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JCL roundtable: Lipids and inflammation in atherosclerosis

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Abstract

Clinical effort in lipidology focuses largely on mitigating effects of atherosclerosis, a pathologic process localized to the intimal layer of larger arteries. This JCL Roundtable brings together 3 leading researchers to discuss the current understanding of pathogenesis in atherosclerosis. We begin by recognizing that low density lipoprotein concentrations in arterial intima far exceed concentrations in other connective tissues, consistent with the response-to-retention hypothesis of atherogenesis. High density lipoproteins facilitate reverse cholesterol transport and also have antioxidant and anti-inflammatory roles. New evidence points to remnants of triglyceride-rich lipoproteins as promoters of atherogenesis, highlighted by deleterious effects of apolipoprotein C-III. The multifaceted role of inflammation is becoming clearer through discoveries related to leukocyte recruitment, efferocytosis, resolution of inflammation, and crystal formation. MicroRNAs represent a new, complex mode of gene regulation bearing on lipoprotein and inflammation biology. Progress in understanding atherosclerosis portends a future in which residual risk related to obesity, diabetes, and other factors will yield to new targeted therapies.

Keywords

atherosclerosis; lipoproteins; response-to-retention hypothesis; inflammation; resolution of inflammation; efferocytosis; microRNAs

Dr John Guyton In this JCL Roundtable we'll dive into pathobiological mechanisms of atherogenesis, particularly as they relate to lipoproteins and inflammation. I'm joined by 3

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renowned atherosclerosis researchers. Karin Bornfeldt is the Edwin L. Bierman Professor of Medicine, Division of Metabolism, Endocrinology and Nutrition, Associate Director for Research and Director, Diabetes Complications Program, University of Washington Medicine Diabetes Institute, and Professor of Laboratory Medicine and Pathology, at the University of Washington in Seattle, WA. Mac Linton is the Stephen and Mary Schillig Professor of Medicine and Pharmacology, Director, Vanderbilt Lipid Clinic, and Director, Atherosclerosis Research Unit at Vanderbilt University in Nashville, TN. Ed Fisher is Leon H. Charney Professor of Cardiovascular Medicine, Founding Director of the Center for the Prevention of Cardiovascular Disease, and Director of the Marc and Ruti Bell Program in Vascular Biology (a component of the Cardiovascular Research Center) at New York University Grossman School of Medicine in New York City.

Let me start the discussion with a curious fact sometimes overlooked. As far as I know, the arterial intima is the only tissue in the body that commonly develops deposits of cholesterol by midlife. So what accounts for the particular susceptibility of the arterial intima to cholesterol deposition?

Dr Edward Fisher I'm trying to think of other sites. When you say commonly, of course xanthomata are not common. Obviously, you have thought about this.

Dr Guyton It's almost an engineering approach. How does the body protect connective tissues from build-up of excess macromolecules and macromolecular assemblies like lipoproteins? The answer is that lymph vessels collect those macromolecules and drain them away. In 1883 the Hoggans found that lymph vessels are absent from human arterial intima and most of the tunica media. Incidentally, this apparently was a wife and husband team, and Elizabeth Hoggan would be the first female M.D. I've read about.

It makes sense that lymph vessels don't normally penetrate the tunica intima, because it's a tissue constantly squeezed between arterial blood pressure on one side and the tunica media on the other. No other tissue in the body has this constant structural pressure, not even the heart or spine. Lymphatics operate at low pressure, and they don't grow into this space with high structural pressure.

In the 1960s along came the great Scottish biochemical pathologist, Elspeth Smith. As I understand it, she took little plugs of normal human arterial intima and inserted them into holes in an agarose gel loaded with anti-apoB antiserum. By immunoelectrophoresis she found that the concentration of apoB that could be mobilized in this manner, interpreted as low density lipoproteins (LDL), was essentially equal to plasma LDL concentration. In contrast, LDL concentration in lymph measured by others is one-tenth of plasma concentration. So the logical sequence would be that high structural pressure in the intima – a unique tissue characteristic - leads to absence of lymphatics which leads to interstitial LDL concentration ten times higher than other connective tissues in the body.

Dr Fisher Well, it's interesting, in that for the medial layer, there's old literature that Gwen Randolph (Washington University) quoted to me once–old meaning in the sixties. Of course, back then, I didn't think I was old. But it was concerning the concept of immune privilege of the medial layer, which meant that there was not much in the way of lymphatics that penetrate into the arterial wall from the adventitia. So certainly, the adventitia has lymphatics, but that they do not penetrate as deeply into the tissue has been noted. Now, Gwen has gone on, and I don't know if Mac and Karin remember this–I'll let them comment–but I remember she had a paper in *JCI*. Of course, it's a mouse model. But the point was that the HDL that is entering the plaque from the lumen, from the blood, actually was exiting across into the lymphatics. But HDL is small, so it doesn't mean that the lymphatics were deep in picking up the HDL. But at some point, even if they were in the adventitia, the draining lymphatics–well, just like in the blister model, HDL can be carried off in the lymphatic circulation. So I'll let them comment.

Dr Karin Bornfeldt Another observation that might be relevant in this context is that cholesterol or LDL doesn't get trapped evenly in the arterial tree. There are certain sites that are more prone to lipid accumulation or lipid retention, predisposing to later development of lesions of atherosclerosis. At these sites of turbulence, the extracellular matrix appears to be altered is such a way that it traps apoB-containing lipoprotein particles.

Dr Macrae F. Linton You know, the concept of endothelial dysfunction, which can be caused by a lot of different insults, is that it increases permeability to LDL, turns out to be an oversimplification, because you have to have altered matrix to trap the LDL in the intima. And then, Ed, I figured this question was actually for you, because you published the paper on SR-B1 transport of LDL across the endothelium. So, I'd be interested to know how much of a contribution you think that is making to getting the LDL into the intima.

Dr Fisher Well, I'll address that in a second. I just want to go back to sort of an extension of John's question to us, in that even if this is true that there's this sort of unique feature, it's not the only factor–and Karin brought in the idea of the turbulence, the flow, and branch points and inner curvature, greater curvature of the arch, that have all been investigated, and there's good evidence to support these as factors. But as those of us, the three of us who are in lipid clinics and have seen a variety of patients, what's striking to me is that some patients will have clean coronaries, clean carotids, and have bad peripheral arterial disease. Some patients will have bad coronaries and clean carotids and peripherally fine femorals. And some people, of course, have very bad carotids, have stroke, and don't have coronary or peripheral disease.

So even within the arterial tree, and even with relatively similar flow and turbulence, like in the femoral versus the carotid, there is a vast difference in the amount of deposition and in the sequelae of that deposition, which I've brought up, and I know Karin in the mouse model has done a number of studies on the brachiocephalic versus aortic root, in which there are large differences. But there anatomically, you can start thinking, oh, well, it's clear there's a branch point where the brachiocephalic is. The turbulence is different. The laminar flow is different. But if we take even the larger arteries, that are relatively straight and in the non-branched portions, there's big variation from anatomical sites in atherosclerosis.

Dr Bornfeldt Do you think the variation in susceptibility in individual patients and in parts of the vascular tree you mentioned earlier is explained in part by different cardiovascular risk factors affecting different types of endothelial cells or vascular extracellular matrix deposition through distinct mechanisms?

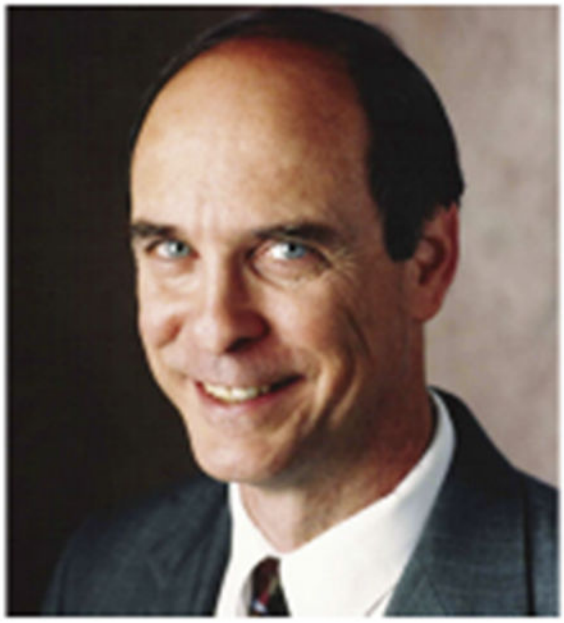
Dr Fisher Well, I guess there's that, and there's also what we know about the smooth muscle cells, which are taking on more of a mysterious contribution to plaque development in recent years because, as you know, they can become macrophage-like. The embryonic

origin–Mark Majesky and others have done beautiful work showing that the embryonic origin of smooth muscle cells varies. And certainly, the properties of endothelial cells vary, depending on what vessel they're in.

But Mac brought up a point about SR-B1, and Bill Sessa has his factor, bringing LDL into endothelial cells. Ira Goldberg here at NYU has some unpublished work that he's doing with Bill Sessa showing different ways in which LDL can get across endothelial cells into the intima. And other people have shown that there's transcytosis using, we'll say, lower resolution techniques than the more recent studies. These investigators that I just mentioned are putting names on specific factors. But it had been thought historically, as you well know, from Russell Ross's work in particular, that with endothelial damage, there is leakage, and the LDL is coming in through the gaps between the endothelial cells. And that was the major route.

And again, I don't know how to put a number on it in people, because those studies haven't been done. But in the mice, the number that I hear bandied about is half of it–at least half of LDL particles coming across the endothelial cell, and the other half coming in through the sides.

Dr Guyton Between the cells.



Dr Fisher Yeah, between the cells. And the quantitative hierarchy, to address Mac's question– the quantitative hierarchy of which pathway is contributing the most, I think that each group, including the group I was in with Phil Shaul (UT Southwestern) and the one led by Bill Sessa (Yale), is looking at one factor in isolation of the other. And so what you really need, of course, are people who will now, in a single experiment, be inhibiting either pathway and both, and see what the quantitative impact is. I don't think the data are there to answer Mac's question.

Dr Guyton Yeah. I think one other thing we have to think about is what is the permeability of the tunica media, because if you do an immunostain for apoB, you just don't see apoB in the tunica media. You can see some faint staining for apoA-I. So as you said, the HDL can move on through. But the LDL may get hung up, potentially simply as a result of pore size.

Dr Fisher It relates to your next question about the retention. I do believe retention is a major contributor. And as Ira Tabas and Kevin Jon Williams have concluded based on a number of studies, including those of Joy Frank and Dawn Schwenke, who did beautiful structural studies of human plaques, as well as from animal models, that you have these great clusters. They look like clusters of grapes of LDL that's all glopped together. And yeah, I could see where that's not going to have much chance of getting into the tunica media. I mean, LDL is big enough. But once you start aggregating it and glopping it together, it's going to be stuck there.

Dr Guyton When LDL aggregates, the particles tend to fuse.

Dr Fisher Correct.

Dr Guyton And so you get lipid droplets forming there. And do you agree that retention of LDL, as described by Kevin Williams and Ira Tabas in the early nineties, could provide the major driving force for early atherosclerosis?

Dr Fisher Well, the first paper was authored by just the two of them, and that came out in 1995. And then in updates, they've added Jan Boren, because Jan had a beautiful paper in *Nature* that tested the retention hypothesis in mice and had beautiful data that if you eliminated the part of the apoB molecule that couldn't bind to heparin sulfated proteoglycans, then there was less atherosclerosis. So it was indirect evidence to support this retention hypothesis.

Dr Linton Complementing Jan Boren's work there are also studies showing that knocking out proteoglycans like perlecan decreases retention of apoB-containing lipoproteins and reduces atherosclerosis. Also, treatment with monoclonal antibodies to block binding sites for apoB on glycosaminoglycans reduces retention of apoB-containing lipoproteins and atherosclerosis.

Dr Bornfeldt I also agree that the retention of LDL in the artery wall is an important driving force of lesion initiation. This is what I alluded to earlier, and actually our work on diabetic mouse models suggests that the retention is accelerated in the setting of diabetes. But I'd also like to add that this retention is not specific to LDL. Subsequent studies showed that other apoB-containing lipoproteins, those derived from the triglyceride-rich lipoproteins - chylomicrons and VLDL - can also be retained by this mechanism. So we think a lot about these remnant particles derived from triglyceride-rich lipoproteins by lipolysis. These particles are also likely being trapped through a positively charged apoB to negatively charged glycosaminoglycans. And so it is interesting to widen this discussion to larger triglyceride-rich lipoprotein particles and their remnant lipoprotein particles. Actually one can think of LDL as a remnant of triglyceride-rich particles as well.

Dr Linton Definitely, LDL comes from lipolysis of VLDL, but it lacks apoE, so it has to rely on apoB100-mediated clearance by the LDLR; whereas the other TG-rich remnants can be cleared by the remnant receptor pathway via apoE.

Dr Fisher Right, all the remnants have apoB.

Dr Bornfeldt Yes, although chylomicron remnants carry the smaller apoB48 rather than apoB100, which can be trapped by negatively charged glycosaminoglycans, just like apoB100 present in VLDL and its remnants.

Dr Guyton What components in LDL, which contains cholesterol, phospholipid, oxidized phospholipids, oxysterols, apoB and other peptides perhaps, drive lesion initiation and progression?

Dr Fisher Okay, well, lesion initiation and progression are different. That's why you separate them out.

Dr Guyton Okay.

Dr Fisher Once you're in progression, I think–again, a lot of this is based on either descriptive work from human plaques or the work in the preclinical models. And these days, that usually means mice, but of course, there's a literature of pig and rabbit, and even nonhuman primate, atherosclerosis studies. But certainly, once you start beating up LDL, you create fragments of apoB that are crosslinked. You have the modified phospholipids. You have oxysterols. A lot of these can be simply considered as DAMPs, the Damage-Associated Molecular Patterns, that would stimulate the toll-like receptor (TLR) system for inflammation. And some things, like oxysterols or modified lipids, people have shown in cell cultures–George Rothblat and Guy Chisholm as well as many other people historically have shown that you put these things into cultures of macrophages, and the macrophages croak. Nowadays we call it apoptosis or necroptosis. So in direct investigation in people, I'd be interested in knowing what Karin and Mac think, because we just have the data from Joe Witztum and Dan Steinberg with the antibodies to oxidized LDL components that give evidence that there's been modification of the phospholipids in particular. But I don't know what other evidence we have in people for what is particularly atherogenic about LDL.

Dr Guyton I'm interested in knowing what you think about cholesterol itself, because you've got an environment where what might be called the chemical potential of free cholesterol is so high that you're actually forming cholesterol monohydrate crystals. And what's that going to do to a cell membrane? The cell membrane is going to pick up cholesterol if it's in an environment that's so rich in free cholesterol. And it's not clear to me that's been sufficiently studied. I think Ira Tabas has done great work in this area, but has the role of cholesterol itself been clearly defined?

Dr Linton Well, I think, like you're saying, the cholesterol, at least, once it gets into the macrophages, cycles between being free and being esterified by ACAT1. Our studies with Bob Farese, showed that if you knock out ACAT1 in *Ldlr* or *ApoE* deficient mice, you get increased formation of cholesterol crystals that pierce the macrophages, which makes the point in vivo that excess free cholesterol leading to extracellular crystals is toxic to the cell and promotes atherosclerosis. More recent studies show that extremely small cholesterol crystals are present in early atherosclerotic lesions and play a critical role in activating the NLRP3 inflammasome in macrophages, which promotes secretion of interleukin-1-beta (IL-1β) and IL-18 cytokines and the development of atherosclerosis.

Dr Bornfeldt The ability of the macrophage to deal with the ingested cholesterol, whether it has the ability to convert a sufficient amount of free cholesterol into cholesteryl esters in lipid droplets, where it can be safely stored, or to export it through ABCA1 and ABCG1, rather than being overloaded by free cholesterol appears to determine the detrimental effects of cholesterol loading.

Dr Fisher Yeah, I think cholesterol itself is not particularly inflammatory. We did some limited experiments we published in PNAS in 2003. If you take pure cholesterol, and you deliver it in the form of a cyclodextrin, that it's a fairly neutral substance from the inflammation perspective. And the macrophage does fine as long as it can keep esterifying

it into just a lipid droplet. And as Mac just brought up, when you start interfering with that, there are problems. In the Tabas model system published in 2003, cholesterol in macrophages caused ER stress. He had to overload the cells and block esterification at the same time. So esterification is a protective mechanism to keep the cholesterol in a relatively inert depot.

And as Mac and Karin just brought up, and John, as you brought up as well, that once you can't handle that free cholesterol, since the plasma membrane can only accommodate a few mole percent, then it comes out of solution and forms those crystals. And Mac, you remember, of course, the work that Rothblat did years ago, with I think Jay Jerome, when they had these beautiful scanning electron micrographs of those crystals–

Dr Linton Right, those studies showed that free cholesterol crystals can form from the hydrolysis of cytoplasmic stores of cholesteryl esters in macrophages when free cholesterol is increased by ACAT inhibition. The crystals were found extracellularly in contact with live macrophages and dying macrophages. They also showed that cholesterol crystal formation can be modulated by the addition of extracellular cholesterol acceptors, such as apoE or apoA-I, or by affecting the cellular rate of cholesteryl ester hydrolysis.

Dr Guyton George Abela and his group have published a JCL review on this recently.

Dr Fisher And then the work with oxysterols and the ketosterols that are thought to be toxic to the cells, they start to be made in higher concentrations when the cell is overwhelmed by cholesterol. So cholesterol, a fair amount, can be accommodated in a cell, including a macrophage, without having much of an inflammatory effect. But then when you overwhelm the ability to store it as a droplet, then it's bad news.

Dr Bornfeldt And, as shown by really nice work by Alan Tall's group, when you inhibit the cell's ability to export phospholipids and cholesterol through ABCA1 and ABCG1, it also becomes overloaded with cholesterol.

Dr Fisher Right. And one of those papers I think you're recalling was in the Journal of Clinical Investigation (JCI), and they showed that one of the things that accumulated was ketocholesterol, which was particularly toxic. So that couldn't come out either.

Dr Guyton Okay. I think that Howard Kruth has looked at oxidation of cholesterol as one of the mechanisms of reverse cholesterol transport of getting cholesterol out of the cell, because the oxysterol in small quantities may not be especially toxic to the cell, but can actually leave the cell. It's more soluble.

Dr Fisher Well, it's water-soluble. It also is an activator. The oxysterols activate LXR (liver X receptor), which turns on the cholesterol efflux pathways. So it works out very nicely. It induces its own exit.

Dr Guyton Let's go on to high density lipoproteins (HDL). Is there general agreement that a major function of HDL, which we just cannot dismiss, is reverse cholesterol transport?

Dr Fisher I certainly think so.

Dr Linton And I think so. I think with HDL, it's hard to say there's general agreement about anything anymore. But I think definitely, there's evidence that reverse cholesterol transport is important.

Dr Fisher Certainly, you put HDL into any system, it pulls cholesterol out. That's for sure.

Dr Bornfeldt Especially small HDL and apoA-I effectively pull phospholipids and cholesterol out of cells. However, the level of HDL cholesterol appears to not be a good measure of the cholesterol efflux capacity of HDL. This makes sense because large HDL contains the most cholesterol. Dan Rader and others have shown that HDL's cholesterol efflux capacity is a better predictor of cardiovascular disease protection than is HDL cholesterol. The number of HDL particles, which vary in size and carry different cargo, also appears to associate with cardiovascular protection.

Dr Guyton All right. What are some of the other key functions of HDL that are of current interest?

Dr Fisher Well, we published a paper–again, it's pre-clinical–that HDL can suppress some of the effects of hyperglycemia in diabetes on myelopoiesis and macrophage inflammation. But this area, as you allude to, is chock-full of contradictory reports, and very system-dependent. We talked a little bit before about the accumulation of modified lipids that are toxic–it could be from oxidized LDL; it could be from what the cells are making as they get loaded up. Years ago, I learned the concept that HDL does reverse lipid transport. It can potentially pull out all sorts of stuff. I have a project where I'm trying to do some lipidomics to determine what HDL is pulling out of mouse plaques.

Also remember that Karin's neighbor, Jay Heinecke, threw us all a very big question in his JCI paper years ago, where he did shotgun proteomics of HDL and showed that there was a total of about 200 identifiable proteins that are carried on HDL subspecies. And Kasey Vickers, Mac's neighbor, when he was with Alan Remaley at the NIH, had the beautiful paper in *Nature Cell Biology*, that HDL carries micro-RNAs to cells. And so I think the big question is, what are all these other things doing, right, Karin? You're in the center of this up in Seattle.

Dr Bornfeldt Yes, right. So the HDL protein cargo might relate to functions of HDL distinct from cholesterol efflux. For example, we showed recently that if HDL carries more PON1, paraoxonase 1, that it associates with long-term protection from cardiovascular complications in people with type 1 diabetes. That protective effect did not seem to be related to the cholesterol efflux capacity of HDL, but was associated with increased PON1 in larger-size HDL particles. While the exact mechanism is still unclear, it's believed that PON1 can protect from oxidation of lipids. On the flip side, our collaboration with Jay Heinecke showed that low concentrations of PON1 in HDL associate with both kidney disease and coronary artery calcification in people with type 1 diabetes, raising the possibility that low PON1 levels in HDL might contribute to the known association between kidney disease and heart disease. Of course, as Ed mentioned, many proteins in addition to PON1 are carried by HDL. And so the cargo of HDL could potentially have important effects that are independent of its cholesterol efflux capacity.

Dr Linton I think that's definitely an important theme. Sean Davidson also has done a lot of work on the proteomics of HDL and likes to emphasize the point that HDL isn't one thing, because the particles really vary by what they carry. But to carry what Ed mentioned about Kasey Vickers a little further, since he's moved to Vanderbilt, we've discovered that not only does HDL carry micro-RNAs, but essentially all of the lipoproteins carry small, non-coding RNAs. And actually the vast majority of them are non-human in origin. And so we're actively studying now the role of these mostly bacterial, and they can also be fungal or viral, small RNAs that are carried by HDL, as well as LDL, in atherosclerosis.

Dr Guyton Well, some people think everything goes back to the intestinal microbiome.

Dr Linton Yeah. Interestingly, these small, non-coding RNAs are not all from the intestine. And we think quite a bit of it comes from the lungs.

Dr Fisher When we breathe, right?

Dr Linton It's incredible to think about the number of microorganisms and viruses we inhale and carry around with us. So, we're very interested in trying to understand where these foreign small noncoding RNAs carried by HDL and other lipoproteins come from, how they get onto our lipoproteins and what biological effects they have.

Dr Fisher And it's possible that some of this is to get rid of some of those bacterial and fungal products.

Dr Linton That seems likely. Just think about the overwhelming burden of bacteria and microorganisms that our body carries around and encounters, and when the bacteria die, you have to deal with the components. And one of these components is their nucleic acids.

Dr Guyton I wanted to interject this, that HDL is in evolutionary terms the ancient lipoprotein. It's found in primitive species. LDL is found only in larger animals and particularly in mammals. But HDL is a lipoprotein in insects. And so you might expect HDL has been around long enough to accumulate a whole number of functions that LDL does not share.

Dr Bornfeldt Yes, that's also interesting in the context of this idea that HDL might be a storage pool for apolipoproteins, such as apoCs, and other molecules, like the RNAs Mac talked about. Perhaps HDL, at least in part, serves the role of a circulating storage pool for lipid-associated molecules or molecules that need to be protected by a phospholipid layer, and that some of the changes in HDL might be biomarkers of cardiovascular disease risk, rather than directly mediate increased risk.

Dr Fisher That's a good point.

Dr Linton We've been interested in other types of modification that affect HDL function. Some are related to things that had been shown before by Jay Heinecke and others, like malondialdehyde. But there's a whole group of reactive aldehydes or dicarbonyl molecules that can modify all types of proteins. There's quite a bit of evidence now that in familial hypercholesterolemia (FH), where we know that the main problem is really high LDL,

that their HDL is also dysfunctional. And we have found that the apoA-I and HDL and also phospholipids can be modified by these different reactive molecules and that HDL in patients with FH versus controls has increased modification with malondialdehyde (MDA), and also isolevuglandins (IsoLG), which are reactive molecules that come from breakdown of arachidonic acid, basically, through non-enzymatic free radical-mediated pathways. In addition, modification of HDL with 4-oxo-2-nonenal (ONE) is increased in individuals with FH. And we have evidence that the type of modification can also impact the actual function. For example, modification of HDL with ONE doesn't impact cholesterol efflux capacity, whereas modification of HDL with MDA or IsoLG dramatically impairs it.

And in that line, we also are studying some small molecule scavengers, which can prevent modification of HDL and in mouse studies have evidence that it improves HDL function and reduces atherosclerosis. We have recently reported in *Nature Communications* that treatment of *Ldlr* deficient mice with the dicarbonyl scavenger 2-hydroxybenzylamine (2-HOBA) reduces modification of HDL and LDL, improves HDL function and dramatically reduces atherosclerosis without impacting plasma lipid levels. We are planning to do a Phase II study in patients with FH to see if treatment with 2-HOBA reduces modification of HDL and improves HDL function in humans.

Dr Bornfeldt Mac, that is an excellent point, modification of HDL can also alter its functionality. Before we move on from the HDL theme, Ed, you're finding that HDL can prevent the effect of hyperglycemia in a mouse model of diabetes. This is very interesting in light of the increased cardiovascular disease risk in individuals with diabetes. Can you tell us a little more about how you think that might be working?

Dr Fisher Well, Alan Tall with his post-doc, Laurent Yvan-Charvet, and other authors had a paper in *Science* that I'm sure you are familiar with, showing that the amount of cholesterol in the membrane of a monocyte and neutrophil precursor is a regulator of proliferation of the cells in the bone marrow. And they go into some potential mechanisms in there, which I won't take time to cover at this point. So it's known that hyperglycemia will down-regulate the expression of ABCA1 and ABCG1. But you can overcome the decreased efflux of cholesterol out of cells through the specific pathways of ABCA1 and ABCG1 when they are downregulated by providing more HDL, which would pick up the cholesterol from the nonspecific aqueous diffusion pathway.

So the specific transporters are sort of sexy and jazzy, but Mike Phillips and George Rothblat, who were my chairs in Philadelphia, and who did a series of incredibly detailed, rigorous studies, have shown that actually, in a cell that's not particularly cholesterol-loaded, under normal homeostatic mechanisms, there's efflux to HDL, but 80% of that–up to 80% of that can be explained through this aqueous diffusion pathway, which is a physicochemical phenomenon.

Going back to hyperglycemia, you do accumulate cholesterol. We show that the cell membranes, where the cells become cholesterol-enriched in a hyperglycemic setting, are enriched in part because they can't do efflux through ABCA1/G1. So if we provide more HDL, functional HDL, either through transgenic means, or through just infusing HDL, we

can pull out the cholesterol from these precursors in the bone marrow and reduce their proliferation and reduce the circulating levels of neutrophils and monocytes.

Dr Guyton I wonder if there may be regulatory steps in the lymphocytes and the macrophages in the artery wall as well. The bone marrow is a nice place to look, but could it represent some similar regulatory steps in arterial intima?

Dr Fisher I think it can, because another buzzword in the macrophage field is epigenetics. And we know that diabetes and other metabolic conditions will imprint changes in the chromatin, the DNA protein-histone complex, that start in the bone marrow and are stable in monocytes and in the macrophages that they become in the tissues. So what we're now going to do is follow the changes in the monocyte bone marrow precursors out to the circulating monocytes and into the macrophages and the plaques. I think the evidence in other contexts that there are epigenetic changes associated with various inflammatory diseases involving macrophages, monocytes and their precursors, is exactly what you are implying, John, that there can be changes in programming of the cells by factors that would be in not only in the final tissue site (the plaque), in the macrophages, but also, these changes get programmed in their precursors as well.

Dr Guyton That's fantastic. Now let's think about an apolipoprotein that we haven't talked about very much, but it's very much in the news–apolipoprotein C-III (apoC-III). If someone could briefly summarize some of the evidence that apoC-III is a bad player and a promising target.

Dr Bornfeldt I'd be happy to start, and I'm sure Ed and Mac have additional thoughts on apoC-III. There is a lot of interest in apoC-III, as well as other proteins that regulate clearance of triglyceride-rich lipoproteins right now. We are very interested in apoC-III as a coronary artery disease risk factor and mediator especially in relation to diabetes. In 2008, Alan Shuldiner's group showed that loss of function mutations in *APOC3* were associated with lower plasma triglycerides and apparent cardio-protection in an Amish population. Several subsequent studies have confirmed these initial observations, and now there's very strong evidence in humans that apoC-III is important in regulating triglyceride metabolism and worsening of cardiovascular disease risk.

Dr Guyton It's an inhibitor of lipoprotein lipase, as has been known for quite some time.

Dr Bornfeldt Yes, but it appears to delay clearance of triglycerides through at least two different mechanisms of action, and the relative contributions of those mechanisms might vary depending on other factors or disease states. It does indeed inhibit lipoprotein lipase. But inhibition of apoC-III also results in triglyceride lowering in humans who are deficient in lipoprotein lipase, and in mouse models deficient in lipoprotein lipase. The explanation is that apoC-III also prevents hepatic clearance of triglyceride-rich lipoproteins and their remnants through interfering with binding to hepatic receptors of the LDL receptor family, LDLR and LRP1, which mediate hepatic uptake of these particles.

Dr Linton And that could be through an apoE-mediated mechanism.

Dr Bornfeldt Yes, that's believed to be through an apoE-mediated mechanism. Both of these actions of apoC-III might be important in the setting of diabetes. We recently investigated apoC-III as a risk factor for coronary artery events in people with type 1 diabetes. This study showed that elevated levels of serum apoC-III predicted who was going to have a coronary artery event later in life. Very similar conclusions were reached by Dr. Lyons, studying a different population of adults with type 1 diabetes. We also found that the increased coronary artery disease risk associated with elevated apoC-III was independent of traditional risk factors, such as LDL cholesterol, and also independent of glycemic control. And then, to investigate causality, we used an apoC-III antisense oligonucleotide, which is similar in mechanism to volanesorsen, the antisense therapeutic that's been shown in humans to reduce triglycerides, to treat our mouse model of type 1 diabetes-accelerated atherosclerosis. These studies revealed that silencing hepatic apoC-III prevented the effect of diabetes on both early and advanced lesions of atherosclerosis, and also prevented accumulation of apoC-III and apoB in the artery wall. This brings us back to the previous discussion on apoB lipoprotein retention. We believe that apoC-III is particularly important as a cardiovascular disease risk factor in diabetes because it's upregulated by lack of sufficient insulin or by insulin resistance. That's what we're working on currently.

Dr Fisher So, Zemin Yao years ago had some nice papers that another possible contribution of apoC-III was through the promotion of the secretion of VLDL, which, of course, carries triglyceride out. So that was a cell culture model. But I was co-author of Jan Breslow's paper in the early nineties in *JCI* in which he made an *APOC3* transgenic mouse, which showed hypertriglyceridemia. And my particular contribution was to look at the production of triglycerides coming into the circulation, either from the intestine on chylomicrons or from the liver on VLDL. And we show in our paper that there was a minimal effect on triglyceride production from either organ. The majority of the effect for the hypertriglyceridemia was on the clearance of the triglycerides, which is in agreement with the more modern studies. But this mouse model actually gave the phenotype that Karin just reviewed, and it seemed to be much more from the clearance of the triglyceride-rich lipoproteins, rather than their production. But there is a literature that there might be some small increase also in the production of VLDL TG.

Dr Linton ApoC-III content also plays an important role in the retention of apoB-containing lipoproteins in the arterial wall, so that is another potential mechanism by which it can promote atherosclerosis.

Dr Guyton Interesting. Let's get back into the artery wall, and my first question is this: what is it that kills the cells in the necrotic, lipid-rich core of the plaque? Do we have a clear idea? I think we've already spoken about this a little bit. We've talked about cholesterol crystals. We've talked about cholesterol overloading cell membranes, but I wonder if you have any further specific ideas about what kills the cells.

Dr Fisher Well, some of it is hypoxia. I'll just throw that in, because we did a study in hypoxia, and certainly human plaques have hypoxia. The further you go in away from the adventitia and the lumen, we show that there's actually almost a belt-like zone of hypoxia in

large mouse plaques. And people have seen evidence of hypoxia in human plaques for a long time. So chronic hypoxia is not going to be good and it will be more in larger plaques.

Dr Guyton Well, what's fascinating is that it's the wall of the artery, but it's an extraordinarily poorly perfused tissue, sometimes 500 μm, 700 μm of tissue without any capillaries, without any lymph vessels. It's a very stagnant area and, as you say, it's a hypoxic area as well. The intima forms part of the wall of a blood vessel, but the intimal tissue itself is very poorly perfused.

Dr Fisher Right. And Mac brought up before, and you just alluded to, the crystals form. They activate the inflammasome. They directly kill the cells. And that's certainly a factor.

Dr Linton There are multiple triggers for macrophage cell death in atherosclerotic lesions, including saturated fatty acids, free cholesterol loading, oxidative stress, death receptor activation, nutrient deprivation, and defective insulin signaling. Many of these triggers induce endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR), leading to activation of apoptosis signaling pathways. Oxidized phospholipids can induce apoptotic signaling by binding scavenger receptors (CD36 and SRA) or toll-like receptors (TLR2 and TLR4). Suppression of survival pathways such as Akt and NF-κB can lead to macrophage death. In contrast, HDL can reduce ER stress and prevent macrophage apoptosis by promoting cholesterol efflux and via its anti-oxidant functions.

Dr Guyton What's the role of cellular proliferation in atherosclerosis? Mac, you had an interesting paper about this recently.

Dr Linton The cellular growth of atherosclerotic lesions occurs via recruitment of bone marrow–derived monocytes and vascular smooth muscle cells (VSMC) from the media as well as local proliferation of lesional macrophages and VSMCs. In collaboration with Jonathan Brown and Matt Steinhauser, we used a quantitative imaging technique called multi-isotope imaging mass spectrometry (MIMS), to measure cell division and glucose utilization simultaneously, using 15N-thymidine and 2H-glucose labeling, in atherosclerotic plaques. This technique allows suborganelle resolution. Surprisingly, using MIMS we identified higher rates of proliferation in foam cells compared to prior studies. In contrast, the frequency of proliferating foam cells declined dramatically in advanced lesions. Interestingly, we did detect lipid-laden VSMCs directly underlying the intima, supporting foam cell–like changes. In contrast to foam cells, proliferation of medial VSMC was detected in small subpopulations of cells. Dividing foam cells demonstrated increased glucose labeling. However, the glucose label in medial VSMCs underlying plaques was greater than that in foam cells, but this pattern was lost in advanced plaques.

Filip Swirski has done studies that really support the idea that you have local proliferation of macrophages versus just monocyte recruitment, contributing to the growth of atherosclerotic lesions. However, there's still a question about how much of the macrophage increase in numbers in the lesions is due to proliferation versus recruitment. And one of the things, at least in mouse models, in bone marrow transplants, you essentially see complete replacement of the macrophages in the artery wall with donor phenotype, which really suggests in that model, there's not much proliferation of local macrophages in the lesions

going on because otherwise you would see a significant contribution of the recipient phenotype to the foam cells. It is possible that the radiation may knock off the resident macrophages that are prone to proliferation. But I think there is quite a bit of evidence now to support an important role for local proliferation.

Dr Guyton In some ways, some of the smooth muscle cell proliferation could be protective. If you want to prevent a vulnerable plaque, you want to have a good fibrous cap overlying the necrotic core.

Dr Fisher Well, the proliferation of smooth muscle cells, of course, has been known for a while. They proliferate in the media, and they migrate out into the intima. And then some, of course, assume a position as subendothelial. There's beautiful work using what's called confetti mice, in which you can lineage mark cells. It turns out that–again, this is mouse work, but it turns out that all of the smooth muscle cells that wind up in the intima are derived from just one or two particular cells in the medial layer. And it's not clear why is it such a small number and how they're different in some way. They give rise to all the smooth muscle cells that are in the intima.

Swirski updated the classic work of Earl Benditt from years ago, where he used radioisotopes, like thymidine incorporation, and showed that there was radioactivity in, I think it might have been, rabbit plaques back then. And so that was the first hint that proliferation can occur locally in the plaques. We published a single-cell RNA Seq paper in *JCI Insight* about a year and a half ago, in which we show there is a local population of monocytes that enter the wall of plaques in mice that can actually proliferate, then differentiate into macrophages. But this was a very small population. Quantitatively, at least in a snapshot view, it maybe gave rise to 4% or 5% of the macrophages in the plaque, with the others coming from the circulating pool of monocytes that entered.

The work that Swirski did had some rather phenomenal estimates that I have not seen confirmed. Maybe my colleagues have seen this. But he was estimating in an apoE knockout mouse that if the plaque advanced, 80% of the macrophages in those plaques could be explained from local proliferation. And I have not seen work since then–and correct me if I'm wrong, because I don't want to be critical if I'm wrong, that estimate has been confirmed. What do my colleagues think?

Dr Linton I think it's variable. In our MIMS study mentioned above, atherosclerotic plaques in the proximal aorta of *Ldlr* deficient mice fed western diet for 9 weeks, 65% of intimal foam cells and only 4% of medial VSMCs were labeled with 15N-thymidine after 1 week of isotope treatment, indicating proliferation. However, it is important to understand that this technique did not distinguish whether cells were initially labeled locally in the lesion or in the bone marrow, so it does not address whether the macrophages are resident or derived from monocytes recruited to the plaque. Some of Filip Swirski's studies show less of a contribution of proliferation of arterial macrophages to the foam cells in atherosclerotic lesions than others. So, I think that 80% is too high.

Dr Fisher Though it might have been an estimate on the high side, I'd say there's certainly proliferation. It depends on the cell type, too. There are endothelial cells proliferating. There

are certainly smooth muscle cells. And there is some level of macrophage proliferation. All of us use something called KI-67, which is a proliferation marker. You can see always there's a level of positivity. But it tends to be certainly less than 10%.

Dr Guyton All right, wonderful. Now. Help me understand inflammation.

Dr Fisher Good luck.

Dr Guyton That's exactly my feeling about it. It seems to me very complicated with perhaps multiple different processes of inflammation.

Dr Fisher You got it right. I was just talking with my former fellow, who is now at Oxford, Robin Choudhury, about this. But macrophages are–yes, they're inflammatory cells, and so are neutrophils. But there's TH1 lymphocytes. There's TH2 lymphocytes. We now know there are B cells that are pro-inflammatory. There are different chemokines, cytokines, NF-kappa B pathways. It's like the three blind men with the elephant. There are just so many components to it. You're looking at the tail, and you think it's a rope, and that's all there is to it, but there are all these other pathways, it's mayhem. That's the way I look at it as a plaque in terms of the site of inflammation. It's mayhem in there. I don't know what Mac and Karin think.

Dr Linton There is a lot of evidence now that there are multiple separate pathways that are important. And just in a very simplistic type of thinking from clinical studies, COX2 inhibitors, which are anti-inflammatory, we have associated with increased cardiovascular events. So, clearly, inhibition of some inflammatory pathways is not beneficial in terms of reducing risk of atherosclerotic cardiovascular events. However, the CANTOS trial (Canakinumab Anti-inflammatory Thrombosis Outcomes Study) demonstrated that inhibition of IL-1β can reduce cardiovascular events, providing strong support for the critical role of inflammation in atherosclerosis. Unfortunately, there was also an increase in deaths due to infection, highlighting the challenges of intervening in pathways critical for our defenses against microbial pathogens.

And another interesting point I would like to raise relates to high sensitivity C-reactive protein (hsCRP), which is an independent risk factor for cardiovascular events. And it usually tracks with the other inflammatory cytokines as well.

Dr Guyton Mendelian randomization with CRP turned out to be negative. So genetic variation in CRP levels did not track with cardiovascular events.

Dr Linton Those results may have more relevance as to whether CRP is directly mediating the proatherogenic inflammation or a marker of other critical inflammatory pathways. But the point I was going to make relates to whether LDL drives the inflammation in atherosclerotic lesions. We've always believed the LDL gets modified and taken up by macrophages causing an increase in inflammatory response from macrophages and other cells critical to the inflammatory response. But I think it's interesting that statins lower hsCRP, and PCSK9 inhibitors don't. I don't know what you guys think about that, but it

says that just lowering LDL levels alone isn't really anti-inflammatory, at least in terms of looking at the impact on hsCRP levels.

Dr Fisher Well, the statins being chemical compounds, Stan Hazen and others have shown that there's some antioxidant properties to some of the statins. And certainly, the pathway to cholesterol has the isoprenylation-mediated modifications of proteins and promotes NADPH oxidase complex assembly. So that's a very good point that some of the effects of the statins aren't just from lowering LDL.

But the CANTOS trial brings up the issue. That's looking at blocking one particular inflammatory factor. It did have an effect. But remember; it was only 15%–20% reduction of events. There are all these other inflammatory pathways–there's a whole list; I won't try to enumerate them all–that would not be affected by that particular therapy. We've shown in animal models of regressing plaques that you need some broad change in the inflammatory state of the macrophages to get the full benefits of the lipid lowering.

What we did was something that cannot be done in people yet, but it really emphasized the principle that lipid lowering alone, without changing the general state of inflammation in the plaque, was not going to be optimally successful. When we prevented macrophages from being able to become anti-inflammatory by knocking out a particular key pathway, and we lowered the lipids tremendously, we didn't do much to the plaques. So it doesn't really answer your question about what is inflammation. But it sort of reminds me of the Supreme Court Justice years ago, when asked to define "pornography," he said, well, I can't really define it, but I know it when I see it. And so it's like inflammation. I know it's bad, and we have to get rid of it in the plaques. But there are so many factors and pathways–it's like the game whack-a-mole that we might have played at the carnivals, where as soon as you knock down one head, which could be IL-1, there's another one that's over there. And I think the ability to respond to invaders using inflammatory responses is so important through evolution, that if we were to globally knock out the innate immune system, we would die very quickly. So we are so geared to having multiple varieties of, quote/unquote, "inflammation," that by attacking one or the other, it may not have the intended benefits and may produce an incomplete therapeutic effect in people.

Dr Guyton I think that's a very good point. What's beautiful about LDL is that lowering LDL to extremely low levels is very safe. But knocking out inflammation is not necessarily safe.

Dr Fisher Right, but if you now look at those PCSK9 trials by intravascular ultrasound (IVUS), having ontreatment LDLs in the low 30s for almost, what, two years, there was a 0.1% change in plaque volume. The trials also show that the residual risk, if you want to call it that, if you look at the actual absolute numbers-and John, you're more attuned to this than probably I am-there's still a significant number of those people with LDLs in the 20s and 30s that have an event and still don't have any change in their plaques.

Dr Guyton Yes, I think you're right. I think there's a limit to what you can do with LDL alone.

Dr Linton Those outcomes trials did show, though, that PCSK9 inhibitors did reduce events.

Dr Fisher Yeah, by 11% from LDL-C dropping from 90 to 30 mg/dL. In the statin years, we were taught for every percent lowering in LDL, you had a percent reduction in risk. So there's clearly a threshold effect, and I think we might actually be on the J-shaped curve that Scott Grundy used to talk about.

Dr Guyton Another way to look at it, according to the Cholesterol Treatment Trialists group, is that for absolute reduction of LDL-C by 1 mmol/L, or 38.7 mg/dL, you get 22% relative risk reduction. One of the implications of that relationship is that getting LDL-C close to zero does not eliminate atherosclerotic cardiovascular risk.

Dr Bornfeldt I think Ed is exactly right. There are so many pathways involved in inflammatory processes, even if we limit the discussion to macrophages. I strongly believe we should think about "inflammation" not as one overall entity, but as different pathways, which are activated to different extents by different cardiovascular disease risk factors and depending on the environment surrounding the macrophage. We know from CANTOS that the IL-1β pathway seems to be important in high-risk patients who had had a previous myocardial infarction and had high levels, 2 mg/L or more, of high-sensitivity CRP, but we don't really know about the role of other pathways or even if the IL-1b pathway is equally important in other groups of patients with different sets of risk factors.

Dr Guyton Sam Tsimikas and colleagues have found an interesting interaction between IL-1 genotypes and lipoprotein(a) levels. He found the cardiovascular risk of lipoprotein(a) to depend strongly on IL-1 genotypes in two reasonably large studies. So there might be anti-inflammatory drugs that are specifically more helpful in patients with high levels of lipoprotein(a), which as you know are the particles that carry most of the oxidized phospholipids.

Let me ask, what is efferocytosis? Ira Tabas has done a lot of work on efferocytosis. What is it?

Dr Fisher It's the ability of a healthy macrophage to eat up a dying one. So it's a way of clearing those dead macrophages–we talked before, however the cells die in the core, if you can clear them before they give up their innards, which is full of cholesterol, which can crystallize, and release tissue factor, which could promote the thrombosis in a ruptured plaque, then this would be a good thing. And so Dr. Tabas, and then more recently Nick Leeper, looked at manipulating particular efferocytosis factors. They've shown nicely that in the preclinical models, if you inhibit efferocytosis, then you do promote not only progression of atherosclerosis, but an increase in the necrotic core in particular.

Dr Guyton Fascinating.

Dr Bornfeldt A concept I think is very interesting is continual efferocytosis – the ability of a macrophage to ingest one dead or dying cell and then go on to ingest another one or maybe several more cells. That process might fail at some point, due to cholesterol overaccumulation or other factors, which could contribute to necrotic core expansion as

well. Ira Tabas has recently shown that continual efferocytosis has an interesting link to immunometabolism in that it is fueled by arginine from the ingested cell, and that lesion regression can be enhanced by increasing this continual efferocytosis mechanism by supplementation of putrescine, an arginine metabolite, in a mouse model.

Dr Linton There are a several different receptors and pathways that are involved in efferocytosis. Ira Tabas has done a lot of work on the role of merTK as an important receptor for mediating efferocytosis, but there is a lot of redundancy and several other receptors are involved. We have shown that LRP1 and SRB1 both play important roles in macrophage efferocytosis in atherosclerosis. These receptors bind to ligands on apoptotic cells such as phosphatidylserine, and there are also bridging molecules such as apoE and MFGE8 that can improve efferocytosis efficiency. Impaired efferocytosis is associated with increased necrotic core formation and promotes characteristics of unstable plaques leading to plaque rupture. And I think that the study that Ed mentioned, which was Nick Leeper's looking at the ability of a monoclonal antibody to CD47 to mask a "don't eat me" signal, as a potential therapeutic approach for improving efferocytosis is really an interesting approach.

Dr Fisher But efferocytosis is linked to inflammation, because what Tabas showed in his recent paper that Karin alluded to, this ability to do a succession of uptake events involved the metabolites from the enzyme arginase 1. And we've shown–and he cites this work in his paper–that in the preclinical models, if you can shift the macrophages to the so-called M2 state, in which there is an upregulation in arginase 1, plaque inflammation will decrease. It's known that M2 macrophages are better at efferocytosis, so there is further support for the connection between efferocytosis as part of the inflammation resolution process.

Dr Guyton So this is important, because an expanding necrotic core is what makes the plaque vulnerable and prone to rupture, resulting in coronary thrombosis or perhaps resulting in atherothromboembolism from a carotid plaque and then a stroke. So macrophage biology is important.

I think that's a good segue into resolution of inflammation. What does resolution of inflammation mean, and do we have opportunities for therapeutic intervention there?

Dr Fisher Well, we've had a series of papers going back for years showing that in the preclinical models, it is possible to have resolution of inflammation as judged by how one defines inflammation. So we have focused on the general inflammatory state of the macrophages using this convenient M1/M2 classification.

But more broadly speaking, we have the whole area of resolvins, which of course are given that name because they resolve inflammation. And Charlie Serhan has pioneered this area, and there are a number of studies now in the preclinical literature that if you can increase the production of these resolvins or administer them through nanotherapies, you dampen the fire in a plaque. Esther Lutgens in Amsterdam has partnered with Willem Mulder, and I've been a collaborator on this, where she has a specific inhibitor of pathways downstream of the toll-like receptors that activate the inflammatory master factor NF-kappa B.

Dr Guyton These resolvins are lipid mediators, are they not?

Dr Fisher Yeah, and they're mainly made from omega-3 fatty acids. So some people think, like me, that the REDUCE-IT trial, which used highly purified EPA, that resolvins may be the basis for reducing the event rate by what, 25%, without really doing much to the triglycerides. And in fact, as the investigators report, the decrease in events was independent also of the effects on LDL-cholesterol or CRP, for that matter. And so I think that it's very plausible that the EPA was converted in the plaques to resolvins, and these promoted decrease in the inflammatory state.

Dr Linton There are several specialized pro-resolving mediators (SPMs), and it will be important to discover whether resolvins and other SPMs derived from EPA and DHA have different impacts on the cardiovascular system. Based on what we know from eicosanoid biology, small structural differences can result in large differences in biological effects.

Dr Bornfeldt And the overall hypothesis is that stimulating resolution of inflammation would have fewer side or serious side effects than inhibition of the inflammatory process, which I think makes sense.

Dr Guyton That's a great point.

Dr Fisher Also, going back to epigenetics, Mulder has with other colleagues a very nice paper in one of the *Nature Review* journals. These epigenetic effects give rise to what's called immune memory to macrophages so that they respond even more avidly to an inflammatory signal that they see again. As he reviews, you could interfere with that with inhibitors of mTOR.

So what they did, they delivered with nanoparticles mTOR inhibitors and reduced macrophage inflammation, and they used a fascinating model. It was actually a transplanted heart. And they showed that it kept beating longer. It was not rejected by the immune system for a significantly longer period of time. And so I think nanotherapies are going to be an important therapeutic opportunity or avenue, because of what happens when you globally interfere with inflammation. The CANTOS study was very important conceptually, but as we all know, there was an increase in fatal infections when you broadly inhibit the IL-1β. So when we talk about resolving or dampening inflammation or preventing inflammation, if we do that globally, of course that's not likely to be risk-free. But if we can deliver potent agents to the plaque directly, certainly in the peri-acute coronary syndrome setting, then you have a much more likely chance of dampening the inflammation in a plaque without having systemic consequences.

Dr Guyton I see. That would be a way of localizing your anti-inflammatory effect. Let's turn to obesity. We know it increases diabetes, hypertension, and hypertriglyceridemia. But does obesity influence atherosclerosis in other ways?

Dr Fisher Yeah. Well, as you know, the American Heart Association has declared it an independent risk factor. But not on the basis of, I think, settled science. I just had a meeting this morning where one of our junior faculty is proposing in his NIH RO1 application that obesity is an independent regulator of platelet function, which implies a connection with thrombosis. In general, I think it's hard to formulate a simple model of why obesity is an

independent risk factor. There are likely many and separate contributions of the adipocytes and the different types of immune cells (macrophages, T and B cells, etc.), in fat depots. For example, each type of cell is known to secrete factors that can exert systemic inflammatory effects.

Dr Guyton I think that Alan Chait has–and maybe others have–shown clusters of macrophages in adipose tissue. The idea seems to be that a fat cell dies and leaves all this triglyceride out in the extracellular space, and then you have a cluster of inflammatory cells around that.

Dr Bornfeldt Yes, Alan Chait has done some very important work in this area, as have many others. A remaining question is to what extent accumulation of adipose tissue macrophages contributes to atherosclerosis. One possibility is that inflamed adipose tissue can result in myelopoiesis, as shown by Ira Goldberg and you, Ed, in several different mouse models. Monocytosis in turn can cause increased monocyte and macrophage accumulation in lesions of atherosclerosis. This is one process that links adipose tissue macrophages and atherosclerosis.

Dr Guyton We have insulin-sensitive fat cells and insulin-resistant fat cells that may have different origins. If you have someone who comes in with very little fat on the thighs and the buttocks, many clinical lipidologists have the idea that there's really quite a difference in atherosclerotic risk depending on how the fat is distributed. Waist-hip ratio was advocated once, and now it's more waist circumference. I'm not sure we really know even today how to categorize the lipodystrophy-like pattern that we see. And this perhaps is what you're talking about. The visceral fat may be more inflammatory, whereas the fat on the buttocks and the thighs is less inflammatory and more insulin sensitive.

Dr Fisher Well, it's certainly a relevant theory for explaining why many South Asians, in spite of being relatively thin, have insulin resistance because when they do imaging, they have more visceral fat. So the concept of the visceral fat being more diabetogenic or inflammatory is there. But just to go back to that paper that Karin brought up that we were coauthors on–there, the model was that the macrophages in the adipose tissue secreted a factor that stimulated in the adipocytes themselves some IL-1β that actually, in the bone marrow, led to the proliferation of the monocyte and neutrophil precursors. So there would be an effect of obesity that was independent of diabetes and hypertension. Adipocytes or the adipose tissue macrophages secrete inflammatory factors that become systemic.

Dr Guyton Back in the 1960s we had a television quiz show called the $64,000 question. I'm revealing my age. Of course, today it's literally a billion-dollar question: How does diabetes drive the atherosclerotic process? What are the main concepts in the role of high glucose levels? Or is it something else about diabetes? Karin, I'm going to throw this one at you.

Dr Bornfeldt I think that it's a multifactorial process, but I'm not a proponent of glucose as a critical mediator of cardiovascular events. Human data on a direct causal role of glucose are not very strong, in my opinion. There is definitely a correlation between improved blood glucose control and reduced cardiovascular disease risk in several human studies, but that

does not mean that glucose per se is the culprit. Denis McGarry wrote a thoughtworthy article in Science in 1992 entitled “What if Minkowski had been ageusic? An alternative angle on diabetes.” He basically argued that we view diabetes primarily as a disorder of glucose metabolism (which could, according to legend, be detected by Minkowski by tasting the urine from diabetic dogs in the late 1880s), but that we are not paying enough attention to the fact that diabetes is also a disorder of fat metabolism because insulin affects homeostasis of several fuels. Based on our studies, we believe that remnants of triglyceride-rich lipoproteins play a more important role in accelerated atherosclerosis and increased cardiovascular disease risk associated with diabetes than does glucose. Indeed, in the apoC-III silencing experiments mentioned earlier, diabetic mice remained severely hyperglycemic but were protected from increased atherosclerosis. It is possible, though, that hyperglycemia plays a supporting role. For example, Trevor Orchard has suggested that elevated glucose levels can result in stiffening of the artery wall, which might exacerbate the effects of other risk factors. But certainly, there are many different cardiovascular risk factors, especially in type 2 diabetes. And so all those risk factors are likely to contribute in people with diabetes as well as in people without diabetes. But overall, I think of lipids and apolipoproteins as an area that we should emphasize more, even in relation to type 1 diabetes, which is not usually thought of in terms of dyslipidemia. Enhanced activation of inflammatory pathways, and to what extent lipids and glucose play a role, is another area of interest.

Dr Fisher In that meta-analysis that was published in *Lancet* in 2010 or 11, which covered about 200,000 patients in the statin trials, it clearly shows that although the relative risk in both type 1 and type 2 diabetics went down as in non-diabetics with statin treatment, if you look at the actual absolute rate of events, it still was about two times elevated versus the control group. So the lipid lowering in people and in mice with diabetes, as we have published in a series of papers, does not allow the full benefits of lipid lowering on the plaque.

And so in general, you go back to inflammation, even though I claim not to really understand it fully, but I know it when I see it. When we lower the lipids in the mice, the inflammatory state of the macrophages is sustained in the type 1 diabetic. And also now, we’re getting some similar evidence in the type 2 models of diet-induced obesity.

One factor that is coming on to be recognized is the neutrophil extracellular trap (NET), which has been found to be in human tissues, and increased in those with diabetes. NETs impair wound healing in mouse models of diabetes, and they promote atherosclerosis in mice. And Peter Libby and others have had papers in which they have correlated ruptured plaques with NET contents, which makes sense because NETs are considered to be inflammatory.

The reason I bring this up in the context of diabetes is that diabetes definitely increases the susceptibility of neutrophils to form these NETs. I think that one of the contributing factors to the increased cardiovascular risk and disease in the diabetic setting is these neutrophil extracellular traps. And I would be remiss if I didn’t bring up, since Ann Marie Schmidt is my colleague here at NYU, who discovered RAGE, the receptor for advanced

glycation endproducts (AGEs). When you look at human plaques in people with diabetes, the immunohistochemical staining for RAGE is very high. And RAGE feeds into the NF-kappa-B pathway. So I don't know about my colleagues' thoughts about RAGE, but certainly here at NYU, we think it's a contributing factor to the cardiovascular disease that's seen in diabetes.

Dr Bornfeldt It's clear that RAGE is important in several different conditions characterized by inflammation and metabolic stress and that it has a range of ligands not limited to diabetes. I think of RAGE not as a receptor with a limited role in diabetes, but as a receptor with a wider role in immune regulation and metabolism.

Dr Guyton When we think about advanced glycation end products, I recall how my biochemistry professor in medical school said that the cyclic form of glucose protects you from the glucose aldehyde, but that 1 out of every 100 glucose molecules is an open-chain aldehyde. That's the way the chemical equilibrium of glucose conformation works.

So glucose is a weak aldehyde and forms the glycation products via aldehyde reactions. And RAGE, the receptor for AGEs is basically protecting against the aldehyde effect of glucose.

Dr Fisher Yeah, I hadn't heard that. So even though I have a PhD in biochemistry, I clearly nodded off during that part of the lecture.

Dr Guyton There's another current area we need to touch on. Could someone summarize what are microRNAs, and what are the prospects for therapeutics derived out of microRNAs?

Dr Fisher Yeah. So here again, all politics is local, as Tip O'Neill used to say. So I'm next door to Kathryn Moore, who co-discovered miR-33 with Carlos Fernandez when he was also here. I'm coauthor of that paper in *Science* that they had. Kathryn went on to show in a *Nature* paper that if you use anti-miR-33, you were able to increase the level of HDL–this is back when HDL was considered a good thing–in nonhuman primates, which suggests translational potential. There are a number of other miR candidates, as I'm sure Mac knows from working with Kasey Vickers, that are thought to have some beneficial effects on one or more of the major cell types in a plaque.

The question is delivery. And I think in terms of the antisense technologies mentioned before, Ionis Pharmaceuticals is very aware of needing to target their therapies, and have been successful in the liver. So first of all, you've got to think of your agent that you want to use. Then being able to target it specifically, I think, is really key. It's very analogous to the cancer field. You want to deliver your substance to the cells that you want to kill and not get the innocent bystanders.

Dr Guyton Right. I have read that of all the proteincoding genes, 60% of them are regulated by a microRNA, which just sounds stunning, when you think that it's an entirely novel mode of regulation.

Dr Fisher So any microRNA has at least 60 or 70 targets. Take miR-33 - although it has this beneficial effect in the mouse and nonhuman primate models on HDL levels, and promotes regression of plaques in mice, Carlos Fernandez published a paper that anti-miR33 promoted cell proliferation and also promoted increased production of VLDL triglycerides. And so that's why you want to make it very specific, as specific as you can to the cell type you want to influence, because they have 60 to 70 targets. And they are not all necessarily going to go in the good direction.

Dr Guyton A note of caution.

Dr Fisher Exactly right. But I don't know. What are some promising pathways to try to target with microRNA?

Dr Linton There are so many of them now. Potential miRNA targets for atherosclerosis may be organized based on cell type. For example, Kasey Vickers has shown that miR-92a-3p is a pro-atherogenic miRNA in endothelial cells that may hold value as a therapeutic target to reduce atherosclerosis burden, particularly in the setting of chronic kidney disease. In macrophages, miR-155 has emerged as a potential therapeutic target to reduce pro-inflammatory phenotypes in macrophages and could be used as a target to reduce atherosclerosis-associated inflammation. There are potential targets for essentially all of the cell types in the artery and also for dyslipidemia. But I agree that it is hard to wrap your mind around because of the large number of pathways regulated. And there has to be a way to really direct the therapy where you want it.

Dr Fisher Another barrier is, of course, we want to know how things are doing in people. A colleague years ago said "M.D." doesn't mean "mouse doctor." We really want, people like Mac and Karin and me, we really want to translate the findings back to the clinical scenario. The major endpoint that drug companies have used for go/no-go decisions for translational decisions has been intravascular ultrasound (IVUS), because we don't have the great biomarkers yet that are going to really tell us yes or no. And the problem with IVUS, as you well know, is first of all, it's invasive, so it can't be used in primary prevention studies. And it's not a high throughput. And it gives you changes mainly in the plaque volume as estimated by the wall thickness.

And we know from the animal studies that changes in plaque composition really happen in a lot of situations in which the change in the plaque volume are not significant. And we know from the cartoons back in all the statin talks we used to go to where Peter Libby or Valentin Fuster would present, they'd talk about its fewer macrophages and more fibrous content (such as collagen) that give you a stable plaque. Two-thirds of plaques that rupture are less than 50% stenotic. So you can take a plaque that's not particularly stenotic, get rid of all the macrophages you're going to have a buildup of collagen because you don't have the metalloproteinases, and your wall thickness is not going to change. And big pharma would say, well, we have a failure here. We didn't see any change in the wall thickness, but the plaque is likely to more stable.

So at the same time that you asked your question about what are some current areas of arterial biology research, we have to think in parallel how are we going to tell if it's doing

anything in people. And our current methods, imaging methods, are not only invasive, but most of them are not giving us compositional changes, which are very, very important.

Dr Guyton You know, it's tough to combine magnetic resonance (MR) imaging with MR spectrometry. You've got only three dimensions you can work with, and if you take one of those dimensions to get the spectrum, you sacrifice 3-dimensional localization. But I thought about this, and we had some exploratory discussions at Duke about looking for cholesteryl esters, because cholesteryl ester would be fairly specific for atherosclerosis, if you could distinguish it from triglyceride. Could you possibly quantify cholesteryl ester in the carotid artery with a magnetic resonance probe? It seems to me that something along those lines might be possible.

Dr Fisher So going back to candidates–I mean, when I was at MIT, we had Bob Lees, who had done his fellowship at the NIH, which resulted with "Frederickson, Levy, and Lees" an historically important dyslipidemia classification. And Bob went on as a faculty member at MIT to try to use LDL as an imaging agent, but I don't know whatever happened to that approach.

And then if you go back to Don Zilversmit, another famous name in our field, what he showed in the 1970s was that when he took a rabbit atherosclerotic aorta and sectioned it, he can show that the plaque burden paralleled the activity of lipoprotein lipase (LPL). So LPL was a good marker of the plaque burden because it is a major product of macrophages in plaques.

Dr Guyton Interesting.

Dr Fisher Another way of expressing this is that it's a surrogate of activated macrophage activity. So we have a lot of imaging candidates. And the field of molecular imaging is trying to really make that clinically useful. My old boss, Valentin Fuster is a big proponent of this. He recruited Zahi Fayad around the same time to apply MRI to coronary imaging. And it just has not worked yet. Even with slowing the heart down with beta blockers to some degree, there appears to be too little time between beats to acquire all of the information that's needed to construct a good MR image of the coronary artery.

Dr Guyton It certainly works better in the carotids.

Dr Fisher Oh, for sure, or the femorals.

Dr Guyton Well, can atherosclerosis be cured? Can we get regression of plaques and healing of the artery wall? How close are we, do you think?

Dr Fisher Well, how close, I don't know. But again, I go back to mice. We can cure it in mice. If we let these mice go on for a long time after our interventions, the wall of the artery returns to a fairly normal appearance. But what we're trying to figure out is exactly how this happens and look for pathways that can be manipulated in human plaques. But I think certainly from the point of view of stopping progression, we have made a lot of progress by lowering the LDL significantly. And I think, going back to inflammation, if we can combine

the right–see, that's the key–the right anti-inflammatory agent or a potent resolving agent–and deliver it to the right cell in the plaque, in combination with lipid lowering, we could regress plaques.

Dr Guyton Is there agreement?

Dr Bornfeldt Yes, but what pathway we need to target in addition to LDL to get rid of the residual risk could vary depending on the set of specific risk factors in a patient, sex differences and race and ethnicity variables. Statins have clearly worked well. But there is the residual risk. An important question is, is the residual risk caused by the same factors in different populations of people with different risk factors?

Dr Guyton Right. Any final thoughts?

Dr Fisher I would tell all the readers to write to their senators and congressmen to support the NIH research budget, because as we know, again, we're the choir. When I tell the patients is that the best we can do is based on yesterday's research. And they don't really understand how research is supported.

I know a lot of educated people who don't really understand the nature of medical research. They think NYU is giving me most of my money for research. All of us, we're dependent on extramural funding. And if you divide up the NIH budget by the amount of people in the U.S., it's what, about 85 bucks a person per year for all health, disease-related, and basic academic research. You go to a New York Yankees game, a family of four, and each gets a hot dog and a drink combined with parking and tickets, it's, like, 500 or 600 bucks. And so people don't think anything of spending 500 or 600 bucks to go for something entertaining. But if they knew that all of their health care research was essentially $85 a person for the whole year, they might be concerned about how we are going to make progress in this area.

The AHA likes to point out how much progress we've made in reducing heart disease. But the national statistics are now showing that in absolute numbers, the deaths from cardiovascular disease since 2015 goes up each year. This is not a problem that has gone away. We need our health professionals to better convince lay people to keep advocating for more research in this area.

Dr Linton One thing I would say is that after a long period of statins dominating our therapeutic options, we've entered into an era where there's suddenly a lot going on in terms of new approaches for therapies to treat dyslipidemia and atherosclerosis. I mean, we talked about microRNAs, and I think Ed mentioned anti-sense. There are several RNA based therapies for different targets that are in, or have completed, phase III clinical trials: GalNAc-anti-sense oligonucleotides (ASO) to apolipoprotein(a), apoC-III, and ANGPTL3, and GalNAc-siRNA to PCSK9. I think there's a huge potential of these therapies to meet unmet needs for some of our difficult to treat patients. And I think lipoprotein(a) [Lp(a)] is really a huge unmet need for everybody that runs a lipid clinic and the patients with severely elevated Lp(a). It will be interesting now that there will be ways to target Lp(a)levels to see whether that reduces cardiovascular events.

Dr Bornfeldt I agree. I think we can look forward to an exciting era of new therapeutics to tackle residual cardiovascular disease risk.

Dr Guyton Let me say this discussion has exceeded my enthusiastic expectations.

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Suggested reading

1. Libby P, Bornfeldt KE. How far we have come, how far we have yet to go in atherosclerosis research. Circ Res. 2020;126(9): 1107–1111. Introduction to a Compendium Issue on Atherosclerosis in Circulation Research. [PubMed: 32324500]
2. Goldberg IJ, Sharma G, Fisher EA. Atherosclerosis: making a U turn. Annu Rev Med. 2020;71:191–201. Atherosclerosis regression is the next step in reducing residual risk. [PubMed: 31986087]
3. Basu D, Bornfeldt KE. Hypertriglyceridemia and atherosclerosis: using human research to guide mechanistic studies in animal models. Front Endocrinol. 2020;11:504. Targeting hypertriglyceridemia as a risk factor depends on understanding mechanisms.
4. Moore KJ, Koplev S, Fisher EA, et al. Macrophage trafficking, inflammatory resolution, and genomics in atherosclerosis: JACC macrophage in CVD series (Part 2). J Am Coll Cardiol. 2018;72(18):2181–2197. Comprehensive review of the involvement of monocyte-macrophages in inflammation and resolution of inflammation. [PubMed: 30360827]
5. Tao H, Huang J, Yancey PG, et al. Scavenging of reactive dicarbonyls with 2-hydroxybenzylamine reduces atherosclerosis in hypercholesterolemic Ldlr−/− mice. Nat Commun. 2020;11(1):4084. Blocking effects of lipid peroxidation may benefit LDL, HDL, and lesion stabilization. [PubMed: 32796843]
6. Guillermier C, Doherty SP, Whitney AG, et al. Imaging mass spectrometry reveals heterogeneity of proliferation and metabolism in atherosclerosis. JCI Insight. 2019;4(11):e128528. Advanced technology to study leukocyte and smooth muscle cell proliferation in relation to glucose utilization. [PubMed: 31167964]