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Thematic Review Series: Living History of Lipids

The early years of lipoprotein research: from discovery to clinical application

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Abstract This review outlines major milestones in the first four decades of lipoprotein research beginning with their discovery nearly 90 years ago. It focuses on the contributions of some of the key investigators during this era, and findings that set the stage for widespread clinical implementation of lipoprotein testing for evaluation and management of CVD risk.—Siri-Tarino, P. W., and R. M. Krauss. The early years of lipoprotein research: from discovery to clinical application. *J. Lipid Res.* 2016. 57: 1771–1777.

Supplementary key words low density lipoprotein • cholesterol • atherosclerosis • history

LINKING BLOOD CHOLESTEROL TO ATHEROSCLEROTIC CVD

In the early 1900s, animal studies showed that high amounts of meat, eggs, and milk led to increased atherosclerosis, and the aortas of patients with atherosclerosis were found to have increased cholesterol content as reviewed in (1, 2). It was, however, the subsequent work of Nikolai Anitschkow that laid the foundation of what would later be termed the “lipid hypothesis,” or the concept that elevated blood cholesterol concentrations induced atherosclerosis. He showed that feeding rabbits purified cholesterol raised blood cholesterol levels and induced atherosclerotic lesion formation, and that the extent of atherosclerosis was proportional to the absolute amount of and length of exposure to high blood cholesterol (3). In contrast, similar experiments in dogs and rats did not elicit a similar response, results that lent skepticism to the lipid hypothesis. The inability to induce atherosclerosis in these species was later found to be due to their relative resistance to diet-induced hypercholesterolemia (2).

Clinically, a number of conditions were defined by high blood cholesterol concentrations and increased risk of CVD, including nephritis, hypothyroidism, and genetic

hypercholesterolemia. A causal role for cholesterol in atherosclerotic disease was, however, not supported by evidence showing that: 1) not all persons with high blood cholesterol concentrations developed CVD; and 2) the majority of patients presenting with atherosclerosis had “normal” cholesterol levels, although the upper boundaries used at that time were much higher than those subsequently deemed to be desirable. As would come to be known, increased atherosclerosis was not simply a function of high total cholesterol concentrations (2).

IDENTIFYING, ISOLATING, AND CLASSIFYING LIPOPROTEINS

The first suggestions that circulating lipids existed in complexes with proteins came from the experimental observations of Machebouef in 1929 (4). Ultracentrifugal studies of serum over the next decades suggested that a labile lipid-protein complex designated protein “X” existed (5, 6). The Swedish investigator Kai Pedersen, an original student of the Nobel Prize winning chemist Theodor Svedberg who invented the ultracentrifuge, had concluded that serum was not suitable for study due to interference by this protein. This artifact was suggested by a smear in the Schlieren profiles, the optical patterns of substances sedimenting (or floating) with ultracentrifugation. Meanwhile, two major lipid-containing fractions, i.e., α - and β -lipoproteins, were identified in human serum by gel electrophoresis (7), as well as by chemical plasma protein fractionation (8).

It was during this time that John Gofman (Fig. 1), a physician-scientist characterized as “very brilliant” by his Nobel Prize winning PhD mentor, the chemist Glenn Seaborg, entered the arena. In his dissertation work, Gofman had codiscovered isotopes of protactinium and uranium-232 and -233 and would later be recruited to isolate significant quantities of plutonium for the Manhattan Project. Having

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Abbreviations: Lp(a), lipoprotein (a); NHI, National Heart Institute; NIH, National Institutes of Health; S_v, Svedberg flotation rate.

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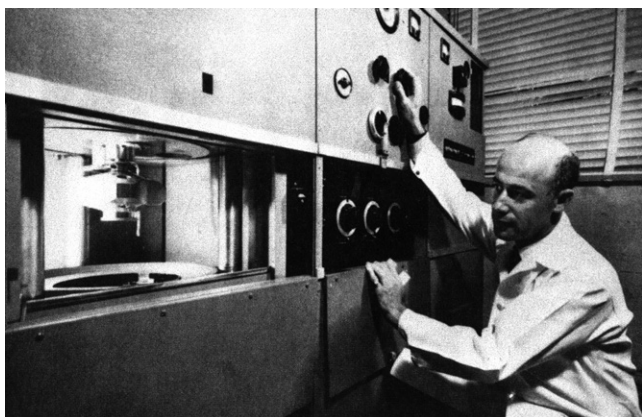


Fig. 1. John W. Gofman. Courtesy of the Lawrence Berkeley National Laboratory.

arrived at the Donner Laboratory of the University of California, Berkeley after completing medical training at the University of California, San Francisco, Gofman decided to focus on studying heart disease. Along with his first graduate student, Frank Lindgren (**Fig. 2**), Gofman began to think about the anomaly presented by Pedersen's work on the analysis of lipoproteins in serum samples.

According to an oral history (9), Gofman and Lindgren utilized the Donner Laboratory-based ultracentrifuges, among the few in the world, to attempt their own analysis of human serum.

"We got the same discouraging results that Pederson got, and we had to say that he was absolutely right. It was a terrible scene. But there was one thing about the ultracentrifuge pictures that was bugging us. It wasn't that the apparent concentration of this lipoprotein was changing with time, it was a dip below the baseline in the ultracentrifugal Schlieren pattern. There was no way that one could interpret this in terms of sedimenting components. I think we talked with Ed Pickels, we talked with each other, and we thought about it, and there was Frank sleeping on the centrifuge while it was running. It was a zany period. I must really give my wife some credit that she put up with us for quite a period there, in our zaniest... We finally came to the idea that there might be a pile up of lipoproteins on the albumin boundary. The pileup would give rise to both an upright pattern and a down pattern, which would explain the dip." (personal communication, 1990).

What Gofman and Lindgren had realized, with the help of Ed Pickels, the inventor of the vacuum ultracentrifuge, was that the incongruity that appeared during fractionation of serum was due to LDLs sedimenting at the density of serum, albeit more slowly, until the concentration of albumin increased, leading to an increased density at the same boundary and the subsequent flotation of the lipoproteins. This migration of LDLs down the tube and then up again explained the apparent artifact observed by Pedersen. Gofman and Lindgren deduced that by adding salt to the serum preparation to control the density, the lipoproteins would remain floating and the accumulation of lipid and protein complexes at the albumin border could be resolved. Repeated and laborious experiments confirmed their hypothesis. Interestingly, their first report (10) was summarily



Fig. 2. Frank Lindgren. Courtesy of the Lawrence Berkeley National Laboratory.

rejected by the editor of the *Journal of Biological Chemistry*, primarily based on the editor's reading of Pederson's earlier observations. After appeal, it was finally accepted, and Gofman received a gracious note from Pederson after its publication congratulating him on solving the boundary problem.

Subsequent to this breakthrough methodological leap, the dedicated and prolific Berkeley team would publish, in short order, a series of papers characterizing the existence of a spectrum of lipoproteins and their variable association with CVD risk (11–13). Lipoproteins were shown to differ in such properties as hydrated density, molecular weight, and chemical composition (13). Further, all of the serum cholesterol, glycerol esters, fatty acids, and phospholipids were accounted for in these lipoproteins, thus making total serum cholesterol necessarily the sum of various members of the lipoprotein spectrum and, as speculated by the group, a less valuable predictor of CVD (11). Classification of lipoproteins was based on their Svedberg flotation rates (S_f), and the densities corresponding to these flotation rates, e.g., VLDLs, IDLs, LDLs, and HDLs, subsequently provided the basis for standard procedures for isolating the lipoprotein classes using the preparative ultracentrifuge, as described further below (13) (**Table 1**). This new classification scheme would ultimately replace the previous categorization system of α - and β -lipoproteins.

Gofman and his team would demonstrate that lipoprotein particles in the S_f 10–20 range were increased with age, male gender, and in diabetes and cases of myocardial infarction (12). Further, lower concentrations of S_f 12–20 lipoproteins were observed with diets restricted in dietary fat and cholesterol (14). Evaluation of the standard S_f ranges, S_f 0–20 and S_f 12–400, which were adjusted for the self-slowness of lipoproteins known to occur with increasing concentration during ultracentrifugation, further showed both of these lipoprotein ranges to be associated with increased coronary disease risk, with the latter range 1.75 times more predictive (11). Gofman et al. (11) used these relative associations to develop an "atherogenic index" to estimate CVD risk.

Building on these initial findings, Gofman and Lindgren set to work to confirm the associations of lipoproteins with

TABLE 1. Classification of lipoproteins

S_f	Density	Current Nomenclature
$S_f > 20$	$d < 1.006$ g/ml	VLDLs
$S_f 12-20$	$d = 1.006-1.019$ g/ml	IDLs
$S_f 0-20$	$d = 1.019-1.063$ g/ml	LDLs
$F_{1,2}$	$d = 1.063-1.21$ g/ml	HDLs

Lipoproteins have been classified based on their Svedberg flotation rates in the analytical ultracentrifuge and their corresponding buoyant densities. The major classes shown are VLDLs, IDLs, LDLs, and HDLs. For VLDLs, IDLs, and LDLs, the flotation rates are designated S_f , and for HDLs, they are designated $F_{1,2}$.

CVD in a larger study that would ultimately evaluate 4,914 men aged 40–59 years, 82 of whom developed clinical manifestations attributable to atherosclerotic disease (15). The study would require the collaboration of several other major research centers, i.e., the Harvard School of Public Health, the University of Pittsburgh, and the Cleveland Clinic. The interesting story of the first rejected National Institutes of Health (NIH) grant application and the influence of Mary Lasker, a prominent political figure, in enabling the “big” study to take place is chronicled in Gofman’s oral history (9) and Daniel Steinberg’s book, *The Cholesterol Wars* (16). Notably, the collaborating research groups were bitterly divided on the interpretation of the findings, and the divergent opinions were expressed in publication, with separate discussions of the data from the Donner team versus the other three centers (15). Whereas the Donner group, led by Gofman, showed that a necessary correction of the ultracentrifuge data was associated with improved CVD risk prediction, the other institutes would claim that cholesterol subfractions lent no more predictive power to assessing CVD risk than total cholesterol alone. Gofman also had the prescient recognition, “something we were realizing as we went along,” that the lipoprotein differences were more relevant in predicting CVD risk in younger people (9). According to Gofman: “We hadn’t realized that or we would have studied a much larger group of younger people. We would have had a bigger effect early” (9).

Over the next several decades, the research evidence would accumulate to overwhelmingly support Gofman’s lipoprotein model of CVD risk prediction. Nevertheless, after the conclusion of the large study, Gofman’s interest in lipoproteins waned and would soon be diverted to the study of radioisotopes, which was the basis of his earlier dissertation work, and later, radiation safety. Left to carry the charge at Donner were, among others, Frank Lindgren and Alexander Nichols (Fig. 3), whom Gofman had identified early on as being able to carry on their own research programs. Lindgren and Nichols would subsequently contribute significantly to investigations into lipoprotein structure, function, and metabolism, as discussed below. They were later joined by Trudy Forte, who was the first to examine lipoproteins morphologically by electron microscopy (17).

Prior to Gofman’s departure from the field of lipoproteins, he and Nichols would work together on a book for medical practitioners entitled *Coronary Heart Disease*, which expanded on their earlier publication related to the dietary



Fig. 3. Alexander V. Nichols (40).

management of hyperlipidemia (18). Of interest was the introduction of the concept that individuals with different lipoprotein profiles respond differently to diets high in fat and cholesterol.

Around the same time period that Gofman and Lindgren were publishing their work on the relationship of lipoproteins to CVD risk, a major heart disease research center was being developed in Bethesda, MD. James Shannon, scientific director of the National Heart Institute (NHI) of the NIH in 1950, had appointed Christian Anfinsen, a protein chemist and later Nobel laureate, to assemble a team of top investigators who could lead and help shape the national agenda for CVD research. A combination of significant clinical and laboratory resources, recently introduced state-of-the-art techniques and technologies, and a team of high level scientists with diverse interests, led to a boom period of productivity in the lipoprotein field from this center. Among the many early accomplishments was, as mentioned above, the adaptation of the findings of Lindgren et al. (13) for the separation of the major lipoprotein classes in the preparative ultracentrifuge by Richard Havel (Fig. 4), Howard Eder, and Joseph Bragdon (19). This method, which was described in a report that was, until very recently, the most cited in the lipoprotein field, enabled the broad implementation of its use in clinical investigations (20). Importantly, the isolated fractions could be analyzed chemically, enabling compositional and structural analyses of the various lipoprotein subfractions (19).

As for lipoproteins at the higher end of the density spectrum, an early retrospective study of myocardial infarction demonstrated lower α -lipoproteins with CVD compared with controls (21). Higher α -lipoproteins were also shown in premenopausal women compared with men, and it was speculated that this physiological difference might explain the differences in coronary artery disease in these two groups (22). Later studies by Gofman et al. (23) showed that consideration of α -lipoproteins analyzed by their ultracentrifugal techniques provided more information than the measurement of α -lipoproteins alone. Specifically, whereas HDL₁ was not associated with CVD risk, HDL₂ and HDL₃ were significantly lower in cases of ischemic heart disease.



Fig. 4. Richard J. Havel. Courtesy of the National Library of Medicine.

The identification of lipoprotein (a) [Lp(a)] as a novel class of lipoproteins was based on the discovery by Kare Berg of the Lp(a) antigen in the early 1960s (24). Lp(a) was shown to be a lipoprotein that floats at a density of 1.050–1.080 g/ml and migrates faster than LDL on paper and gel electrophoresis (24). The atypical pre β lipoprotein band that was demonstrated in cases of hyper- β -lipoproteinemia with xanthomatosis and coronary heart disease was shown to be indicative of high concentrations of Lp(a) (25), and was similar to the “sinking pre β lipoprotein” defined by Rider et al. (26). It was also shown that Lp(a) levels followed an autosomal dominant pattern of inheritance and were associated with increased risk for coronary heart disease (25).

APOLIPOPROTEINS AND LIPOPROTEIN METABOLIC FACTORS

Apolipoprotein identification and characterization

As reviewed in (27), A and B proteins were first differentiated by several groups of investigators who showed differing N-terminal amino acids of these apolipoproteins (27–30). In dog and human chylomicrons, in addition to having the “fingerprints” of the A and B proteins, other proteins seemed to be present, one of which Martin Rodbell and Donald Fredrickson (**Fig. 5**) at the NIH called “C” (31). Another group extracted a third protein from human VLDL that they also called C (32), and subsequently Virgil Brown and colleagues at NIH identified three distinct C apolipoproteins (33, 34), now known as apoC-I, apoC-II, and apoC-III. In the years to follow, Petar Alaupovic, at the Oklahoma Medical Research Foundation, developed a system for identifying apolipoprotein-specific “families” of lipoproteins, e.g., B:E, B:C3, B:E:C3, that could be linked to CVD and other pathologic states (35).

Heterogeneity of the A proteins, now designated apoA-I and apoA-II, was also shown at about the same time, with Angelo Scanu characterizing and defining physicochemical and biological properties of various HDL fractions (36), and Bernard and Virgie Shore (**Fig. 6**), among others, contributing to this work through analysis of the C-terminal



Fig. 5. Donald S. Fredrickson. Courtesy of the National Library of Medicine.

residues of the A proteins (37–39). Alex Nichols of the former Gofman group would further demonstrate that HDLs comprise complex mixtures of multiple subclasses (40). Nichols went on to dissect HDL subclasses and their molecular interconversions; in so doing, he developed a methodology employing native polyacrylamide gradient gels that was widely adopted for HDL subclass analysis, and was subsequently modified to enable the identification of multiple LDL subclasses as well.

The Shores also lent their expertise to identifying what would come to be known as apoE (39). This “arginine-rich peptide” was originally found in VLDL apolipoproteins (41), and a few years later, Gerd Utermann would successfully isolate apoE from VLDL, using isoelectric focusing to separate the three major isoforms, i.e., apoE2, -E3, and -E4 (42).

Notable and rare clinical cases of abnormal lipid metabolism provided early support for the concept that apolipoproteins were critical in maintaining lipid homeostasis and overall health (27). In one case, a young English girl presented with the inability to absorb dietary fats, and analysis of her blood revealed a lack of VLDL, LDL, and chylomicrons as well as very low concentrations of TGs (43). She was the first identified case of abetalipoproteinemia and lacked the protein component of β -lipoprotein, which led to significant clinical abnormalities, including failure to thrive, diarrhea, acanthocytosis, and steatorrhea with possible nervous and musculoskeletal abnormalities. Many of the clinical abnormalities were due in part to the inability to absorb the fat-soluble vitamins A, E, D, and K. The second clinically relevant case was brought to the attention of Fredrickson during his tenure as head of the Molecular Disease Branch at the NHI. In this case, a 5-year-old boy with low total cholesterol, very low HDL cholesterol, and moderately elevated TG concentrations presented with “mammoth amounts of cholesteryl esters in the reticuloendothelial tissues throughout the body.” His bright orange tonsils had been previously removed, and upon further



Fig. 6. Virgie and Bernard Shore. Courtesy of the Lawrence Berkeley National Laboratory.

investigation, it became apparent that his sister also had enlarged bright orange tonsils, and further, that the patient's sister and parents also had very low levels of HDL and α -lipoproteins, providing evidence for a genetic basis for the disease. The disorder was subsequently found to be autosomal recessive in nature and ultimately named Tangier disease, after the Chesapeake Bay island where the family lived (44).

Lipoprotein metabolism

From animal studies in the 1940s, it was known that heparin could render postprandial samples less turbid (45, 46). In elucidating relevant pathways of lipoprotein metabolism, Gofman and colleagues followed up on this observation and showed that the reduction in turbidity resulted from the conversion of VLDL to LDL particles (47). It was speculated that there existed a "post heparin-clearing factor," and this was shown by Anfinsen, Boyle, and Brown (48) to be tissue associated and to have properties that suggested it might be an enzyme. Anfinsen would further suggest that a plasma cofactor might be required for stimulation of the clearing factor. Edward Korn, a post-doctoral student newly arrived in Bethesda, was assigned to work on the project and was able to partially purify the activity, which he named LPL (49). Subsequently, Havel and Gordon at NIH, in the first identification of a genetic disorder of lipoprotein metabolism, demonstrated that deficiency of LPL activity resulted in impaired clearance of intestinally derived chylomicron TG (50). LPL is now known to play a critical role in the catabolism of both endogenous and exogenously synthesized lipoprotein particles. apoC-II was subsequently identified by several independent laboratories as the necessary cofactor for LPL activity (51–53), and apoC-III and apoC-I were found to inhibit LPL (54).

It soon became apparent that multiple lipases existed, although this fact would not be established until the early 1970s, when the most significant of these lipases was identified and shown to be associated with liver cells (55–57). The enzyme was accordingly named hepatic lipase, and one of us worked at the NIH to develop methodologies

that would enable detection of its activity independent of the activity of LPL (58).

Two other early discoveries leading to the identification of key proteins influencing lipoprotein metabolism were made by investigations of the origin and fate of plasma cholesteryl esters. John Glomset (59) identified an enzyme activity subsequently shown to be lecithin:cholesterol acyltransferase, the major determinant of the formation of cholesteryl esters in plasma, predominantly in HDL particles. Shortly thereafter, Alex Nichols reported "that reciprocal transfer of cholesterol esters for glycerides in human serum lipoproteins [between HDL and VLDL] can occur" (60). This observation subsequently led to the identification of cholesterol ester transfer protein, a key determinant of plasma HDL levels, and more recently, a target of drugs that were developed in efforts to reduce CVD risk.

A classification system for lipoprotein disorders

Among the significant achievements at the NHI, by Fredrickson, Levy, and Lees, was the systematic classification of lipoprotein disorders into five categories based on phenotyping analyses performed using paper electrophoresis (27). The series of reports in the *New England Journal of Medicine* describing this system and its scientific background put lipoprotein disorders squarely in the clinical mainstream (61–65). Patients with hyperlipidemias were classified according to which lipoproteins they had in excess, such that those with excess chylomicrons were designated as type I; those with excess LDL, or β -lipoproteins, were defined as type II; and those with excess pre β -lipoproteins were designated type IV. High concentrations of VLDL remnants, or broad- β -lipoproteins, were termed type III, and excess VLDLs plus chylomicrons were categorized as type V. Thus, this system redefined hyperlipidemias as hyperlipoproteinemias, which in many cases had a familial basis.


The categorization of lipid disorders also led to the provision of guidelines related to their dietary management (66), the ground work for which had been laid out a decade earlier when Gofman, Dobbin, and Nichols published their recommendations based on lipoprotein profiles (18). Broadly, restriction of dietary fat to \sim 10% and 30% of energy was recommended for type I and type V hyperlipidemias, respectively. Increases in the polyunsaturated fat to saturated fat ratios were advised for types II, III, and IV and reductions in body weight to ideal were advised for types III and IV in which overweight and obesity usually presented. Many of these same recommendations form the basis for current dietary guidelines, although these have not been closely tied to specific lipoprotein patterns.

The use of lipoprotein parameters to assess CVD risk clinically was greatly facilitated by the development of simplified clinical laboratory procedures, work which began with early studies by Burstein and Samaille (67), who developed a procedure for precipitating β -lipoproteins with heparin-manganese solutions so that unprecipitated HDLs could then be directly quantitated using chemical methods. The NIH group later adapted and extended this procedure to devise a precipitation-based algorithm for the estimation of LDL cholesterol by a formula [total

cholesterol – (HDL cholesterol) + TG/5], also known as the Friedewald equation (68). This equation was developed to approximate the results obtained by determination of cholesterol in the $d > 1.006$ g/ml plasma fraction after heparin-manganese precipitation. However, this fraction also includes IDLs and takes into consideration only levels of lipoprotein cholesterol and not lipoprotein particles (69). Nonetheless, this procedure enabled the widespread utilization of lipoprotein analysis for clinical assessment of CVD risk, as well as for implementation in large-scale epidemiology studies and clinical trials for the evaluation of therapies aimed at CVD risk reduction.

Although beyond the time frame encompassed by this review, the next phase of lipoprotein research was highlighted by the groundbreaking discovery by Joseph Goldstein and Michael Brown of the LDL receptor for which they were awarded the Nobel Prize in 1985. This work triggered an explosion of research in molecular and genetic influences on cholesterol homeostasis and lipoprotein disorders, and the development of new therapies, including statin drugs (70).

CONCLUSIONS

The work and achievements of a multitude of talented and dedicated scientists in the decades spanning the middle of the last century have provided the framework for current practices aimed at optimizing lipid and lipoprotein profiles in the prevention and treatment of CVD. The discovery, classification, and characterization of lipoproteins and some of their key metabolic determinants represent foundational accomplishments in atherosclerosis research. Since these major contributions were made, a strong relationship between LDL and atherosclerosis has been definitively established, and the ability to easily measure lipoproteins has allowed clinicians to appropriately target both lifestyle and pharmacological interventions for CVD risk reduction. Many recent studies, however, have underscored the importance of considering the heterogeneity among LDL and HDL particles, e.g., their quality and number, and their variable association with CVD risk. Going forward, the exploitation of the full power of lipoprotein analysis, building on the accomplishments of the pioneers in this field, hold promise for contributing to the further advancement of clinical science and practice for CVD prevention. 

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