

APPENDIX-E

Some Pathways toward Understanding the Role of Lipoproteins in Atherosclerosis and IHD

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The Lipid Hypothesis of Atherosclerosis and IHD proposes that entrance of certain types of plasma lipoproteins into the intima, from the circulating blood, is the initial step in the whole atherosclerotic process. Between the endothelium and the internal elastic membrane, the plasma lipoproteins (oxidized, or not oxidized) have no physiologic function. They are "out of place," and unless they exit rapidly, they become a "foreign substance" which elicits efforts at removal or isolation --- the inflammatory response (Chapter 44, Part 3).

The hypothesis suggests that, "all other things being equal," the higher is the concentration of the pathogenic lipoproteins in the bloodstream, the greater will probably be their infiltration of the intima. This, then, suggests that the important measure at issue is the blood-level of the culprit lipoproteins, "operating" over time. Of course, back in 1949, all this remained to be established.

● Part 1. Some Remarkable Rabbits Who Made a Lasting Impression

In medical school, I had been quite impressed by the work of Anitschkow (1933) on the cholesterol-fed rabbit. Dietary cholesterol is an exotic substance to rabbits, who are herbivorous animals. The work showed induction of massive elevation in the blood-levels of cholesterol, AND it showed induction of an atherosclerosis-like picture in the rabbit's large arterial blood vessels. In academic circles, the results were disparaged by some professors. How, they asked, could feeding cholesterol to rabbits teach us anything useful, when the human body needs and synthesizes cholesterol on its own (regardless of diet)?

They seemed unable to look past the FEEDING aspect of the work. But if one looks past WHY those rabbits had high blood-levels of cholesterol, one is staring at strong evidence that elevated blood-levels appeared to cause DEPOSITION of cholesterol in the rabbits' arteries.

It seemed quite unreasonable to me that anyone would refuse to look further into the question: "How does the blood level of cholesterol come to be related to the atherosclerotic-like deposits in arterial walls of rabbits, and could a similar mechanism be causing atherosclerosis in humans?"

At that time (1947), two classes of plasma lipoproteins (alpha and beta) had been identified, and there was also some human evidence that in certain families, arterial disease was related to the level of blood cholesterol. But for the general population, the findings were said to be very uncertain (Chapter 44, Part 3b).

Two new sets of rabbits suddenly made the problem irresistible.

In 1948 and 1949, G. Lyman Duff and Gardner McMillan reported on the development of atherosclerosis in rabbits with alloxan-induced diabetes. The results were remarkable: Compared with non-diabetic rabbits which were fed a diet equivalently high in cholesterol, the alloxan-diabetic rabbits developed equal or higher blood-levels of cholesterol, but markedly LESS atherosclerosis. This contrast was intriguing in its own right --- and all the more so because, among humans, the diabetics are MORE prone to atherosclerosis than non-diabetics.

Duff and McMillan made the signal observation that an excessive, visible lipemia (producing a cloudy, creamy look) characterized the blood of the alloxan-diabetic rabbits. These investigators surmised that the physical state of the cholesterol in such blood might have been altered in the alloxanized animals, and that the alteration might account for the lower degree of atherosclerosis.

Atherogenicity: Indication that Size Matters, among Lipoproteins

At the Donner Laboratory, we pursued their speculation --- with our new capability of identifying the spectrum of lipoproteins in their NATIVE states (Chapter 44, Parts 3b + 3g). In the early 1950s, with new sets of rabbits, our group at Donner demonstrated that aortic atherogenesis, at least in the rabbit, was strongly related to the serum level of certain lipoproteins of a limited size-range, and NOT to lipoproteins above or below that size-range (Gofman 1950-a, + Jones 1951, + Pierce 1952).

Even massive elevation, in concentration of lipoproteins larger than a certain size, resulted in little or no incremental atherosclerosis in rabbits. Our interpretation: Probably the larger molecule's size prevented entry into the rabbit's aortic intima. With additional sets of rabbits, Frank Pierce (1952, Tables 2 and 4) confirmed that alloxan-diabetic, cholesterol-fed rabbits on the average developed a much lesser degree of atherosclerosis than normal non-alloxanized non-diabetic rabbits, fed a comparable high-cholesterol diet.

MOST IMPORTANTLY, Pierce (1952, Table 3) showed that the diabetic cholesterol-fed rabbits transported their massive concentrations of cholesterol mainly in large lipoproteins having Sf values above 100. By contrast, normal non-diabetic cholesterol-fed rabbits who develop atherosclerosis, carry the cholesterol mostly in smaller lipoproteins of the Sf 12-30 range.

The various sets of rabbits left us with a strong expectation of a causal relationship in HUMANS between some classes, but not all classes, of plasma lipoproteins and atherosclerosis.

"Man Is Not a Rabbit!" The Necessity of Human Data

Of course, lipid-induced atherosclerosis in rabbits can never establish causal relationships for HUMANS. After all, "Man is not a rabbit!" From the start, it was evident to us that such questions had to be studied in humans. Prospective studies are the most reliable, but by their very nature, they require years for their completion. Meanwhile, our group undertook case-control studies. A question demanded intense investigation by all possible routes:

"Could phenomena, similar to the observed phenomena in rabbits, explain atherogenesis in humans in the coronary, carotid, peripheral, and cerebral arteries?"

● Part 2. An Encouraging Piece of Luck, As We Began Human Case-Control Studies

Very early in our work at Donner, we began to analyze samples of blood from clinically healthy humans. It was clear that the lipoprotein spectrum in humans was VASTLY larger than the range in normal rabbits on their normal diet. Such rabbits have serum lipoproteins in the Sf 5-8 range (Gofman 1950-a, p. 168). Analysis of the blood from healthy young persons (18-30 years of age) revealed lipoproteins in the Sf 6-8 range --- very much like the normal rabbits. But at older ages, the human spectrum grew very much broader.

Where, in the lipoprotein spectrum of humans, should we BEGIN our studies of a possibly causal role of lipoproteins in atherogenesis? We could not simultaneously develop reliable laboratory

techniques with the new methods, and also study everything at once.

In those early days at Donner, we knew (from the work of others) that feeding cholesterol to normal non-diabetic rabbits would produce atherosclerosis in a few months. As our Donner rabbits progressed in the cholesterol feeding program, the blood levels of Sf 5-8 lipoproteins increased. After an even longer time on cholesterol-feeding, an additional peak appeared in their ultracentrifugal flotation patterns (Chapter 44, Box 2). The rabbits' additional peak, in the Sf 10-20 region, announced the development of an additional set of lipoproteins circulating in the rabbits' blood --- while atherosclerosis was developing (confirmed at autopsy).

Luck: Our First Look at the Blood of a Heart-Attack Survivor

Soon after the Sf 10-20 lipoproteins had developed in our rabbits, we decided to find out what the blood looked like in at least one proven case of myocardial infarction. We obtained a blood sample from a lady several weeks after her heart attack. We spun her serum sample ... with immense curiosity.

Her serum lipoprotein pattern looked just like the pattern in rabbits, after several weeks of cholesterol-feeding: A fairly high concentration of lipoproteins of the Sf 0-10 class AND an appreciable elevation of lipoproteins of the Sf 10-20 class.

As we would soon learn, NOT all heart-attack survivors have this pattern. But the fact, that the very first blood-sample from a human heart-attack survivor matched blood from the rabbits who were developing experimental atherosclerosis (confirmed at autopsy), was a piece of good luck which encouraged us to believe that our investigations were definitely worth pursuing.

2a. Results from Our First Case-Control Studies

As a result of this event, we chose serum concentrations of the Sf 10-20 segment of the lipoprotein spectrum, as the parameter for comparison in our first case-control study of humans. In a series of 104 cases of confirmed myocardial infarction, we found elevated concentrations of the Sf 10-20 lipoproteins, compared with controls who had no overt coronary disease (Gofman 1950-a).

By this time also, we had analyzed enough blood samples from clinically healthy males and females, from ages 20 to 40 and from 40 to 70, to perceive the significantly higher mean concentrations of the Sf 10-20 lipoproteins in males than in females before age 40, and to perceive that beyond age 40, concentrations increase in both sexes --- with the females catching up to the males. We noted (Gofman 1950-a, pp.170-171) that these findings --- together with the observation that men (on the average) have a higher frequency of atherosclerosis before age 40 than do women, and that the frequency of atherosclerosis increases with age in both sexes --- were consistent with the hypothesis that the Sf 10-20 lipoproteins are atherogenic.

The parallelism of our rabbit and early human studies created the suspicion that there might be something very special about the Sf 10-20 lipoproteins, and that whatever those special properties might be, they might also cause atherogenesis.

Studying More of the Spectrum

As soon as we were able, we added the Sf 20-100 segment of lipoproteins to our case-control studies. By 1951, comparison of patients having coronary disease, versus normals, indicated that the Sf 20-100 lipoproteins in humans are also and independently atherogenic (Jones 1951, + Gofman 1952-a, pp.125-126 + Gofman 1952-c, p.286). By mid-1953, such findings were based largely on serum lipoprotein measurements of 239 males with clinical Coronary Heart Disease, versus 740 males of corresponding ages (40-59 years) without overt CHD (Gofman 1953).

2b. Correction of an Error Concerning the Sf 0-12 Lipoproteins

From ultracentrifugal work by others, we were aware that migration-rates can be affected by the concentrations of various molecules in a sample (Gofman 1950-a, p.168). Concentration affects rates in two ways: By self-slowng of molecules and by interaction between molecules.

Before long, we realized that our flotation-technique underestimated the concentration of especially the Sf 0-12 lipoproteins --- and increasingly so, with increase in their true serum levels. The result was that the net difference in Sf 0-12 lipoprotein levels, between persons with overt CHD and persons without overt CHD, was underestimated. Hence, we erroneously reported that the Sf 0-12 segment appeared NOT to be atherogenic (Gofman 1952-a, p.122).

During 1952, this error was eliminated from our work by making adjustments which took into account the effects of concentration on the flotation rates, not only of the Sf 0-12 lipoproteins but also on the segments of the lipoprotein spectrum above Sf 12 (technical details in DeLalla 1954-a; discussion in Co-op 1956, p.696, pp.714-715). The improved methodology became our routine practice in 1952 and thereafter, as did the use of "Std Sf" (Standard Sf or, in certain journals, Sf with a superscript of zero) to indicate that measurements were properly adjusted for the effects of concentrations.

With the error corrected, the Std Sf 0-12 lipoproteins were also recognized as atherogenic (Gofman 1953).

Decision Not to Pursue Our Ultracentrifugal Studies above Sf 400

Of course, the entire lipoprotein spectrum needed evaluation, for any relationship with atherogenesis in humans. However, for technical and biological reasons, we soon decided to limit our ultracentrifugal studies of the "Low-Density Lipoproteins" to the Sf 0-400 segment. High variability of lipoprotein findings near and above Sf 400, with respect to meals, convinced us that lipoproteins above Sf 400 would need to be studied in a different manner (Glazier 1954, p.396).

Evaluation of the Lipid Hypothesis in a Rigorous Manner

Case-control studies, excellent for guidance in some general directions, do not suffice for evaluation of the Lipid Hypothesis in a rigorous manner. A prospective study was initiated in 1950 (Part 11, below). And many additional lines of inquiry were required --- some of which are described in Parts 3 through 10, below.

● Part 3. How Do Sf 0-400 Measurements Vary with Food Intake?

If one studies the parameter, "blood levels of the Sf 0-12, 12-20, 20-100, and 100-400 lipoproteins," in relationship with Coronary Heart Disease, one would like to know the nature and extent of variation in those measurements within every individual studied, both acutely over daily cycles and chronically during decades of life. Although this goal is not attainable, one does what one can. Indeed, like many investigators, we began with measurements of the most easily controlled persons: Ourselves. The team at Donner had some pretty sore arms from sampling our own blood, day and night, in relationship with various diets.

From our measurements for the first prospective study (Part 11 of this Appendix), soon we also had data from 1,231 employes of the Los Angeles Civil Service and from 2,105 participants in the Framingham Heart Study. The Los Angeles data are for fasting blood samples; the Framingham data are for non-fasting samples. While we cannot be positive that the two population samples are identical in all other respects, we have taken data from these two population surveys to represent fasting (Los Angeles) and non-fasting (Framingham) lipoprotein levels. For ages below 30, we made non-fasting measurements of 222 members of the university population at U.C. Berkeley, and of 15 clinically healthy children of a Pediatric Clinic.

The measurements, fasting vs. non-fasting by gender and age, are tabulated and graphed in Glazier 1954. Below, we present the major findings just for age 40 and higher.

Mean Values of Lipoprotein Levels: Std Sf 0-12, Std Sf 12-20, Std Sf 20-100, Std Sf 100-400

For Males	Fasting	vs	Non-Fasting
Std Sf 0-12	Fasting samples average about 11 % higher than non-fasting		
Std Sf 12-20	Fasting samples average about 14 % higher than non-fasting		
Std Sf 20-100	Fasting samples average about 5 % LOWER than non-fasting		

Std Sf 100-400 Fasting samples are, on the average, about HALF the level of non-fasting

For Females

Std Sf 0-12

Fasting samples average about 7 % higher than non-fasting

Std Sf 12-20

Fasting samples average about 2-3 % higher than non-fasting

Std Sf 20-100

Fasting samples average about 20% LOWER than non-fasting

Std Sf 100-400

Fasting samples are, on the average, about HALF the level of non-fasting

● **Part 4. From Person to Person, Do the Sf 6-8 Lipoproteins Have the Same Lipid Constituents?**

Having opened up the vast spectrum of plasma lipoproteins for study, the team at Donner Laboratory needed to learn whether a specific class of ultracentrifugally-defined lipoproteins --- Sf 6-8, for example --- had a constant chemical meaning from one person to another, and for one person at different times.

Frank T. Lindgren, Alex V. Nichols, Bernard Shore, Virgie Shore, Thomas L. Hayes, Norman K. Freeman, and Gary Nelson attacked this large and difficult problem with admirable hard work and success, during much of the first decade of the work at Donner. Some of their major findings are brought together in an excellent overview paper in the *Annals of the New York Academy of Sciences*: "Structure and Homogeneity of the Low-Density Serum Lipoproteins" (Lindgren 1959).

Boxes 1 and 2 of this appendix indicate that, WITHIN a specific Sf-range, the lipid composition of the LDL, IDL, and VLDL classes is remarkably similar from person to person.

The constancy of composition was a most welcome feature, as we proceeded with various case-control and prospective studies of the atherogenicity of various Sf-classes of lipoproteins, and proceeded to study the metabolism of these molecules.

● **Part 5. Molecules in a Metabolic Chain? The Steady-State Concentrations**

By 1951, we had developed the view "that all the molecules from Sf 40,000 down to Sf 4 (and possibly into the high density class) represent a sequence of molecules in a metabolic chain involved in the ultimate utilization of glyceryl esters and/or fatty acids" (Jones 1951, p.360). And (also p.360): "From tracer studies now in progress, it appears that the average lifetime of all the lipoprotein species of the low density group is of the order of several hours. Therefore, the most probable explanation of a given concentration of a particular lipoprotein is that it is the steady-state concentration at which the influx and utilization rates are equal." (See also Gofman 1952-c, + McGinley 1952, + Pierce 1954-a, + Lindgren 1956, + and Lindgren 1959.)

● **Part 6. How Do Lipoproteins Move from an Artery's Lumen to Its Intima?**

If certain plasma lipoproteins are the source of the lipids observed in atherosclerotic lesions, we (and others) wanted to learn how the lipoproteins moved from the artery's lumen into its intima. At the outset, we had no preconceived notions. For instance, we did not start in 1949 with the infiltration concept discussed in Chapter 44, Part 5.

In 1949, it was proposed that the lipid material present in atheromatous lesions might be carried there by cells such as macrophages (Leary 1949). In 1949, Kuntz and Sulkin tested the idea by injecting hypercholesterolemic rabbits with dyes. The dyes ended up in visceral phagocytes but not in the lipid-filled foam cells of atherosclerotic lesions (Kuntz 1949). We felt that their experiment did not settle the issue, however, because the dyes had not been injected BEFORE cholesterol-feeding. At the Donner Lab, John Simonton undertook to inject rabbits with a radioisotope which was demonstrated to concentrate in visceral macrophages. Then cholesterol-feeding was initiated. The results confirmed that macrophage migration into the intima was not a significant source of the lipid-filled foam cells in the rabbits' subsequent atherosclerotic lesions (Simonton 1951).

● **Part 7. Why We Should Identify Non-IHD Disorders with Aberrant Lipoprotein Patterns**

The Donner team gave considerable attention to identifying non-IHD disorders and conditions

which are characterized by aberrant concentrations of plasma lipoproteins. These include diabetes (Engelberg 1952-a + Engelberg 1952-b, + Kolb 1955), infectious hepatitis (Pierce 1954-b), infectious mononucleosis (Rubin 1954), biliary cirrhosis (McGinley 1952), obesity (Gofman 1952-b), xanthoma tendinosum, xanthoma tuberosum, nephrotic syndrome, chronic biliary obstruction, myxedema, "essential hyperlipemia," and possibly others (Gofman 1954-e).

When persons have disorders and conditions which can cause unusual concentrations of various classes of lipoproteins, these persons necessarily influence the AVERAGE concentrations measured in population samples. This can cause false conclusions, if the frequency of such persons is appreciably higher in one group than in a second group with which the first group is compared. We and others needed to be watchful for these potential pitfalls, when comparing average concentrations of serum lipoprotein classes in population samples with clinical Ischemic Heart Disease and samples without overt IHD.

Box 3 (from Gofman 1954-e) constituted a warning, about the patterns of extraordinary serum lipoprotein levels which may characterize certain disorders.

● Part 8. A "Smoking Gun": Reversal of Xanthomas by Lipid-Lowering Regimes

In the early 1950s, the Lipid Hypothesis of Atherogenesis was VERY far from accepted. Among the clues ("smoking guns") which convinced the Donner group that our work was well worth pursuing were our clinical observations with respect to xanthomas --- lipid-filled lesions visible on the outside of people with the heritable disorders called xanthoma tuberosum and xanthoma tendinosum.

We soon developed the opinion that visible xanthomatous lesions, of the skin and tendon sheath, are the equivalent of atheromas, and that these lesions --- like atheromas of the arteries --- have their genesis in the deposition of lipoprotein molecules, from the circulating blood, into tissue-sites where deposition should not occur.

Quite early in our work, also, we detected a striking difference in the serum lipoprotein patterns of people with xanthoma tuberosum versus xanthoma tendinosum (McGinley 1952, + Gofman 1954-e). The mean levels, tabulated below, are in mg per 100 ml, and the numbers in parentheses are for matched controls (from Gofman 1954-a, p.432, 434). Because the two disorders typically differ in the distribution patterns of the lesions on the body, we postulated that different serum lipoproteins deposited generally in different tissues because of the molecules' different SIZES. And we wondered why this should not be the case for deposition in arterial walls. too.

	Sf 0-12	Sf 12-20	Sf 20-100	Sf 100-400
X.Tendinosum (18 cases)	793 (336)	150 (65)	128 (92)	36 (56)
X.Tuberosum (23 cases)	206 (358)	128 (74)	616 (105)	650 (72)

Most exciting of all, when we were able to reduce serum lipoprotein levels pharmacologically or by diet, the patients stopped developing new lesions, and we could watch the skin lesions gradually involute and disappear, unless they had been of long standing and were fibrotic (Gofman 1952-d, + Gofman 1958, + Gofman 1959 at pp.210-211).

● Part 9. Early Evidence of Independent Atherogenicity, Sf 12-20 vs. Sf 20-100 Lipoproteins

From the outset, we knew the importance of finding out WHICH segments (if any) of the plasma lipoprotein spectrum are atherogenic (Chapter 44, Part 3a). We faced the possibility that some segments may have elevated concentrations in cases of Coronary Heart Disease (CHD), only because of potentially tight positive correlations with an elevated concentration in IHD cases of one TRULY atherogenic segment of the spectrum. So we began looking at this issue in our early case-control studies (presented in Jones 1951, + Gofman 1952-a, + Gofman 1952-c).

Figure E-1 (which is adapted from Gofman 1952-c, p.286) is one way in which we presented our findings. The plasma lipoprotein measurements on 253 "normals" (males, ages 41-50, NOT having overt CHD) are compared with such measurements in 72 male patients (ages 41-50) having overt CHD. The comparison is based on the lines of best fit from linear regression. Figure E-1 demonstrates that, at any serum Sf 12-20 lipoprotein level (in mg/100 ml, or mg%), the Sf 20-100

lipoprotein level is higher in CHD cases than in normals.

This finding represents evidence for an INDEPENDENT atherogenic contribution by Sf 20-100 lipoproteins. If the presence of Sf 20-100 lipoproteins contributed nothing to atherogenic risk beyond the contribution from the Sf 12-20 lipoproteins, the two lines of best fit (CHD cases and normals) would fall on top of each other, within experimental error.

Although we regard this type of evidence as a strong indication that the triglyceride-rich Sf 20-100 lipoproteins are independently atherogenic, we have pointed out that the issue remains unsettled today, some 47 years later (Chapter 44, Parts 3i + 4).

Separately, we approached the independence issue by using linear regression to evaluate how strongly or weakly blood-levels of various segments of the lipoprotein spectrum correlate with each other, in clinically healthy persons by gender and age (Gofman 1954-a, Tables 8, 9, 10, + DeLalla 1961, Tables 40 through 46). We reasoned that if blood-levels of two segments of the lipoprotein spectrum are not strongly correlated in clinically healthy people, AND if these two segments both are above normal levels in CHD cases, then each segment may be contributing, INDEPENDENTLY, to atherogenic risk. Such findings create the obligation to find out --- but it is surely not easy to do so.

● Part 10. Segregation of Infarcts from Normals: Sf-Class vs. Total Cholesterol

Not long after the Donner team revealed the vast spectrum of plasma lipoproteins, there were voices at some scientific meetings suggesting that serum measurements of the ultracentrifugally-identified lipoproteins would add nothing useful about atherogenesis beyond what could be determined from the simpler chemical measurement of total serum cholesterol.

Their prediction deserved testing as soon as feasible. By 1951, the Donner team published its first results --- from comparing male survivors of Myocardial Infarcts with male "normals."

Results are shown in our Figure E-2 (adapted from Jones 1951, Figure 2, p.364). The results for males ages 41-50 (based on 64 survivors of Myocardial Infarcts and 273 "normals") revealed that there was significant segregation of infarcts from normals by the Sf 12-20 measurement, independent of serum cholesterol. Not shown here are the results for males ages 51-60 (based on 92 survivors of Myocardial Infarcts and 126 "normals"). Those results also revealed that there was significant segregation of infarcts from normals by the Sf 12-20 measurement, independent of serum cholesterol (Jones 1951, p.364).

Our analysis revealed, too, that for males ages 41-50 (but not for ages 51-60), there was independent segregation of infarcts from normals by the serum cholesterol measurement, independent of the Sf 12-20 lipoprotein measurement.

Our Table E-1 (adapted from Jones 1951, Table 3, p.366) demonstrates this finding by the "matched-series" method, for males ages 41-50. Our interpretation is that the independence of the serum cholesterol measurement (independent from the Sf 12-20 measurement) resided in the contributions of extra serum cholesterol from atherogenic lipoproteins of the cholesterol-rich Sf 0-12 segment, as well as some cholesterol from the glyceride-rich Sf 20-400 lipoproteins. However, for the males of ages 51-60, the cholesterol measurement did not independently segregate infarcts from normals, while the Sf 12-20 measurement successfully did so (Jones 1951, pp.366-367).

Of course, the merits of the ultracentrifugal and chemical measurements would not be established by a single study. There was much more to be done.

● Part 11. Do Elevated Serum Lipoproteins Precede or Follow an Infarct?

The initial findings from our case-control data (Gofman 1950-a) created considerable interest, but case-control data could never prove that the observed elevation of Sf 12-20 lipoproteins in survivors of myocardial infarction did not occur AFTER (and due to) the infarction. Only a prospective study could establish which came first.

11a. An Answer from the First Prospective Study

Before the end of 1950 and under a grant from the National Heart Institute, a prospective study was underway at four laboratories: Donner Laboratory at U. California at Berkeley, the Cleveland Clinic Foundation, the Dept. of Biophysics at U. Pittsburgh, and the Dept. of Nutrition at Harvard School of Public Health. The effort was called a "Cooperative Study of Lipoproteins and Atherosclerosis" (Co-op 1956 in our Reference List).

The study did establish that, relative to the base population in which they started, the participants who developed definite new "events" (myocardial infarction, coronary thrombosis, ECG abnormality associated with coronary artery disease, angina pectoris) had above-average serum lipoproteins BEFOREHAND. Total serum cholesterol, transported only by lipoproteins, was of course elevated BEFOREHAND, too.

The total follow-up time was cut off at 2 years or less. Among the 4,914 males, ages 40-59 at entry to the study, 82 "events" developed within that time-frame, according to the Review Committee. These 82 events included 27 "Myocardial Infarctions, definite" and 38 "Myocardial Infarctions, definite, by ECG only" (Co-op 1956, p.709).

Because one goal of the Co-op Study was "to determine the range of values of the Sf 12-20 fraction (lipoprotein) in 'normal' persons of all ages and both sexes," the four laboratories actually measured the serum lipids of approximately 15,000 persons in a period of about 3 years (Co-op 1956, p.692, p.693). Measurements of the total serum cholesterol and the Sf 20-100 lipoproteins were made, too (Co-op 1956, p.695-696).

11b. Disagreement over Converting to the More Accurate Std Sf Measurement

Among the four research teams, major differences of opinion --- much of which is presented in Co-op 1956 --- developed concerning several aspects of the studies themselves and of the results. There was some merit on both sides of the arguments which ensued.

The central disagreement centered on the whether or not to convert the Sf measurements to Standard Sf measurements (Part 2d of this Appendix) --- a conversion which would have been fully consistent with the study's "blinding" protocol. The more refined Std Sf measurements made very substantial differences in levels of both the Sf 12-20 and Sf 20-100 segment of the lipoprotein spectrum --- as can be seen by comparing Exhibit D with Exhibit G in Co-op 1956 (p.735 and p.738). Fortunately, use of the more refined measurement-procedure resulted in no loss of previously made measurements, because the original film could be re-evaluated by the refined procedure (Co-op 1956, p.715).

We at Donner argued vigorously for using the more accurate technique, but we were not in charge and we did not prevail. Therefore, in order that measurements from all four laboratories be comparable, the Donner team agreed to continue providing the less accurate measurements. However, we also provided the more accurate measurements (Appendix-A of Co-op 1956).

Describing the outcome of the Co-op Study, there is a concluding statement by Professor E. Cowles Andrus (Johns Hopkins University) in the report at pp.713-714:

"The participants in this study concur in the presentation to this point. They agree in finding that atherosclerosis, as manifested by clinical signs of coronary artery disease, is associated with a disorder of lipid metabolism and that there is some predictive value in the various lipid measurements examined. However, because of clear divergence of opinion between the Eastern Laboratories and the Donner group with regard to the degree and specificity of this predictive value, and indeed with regard to the significance of certain data in relation thereto, it was agreed that the discussion and conclusions would be prepared independently and presented separately by [the] two groups ..."

11c. Some Valuable Lessons from the Co-operative Study

Not too bad, as an outcome, when we consider that such a project was probably launched prematurely --- only about one year after any technique for separating and measuring serum lipoproteins ultracentrifugally had been initiated. We were still learning about its complexity.

Nonetheless, four separate laboratories undertook this complex new technology, and managed

to carry through the measurements with satisfactory and provable reproducibility. And all four teams had some exceedingly valuable education in two areas of great importance:

(1) We received advice generously from Dr. J. Franklin Yeager of the National Heart Institute (Grants and Training Branch) on how to work together and not to be scientific prima-donnas. And:

(2) We received invaluable advice and firmness from Felix E. Moore of the National Heart Institute (Biometric Research Section) on the rules of credible, blinded epidemiologic research --- rules which did characterize the actual conduct of the project. Sadly, bio-medical research sometimes still permits (when it should disdain) violations of such fundamental rules as "Never tamper with the input data, once the results of the follow-up are known."

● Part 12. Inclusion of Std Sf 0-12 and Std Sf 100-400 in Two Prospective Studies

The Co-operative Study (1956), which did not include consideration of the Sf 0-12 or the Sf 100-400 serum lipoproteins, clearly could not address the question: Are these segments of the lipoprotein spectrum also predictive of de novo Ischemic Heart Disease? Additional prospective inquiry would be required to find out.

12a. 1966: Results from the Framingham Study

During the Co-operative Study, the Donner team had made ultracentrifugal measurements of serum lipoproteins (Std Sf 0-12, 12-20, 20-100, 100-400) and chemical measurements of total serum cholesterol for "townspeople" who were then new entrants to the Framingham Heart Study. We measured the bloods of 2,022 males, ages 30-69 at entry, and 2,487 women, ages 30-69 at entry (Gofman 1966, p.685). Our measurements were permanently "set in concrete," when they were contemporaneously filed by Donner with both Framingham and with Felix Moore at the National Heart Institute.

In 1965, Thomas R. Dawber, M.D. (then Director of the Framingham Study) and his colleagues generously provided us at Donner with a listing of the 319 de novo cases of Ischemic Heart Disease which had occurred during the intervening 12 years among those 4,509 Framingham entrants measured by Donner. In other words, the list represented results for about a 12-year follow-up. In toto, there were 221 de novo cases among the men, and 98 cases among the women. The results for all males combined, all females combined, and for subsets by age, were fully reported in Gofman 1966 (pp.681-685).

Compared with the male base population, all ages combined, the de novo IHD cases had very significantly ($p < 0.001$) higher serum levels of Std. Sf. 0-12, 12-20, 20-100, 100-400 lipoproteins and total cholesterol (Gofman 1966, p.682, Table 1). In other words, EACH of these five measurements was predictive of clinical Ischemic Heart Disease. When examined by age-groups, the differences between base and cases declined with age at entry, and none was significant in the group which had been 56-62 years old at entry (Gofman 1966, p.683, Table 6). This decline in segregation, with advancing age at measurement, had also been observed in our case-control studies (Gofman 1954-d, p.593).

For the females who had been ages 40-69 at entry, the de novo cases had higher levels of each of the five measurements than did the base, but the findings were statistically significant only for the Std Sf 0-12, Std Sf 20-100, and total serum cholesterol (Gofman 1966, p.682, Table 2).

In Appendix-G, Part 4, we show the mean measurements, de novo cases versus base, for the males ages 30-39 at entry.

12b. 1986: Some Later Results from the Framingham Study

In 1986, William P. Castelli, M.D. (then Director of the Framingham Heart Study) authored "The Triglyceride Issue: A View from Framingham" in the American Heart Journal (Castelli 1986). In that paper, he briefly discussed Sf 0-20 and Sf 20-400 measurements made on males entering into the Framingham Study (Castelli 1986, pp.434-435).

His Figure 4 is entitled "CHD risk according to lipid level on entry into the Framingham Study:

Men aged 30–62." It shows clearly that the Morbidity Ratio rises in a steady linear relationship with rising serum levels of the cholesterol-rich Sf 0–20 lipoproteins, and separately, with rising levels of the triglyceride-rich Sf 20–400 lipoproteins.

12c. 1966: The Livermore Lipoprotein Study

In 1954, the Livermore (Weapons) Laboratory asked me to organize its industrial medical facility, where employes at all levels (from the very top to the bottom) would receive complete medical examinations at intervals of approximately 1.5 years.

Nature of This Database

During the years 1954–1957, the Donner Laboratory analyzed the serum lipoproteins and total serum cholesterol from the non-fasting bloods of this population of workers. Oliver F. DeLalla described the study as follows (DeLalla 1958, p.18):

"The healthy subjects for this investigation were employes of the University of California Radiation Laboratory at Livermore, California, all of whom are periodically given routine physical examinations. Venous blood samples --- 30 ml from each subject --- were taken between the hours of 8 a.m. and 3 p.m., without any restriction of diet ... There were certain restrictions, however; no individual was included in the study whose medical records showed any of the following conditions." List follows, verbatim:

- Acute infection at the time of sampling.
- Poliomyelitis with residual deformity.
- Surgery involving removal of part or all of any organ.
- Any condition requiring that the subject take medications such as thyroid, steroids, etc.
- Cardiovascular disease history.
- Cancer history.
- Multiple sclerosis.
- Pregnant women and persons following special diets were also excluded.

"The total number of subjects qualifying was 2,297, of which 1,961 were males and 336 were females. Their ages ranged from 17 to 65 years. The 2,297 samples were collected and analyzed at a rate of about 20 per week over a 3-year period. There were no delays in the analysis of any sample. Whenever the samples were collected, they were immediately put through the complete analysis, which normally takes from 7 to 10 days." The more detailed distribution is as follows (from DeLalla 1961, p.139):

Ages	Males	Females
17-29	585	190
30-39	834	99
40-49	399	37
50-65	143	10
Sums	1,961	336

The database for each person includes ultracentrifugal measurements of serum lipoproteins Std Sf 0–12, 12–20, 20–100, 100–400, HDL-1, HDL-2, HDL-3, plus the Atherogenic Index, total cholesterol, systolic blood pressure, diastolic blood pressure, relative weight. Persons studied twice = 374.

Follow-Up Findings in 1966

In 1966, we conducted a follow-up of the males in this population, which we reported in Gofman 1966. Out of the base population of 1,961 subjects, 38 cases of de novo Ischemic Heart Disease had developed.

Compared with the male base population, all ages combined, the de novo IHD cases had very significantly ($p < 0.001$) higher serum levels of Std Sf 0–12 and Std Sf 12–20 and total cholesterol, significantly higher ($p < 0.01$) higher levels of Std Sf 20–100, and higher (but not significantly) Std Sf 100–400 lipoproteins (Gofman 1966, p.684, Table 10). In other words, EACH of these five measurements was elevated BEFORE the development of clinical Ischemic Heart Disease. The

findings about HDL are discussed in Appendix-G.

12d. Prospective Studies: A Generic Problem

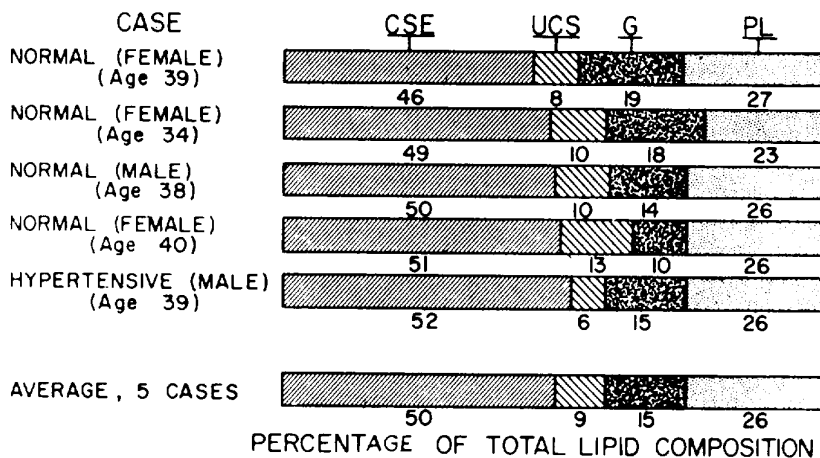
In 1978, we examined another aspect of the Livermore database: The 374 participants for whom we had two measurements about 1.5 years apart. The findings left us greatly impressed that the Framingham and Livermore Studies in 1966 had been capable of discerning statistically significant differences between de novo cases and base populations. The relationship between elevation of certain serum lipoproteins and Ischemic Heart Disease must be very strong indeed, when it remained detectable despite the phenomenon which we quantified in 1978. The 1978 analysis is summarized in Appendix I (eye), because it remains applicable today to prospective studies in many fields of bio-medical inquiry.

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Box 1 of Appendix-E
Comparison of 5 Individuals, Regarding Lipid Composition of Sf 6-8 Lipoproteins.

CSE = cholesterol esters. UCS = unesterified cholesterol. G = glycerides. PL = phospholipids.

• The figure below compares the lipid composition of Sf 6-8 serum lipoproteins from 5 individuals (reproduced from Lindgren 1959, p.834).



Related Text = Part 4.

• Having opened up the vast spectrum of plasma lipoproteins for study, the team at Donner Laboratory needed to learn whether a specific class of ultracentrifugally-defined lipoproteins (say, Std Sf 12-20) had a constant chemical meaning. In short: Is the chemical internal structure the same for Std Sf 12-20 lipoproteins, for one person from time to time, and for one person compared with another person?

• If, for a specific Sf-range of lipoproteins, one could NOT count on internal structure being reasonably constant from person to person, it would be an obstacle to understanding lipoprotein metabolism --- for instance, the apparent conversion of molecules of high Sf values into molecules of lower Sf values (text, Part 3).

• Box 2 shows results, comparable with the figure above, for the combined Sf 0-20 lipoproteins and the combined Sf 20-400 lipoproteins, from the fasting sera of 9 individuals.

• Boxes 1 and 2 indicate that, WITHIN a specific Sf-range, the lipid composition of the lipoproteins is remarkably similar, quantitatively, for both sexes in health and for several major lipoprotein disorders, and across a range of ages. Moreover, the distribution of lipids is distinctly different in the Sf 0-20 lipoproteins vs. the Sf 20-400 lipoproteins --- as already indicated in Chapter 44, Box 1, lower right corner.

• The constancy of composition was a most welcome feature, as the team at Donner proceeded with various case-control and prospective studies of the atherogenicity of various Sf-classes of lipoproteins.

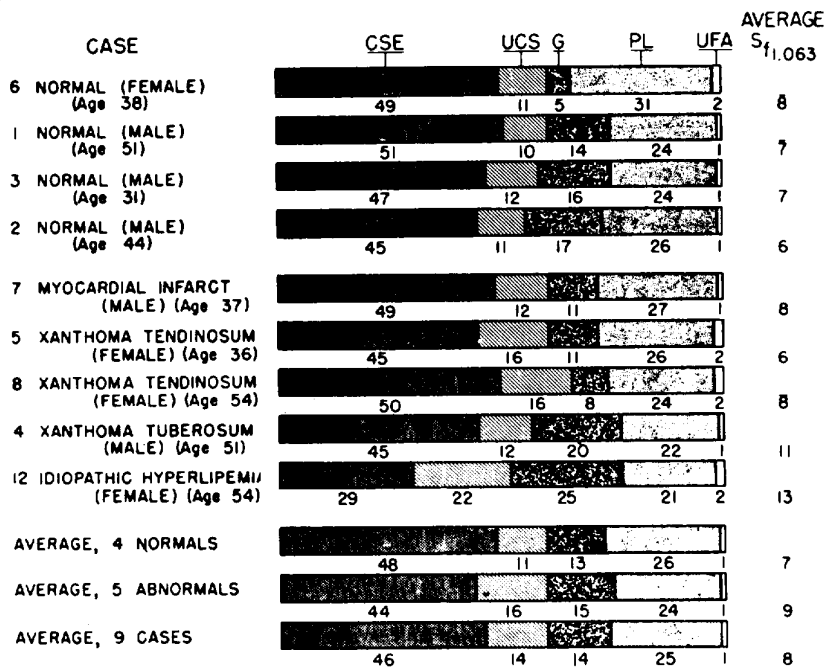
Box 2 of Appendix-E

Comparison of 9 Individuals: Lipid Composition of the Sf 0-20 and Sf 20-400 Lipoproteins.

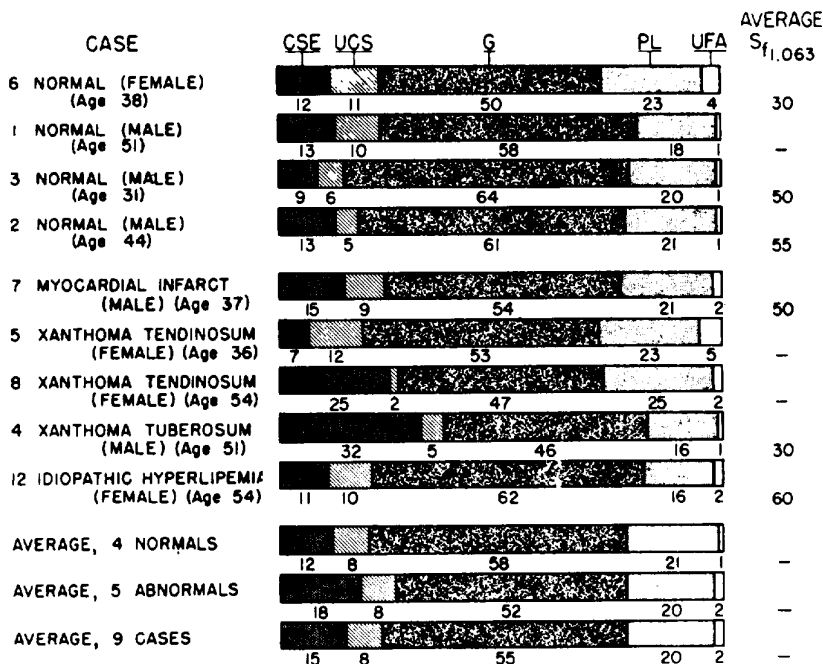
- The figures below compare the findings from the blood lipoproteins of 9 individuals (fasting measurements). Reproduced from Lindgren 1956; also in Lindgren 1957 + 1959.
- Please see text in Box 1 of this Appendix.

CSE = cholesterol esters. UCS = unesterified cholesterol. G = glycerides. PL = phospholipids. UFA = unesterified fatty acids. Far right: Avg flotation rate (Sf) in a solution of 1.063 gm/ml.

Sf 0-20 Lipoproteins: Lipid Composition, Expressed as Percent of Total Lipid.



Sf 20-400 Lipoproteins: Lipid Composition, Expressed as Percent of Total Lipid.



Related text = Part 4.

Related text = Part 7. Reproduced from Gofman 1954-e, p.518.

Hyperlipoproteinemia—Gofman et al.

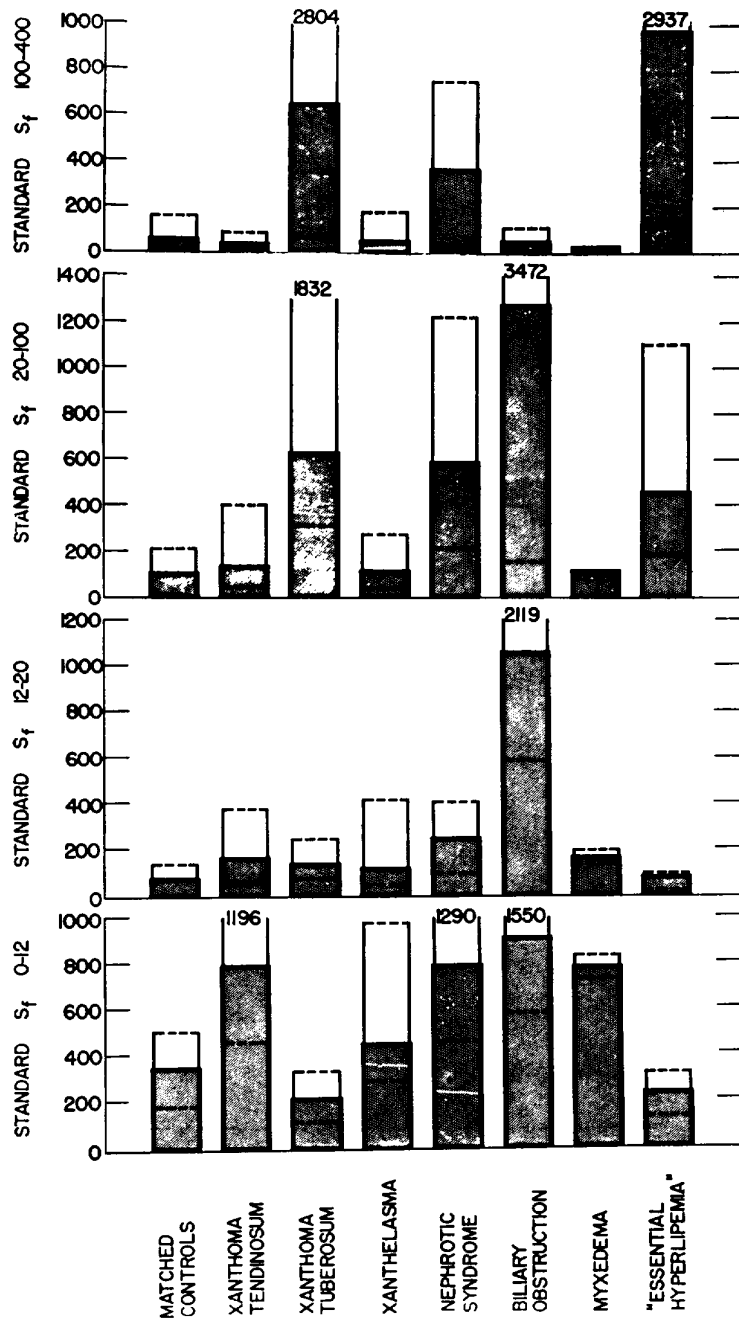


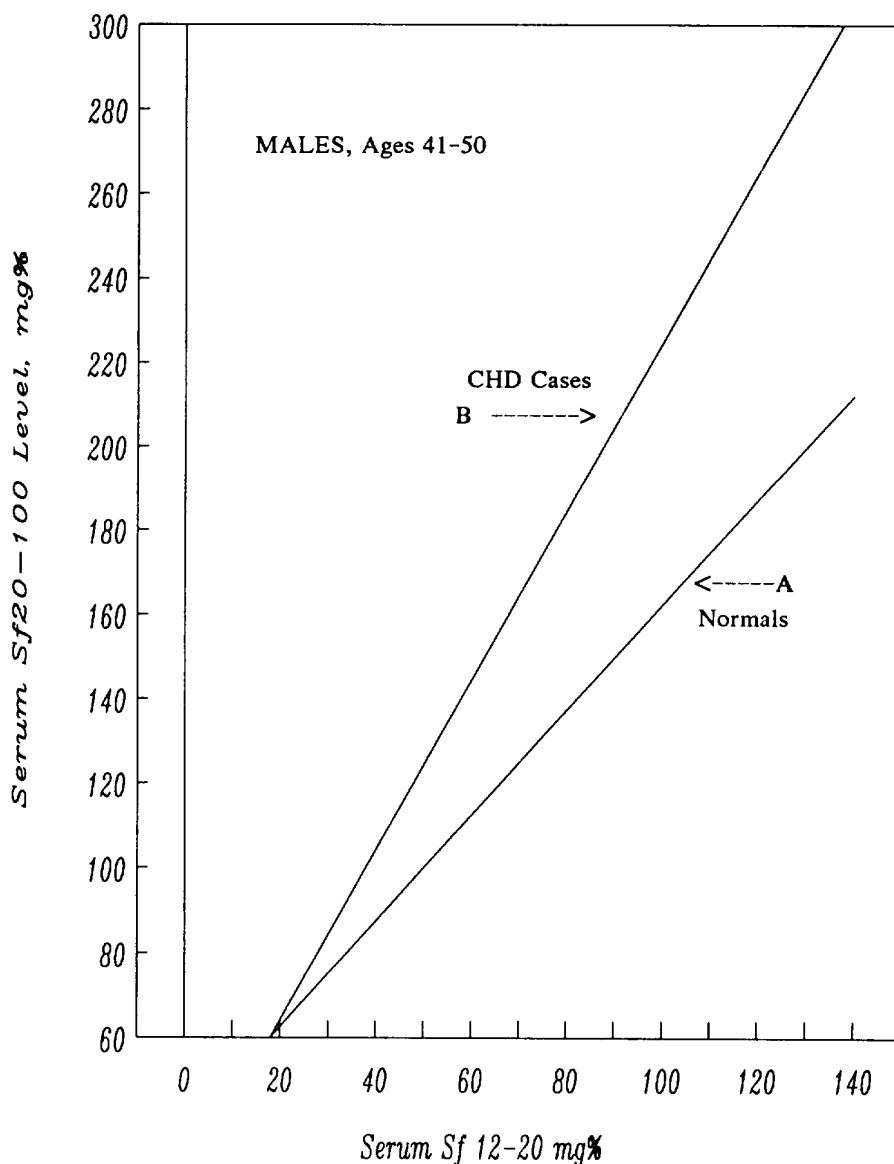
FIG. 1. The mean levels of serum lipoprotein concentration in the various disease states and a control group matched for age and sex for the four lipoprotein classes are shown by the hatched areas. The entire ranges of values found in the patients described here are shown by the interrupted lines. In some disease categories the highest level of lipoproteins found was off the scale of the graph; these high values are written in the appropriate space. For the matched control group the interrupted lines show the levels at two standard deviations from the control mean values.

Related text = Part 7.

Figure E-1

Evidence of the Independent Atherogenicity of the Triglyceride-Rich Sf 20-100 Lipoproteins.
Serum Levels of Sf 20-100 Lipoproteins Regressed upon Sf 12-20 Lipoproteins.

- The figure below is adapted from Gofman 1952-c, p.286 (case-control data).
- Line A in the graph is the regression line for normals (253 men without overt Coronary Heart Disease).
- Line B in the graph is the regression line for 72 patients with CHD.



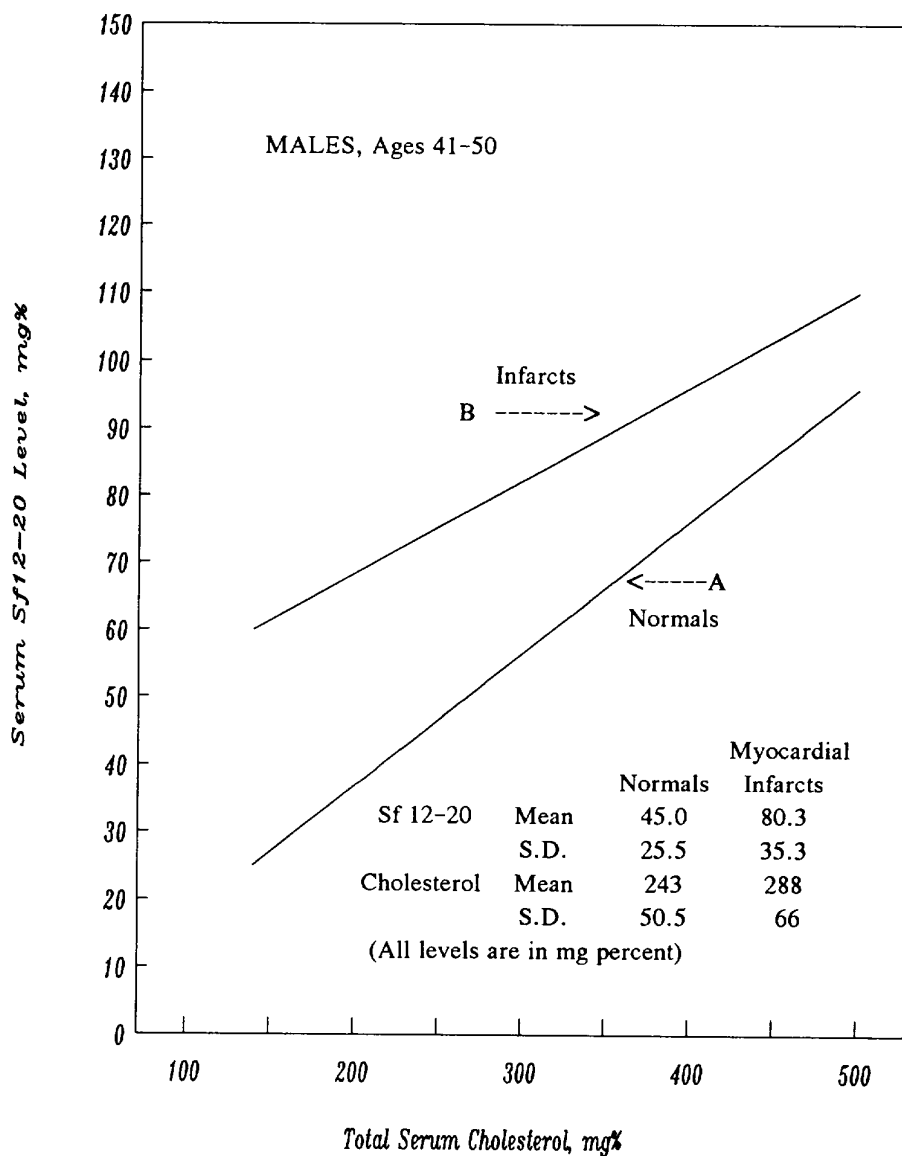
Related text = Part 9.

- At any serum Sf 12-20 level, the mean Sf 20-100 level (in mg/100 ml) can be read for either normals or for CHD cases from the appropriate regression line.
- At every serum Sf 12-20 level, the corresponding Sf 20-100 level is higher, on the average, in CHD cases than in normals.
- This finding represents evidence for an INDEPENDENT atherogenic contribution by the triglyceride-rich Sf 20-100 lipoproteins. If the presence of the Sf 20-100 lipoproteins contributed nothing to atherogenic risk beyond the contribution from the Sf 12-20 lipoproteins, the two lines of best fit (for CHD cases and normals) would fall on top of each other, within experimental error.

Figure E-2

Segregation of Infarcts from Normals, by Sf 12-20 Levels Independent of Cholesterol Levels.
Serum Sf 12-20 Lipoprotein Levels Regressed upon Serum Cholesterol Levels.

- The figure below is adapted from Jones 1951, p.364 (case-control data).
- Line A in the graph is the regression line for normals (273 males without overt Coronary Heart Disease).
- Line B in the graph is the regression line for 64 survivors of Myocardial Infarction.



Related text = Part 10.

- At any serum cholesterol level, the mean Sf 12-20 level (in mg/100 ml) can be read for either normals or for Infarct survivors from the appropriate regression line.
- At every serum cholesterol level, the corresponding Sf 12-20 level is higher, on the average, in the Infarct survivors than in normals.
- This finding represents evidence for an INDEPENDENT atherogenic contribution by the Sf 12-20 lipoproteins, beyond their positive correlation with serum cholesterol. If the levels of the Sf 12-20 lipoproteins contributed nothing to CHD risk beyond the contribution measured by total serum cholesterol, the two lines of best fit (for Infarcts and for normals) would fall on top of each other, within experimental error.

Table E-1
The Matched-Series Method of Assessing Independent Contribution to Atherogenesis.

● The tabulations below, based on case-control data, are adapted from Jones 1951, Table 3, p.366. Tabulations 1 and 2 are based on males ages 41-50. Lipoprotein and cholesterol determinations were done on aliquots of the same serum sample from each individual in the study.

● 1. Myocardial Infarcts Matched with Normals by Serum Cholesterol Levels.

Group	Number of Cases	Mean Serum Cholesterol	Mean Sf 12-20 Level
Infarcts	55	291.3 mg. %	73.8 mg. %
Normals	55	291.5 mg. %	53.7 mg. %

Difference in Sf 12-20 = 20.1 mg. % (+, - 5.5). Significance: $p < 1\%$.

● 2. Myocardial Infarcts Matched with Normals by Sf 12-20 Levels.

Group	Number of Cases	Mean Serum Cholesterol	Mean Sf 12-20 Level
Infarcts	55	292.3 mg. %	74.3 mg. %
Normals	55	273.2 mg. %	74.3 mg. %

Difference in Serum Cholesterol = 19.1 mg. % (+, - 6.4). Significance: $p < 1\%$.

● Quoting from Jones 1951 (p.365): "Matched series [above, in Part 1] have been used to assess the relationship of Sf 12-20 lipoproteins to atherosclerosis, while nullifying the effect of serum cholesterol PER SE, and conversely [in Part 2, above] to assess the relationship of serum cholesterol to atherosclerosis, while nullifying the effect of Sf 12-20 lipoproteins." And (p.365):

● "Thus [for Part 1], the cases of myocardial infarction were listed by cholesterol levels. From the normal data, cases were matched at random at corresponding cholesterol levels. Then the Sf 12-20 levels for the myocardial infarctions were compared with those for the normals that have been matched with these infarcts at the same age and serum cholesterol." And (p.365):

● "Similarly [for Part 2], the infarct cases were listed by Sf 12-20 levels and matched at random with normals having the same Sf 12-20 levels. Then the serum cholesterol levels were compared for the two groups." And (Jones p.365, referring to Table 3 on p.366):

● "The data in Table 3 may be interpreted as follows: [1] For the 41-50 year age group, at the same serum cholesterol, the myocardial infarctions average 20.1 mg. per cent higher in Sf 12-20 levels than normals. [2] At the same Sf 12-20 levels, the myocardial infarctions average 19.1 mg. per cent higher in serum cholesterol than do normals."

● There is significant segregation of infarcts from normals by Sf 12-20 measurement, independent of the serum cholesterol. And in this age-band, there is also independent segregation of infarcts from normals by serum cholesterol levels. However, for the males ages 51-60, the cholesterol measurement did not independently segregate infarcts from normals, while the Sf 12-20 measurement successfully did so.

Related text = Part 10.