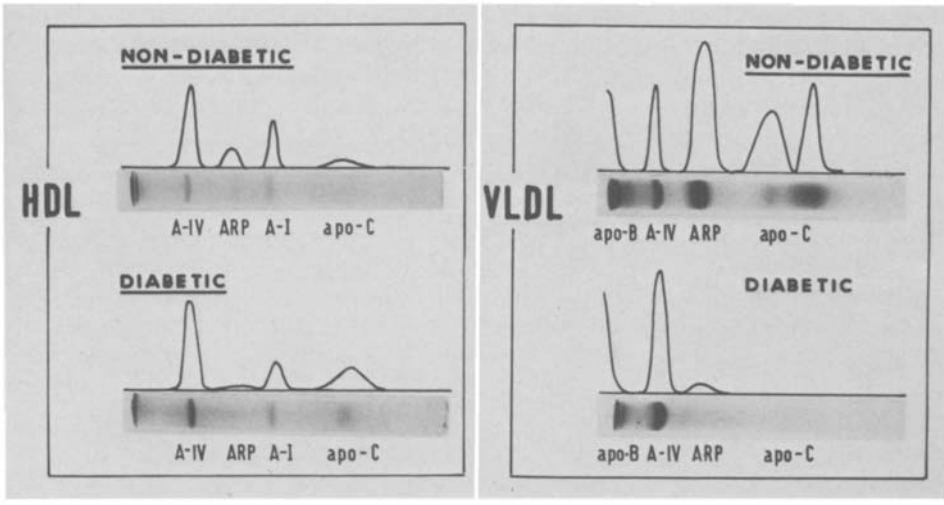

Fig. 2. Sodium dodecyl sulphate electrophoresis of high density lipoprotein (HDL) and very low density lipoprotein (VLDL) isolated from liver perfusates from diabetic and non-diabetic rats. A-IV = apoprotein-A-IV; ARP = arginine-rich protein; A-I = apoprotein-A-I; apo-C = apoprotein C

animals differed from that found in the circulation [31]. It was depleted in arginine-rich peptides and apo-C. There are a number of possible explanations for this. The VLDL synthesized may indeed lack apo-C or be depleted of these apoproteins during recycling of the perfusates. The latter possibility may be supported by the finding of increased C-peptides in the HDL isolated from the perfusates of diabetic livers. This was not found in the non-diabetic preparations. If the apo-C peptides in HDL were indeed derived from VLDL and exchange between these lipoproteins occurs as in normal rats [32, 33] then the converting enzyme was hepatic lipase. This, however, would be contrary to the findings of Elkeles and Hamberley [34], who have reported that hepatic triglyceride lipase activity in the whole animal may be different from the situation in the plasma-free isolated liver perfusion system, since serum is known to inhibit hepatic lipase [35]. The lowered triglyceride : protein ratio in VLDL isolated from the diabetic livers also suggests increased lipolysis of this lipoprotein during perfusion.

In the plasma of sucrose-fed diabetic animals, VLDL levels are raised [1] despite decreased levels in perfusates. It must therefore be postulated that extrahepatic catabolism of VLDL is the prime regulator of plasma levels in experimental diabetes. Reaven and Reaven [36] come to a similar conclusion in studies on the development of hypertriglyceridaemia in acute and chronic streptozotocin-diabetic rats. In diabetic patients, Lewis et al. [37] noted the importance of reduced extra-hepatic catabolism in controlling VLDL levels. In support of this, a reduction in lipoprotein lipase activity has been observed in insulin-deficient diabetes mellitus [38–40] as well as a defect in VLDL removal [41].

In experimental diabetes it is possible that the altered composition of VLDL also influences its own catabolism. Bar-On et al. [31] found that VLDL from diabetic rats possessed both increased amounts of apo-C-III$_3$ which is an inhibitor of lipoprotein lipase [42] and also diminished levels of apo-C-II which is an activator of that enzyme [35, 43]. Since apo-C-III is the only C-peptide which contains carbohydrate residues [44], it is tempting to speculate that deranged glucose metabolism in diabetes mellitus may augment synthesis of this apoprotein and thus lead to reduced turnover of VLDL. If there is also increased glycosylation of apoprotein as shown by ^{14}C-glucosamine relative to ^{3}H-leucine incorporation, then the plasma half-lives of the lipoproteins may be prolonged because of increased resistance to degradation and proteolysis [45, 46].

In the perfusates from diabetic animals, there were increased amounts of HDL protein which differed in apoprotein composition both from controls and circulating HDL. The most prominent alteration was the decrease in arginine-rich protein which is the major apoprotein in nascent HDL and which is transferred to VLDL during the lecithin-cholesterol-acyltransferase reaction [33, 47]. In the diabetic whole animal there is a decrease in total levels of arginine-rich protein, a decrease in its content in HDL (despite raised levels of HDL) and a slight increase in VLDL. This might be explained by either increased lecithin-cholesterol-acyl-transferase activity or decreased synthesis or a combination of the two. Analysis of the apoprotein in the perfusates showed there to be a decrease in arginine-rich protein in both HDL and VLDL, suggesting that synthesis is indeed decreased in diabetes. Arginine-rich protein synthesis may be particularly dependent on insulin but the function of

this apoprotein in the metabolism of VLDL-triglyceride awaits further clarification [28]. It is also possible that arginine-rich protein may be lost to the lipoprotein-free plasma during ultracentrifugation. The deletion of arginine-rich protein from HDL may cause the latter to accumulate in the perfusate, since the uptake and binding of HDL by hepatocytes is dependent on this particle containing arginine-rich protein for receptor recognition [48].

Another difference concerns apoprotein A-IV levels. In the plasma of diabetic rats, total apoprotein A-IV levels are increased [31] mainly due to an increase in VLDL; levels of this apoprotein in HDL are decreased. In the perfusates, however, there was increased apoprotein A-IV in diabetic HDL. The function of apoprotein A-IV is also unknown but it may undergo transfer to VLDL in the circulation.

Whilst the precise significance of these alterations in apoproteins in experimental diabetes is not completely understood, they may indicate a role for apoprotein synthesis and the glycosylation (arginine-rich protein, apo-B and apo-C-III) in the regulation of lipoprotein metabolism. Detailed quantitative analysis of the carbohydrate content of apoproteins in diabetes will be required to verify these suggestions. The changes in hepatic lipid synthesis which undoubtedly occur in diabetes can only be expressed *systemically* through binding to apoproteins [49, 50]. The importance of altered apoprotein glycosylation in relation to lipoprotein function and the pathogenesis of diabetic microangiopathy and atherosclerosis requires further investigation.

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