BIOMARKERS AND COMPOSITIONS FOR USE IN TREATING CARDIOVASCULAR DISEASE

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Background of invention

A. Technical field:

The present invention relates to the use of biomarkers to diagnose and assess the risk for cardiovascular disease, which are important aspects in clinical decision making and setting therapeutic strategies. More specifically, the present invention relates to use of biomarkers as companion diagnostics to assess the efficacy of compounds and formulations to treat vascular diseases particularly thromboembolism.

B. Background art:

1. Human makeup and diseases:

To appreciate the origin of diseases, we start by understanding our make-up (Fig. 1).

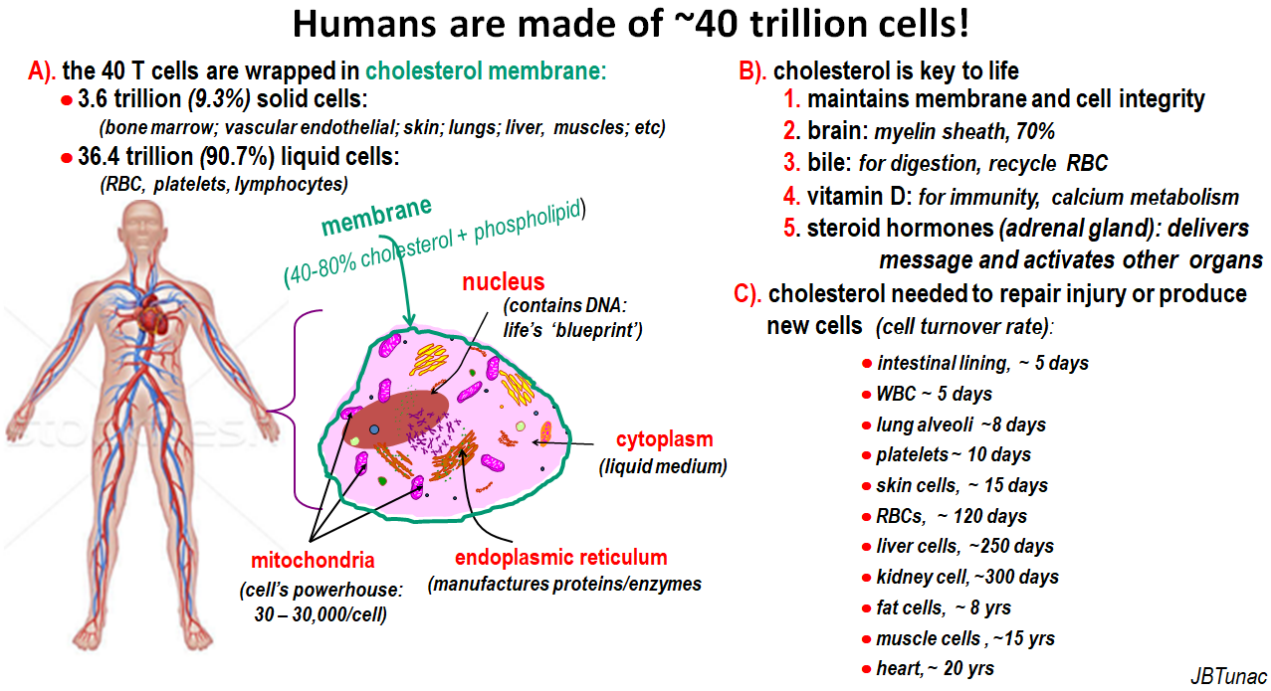


Figure 1. Our body is made of cells that regularly die and regenerate (except the neurons); cell regeneration, growth and repair, need energy.

Nourishment comes from the food that we eat and metabolism extracts electrons from the stored C-H bonds in carbohydrates, proteins, fats, alcohol (Fig 2).

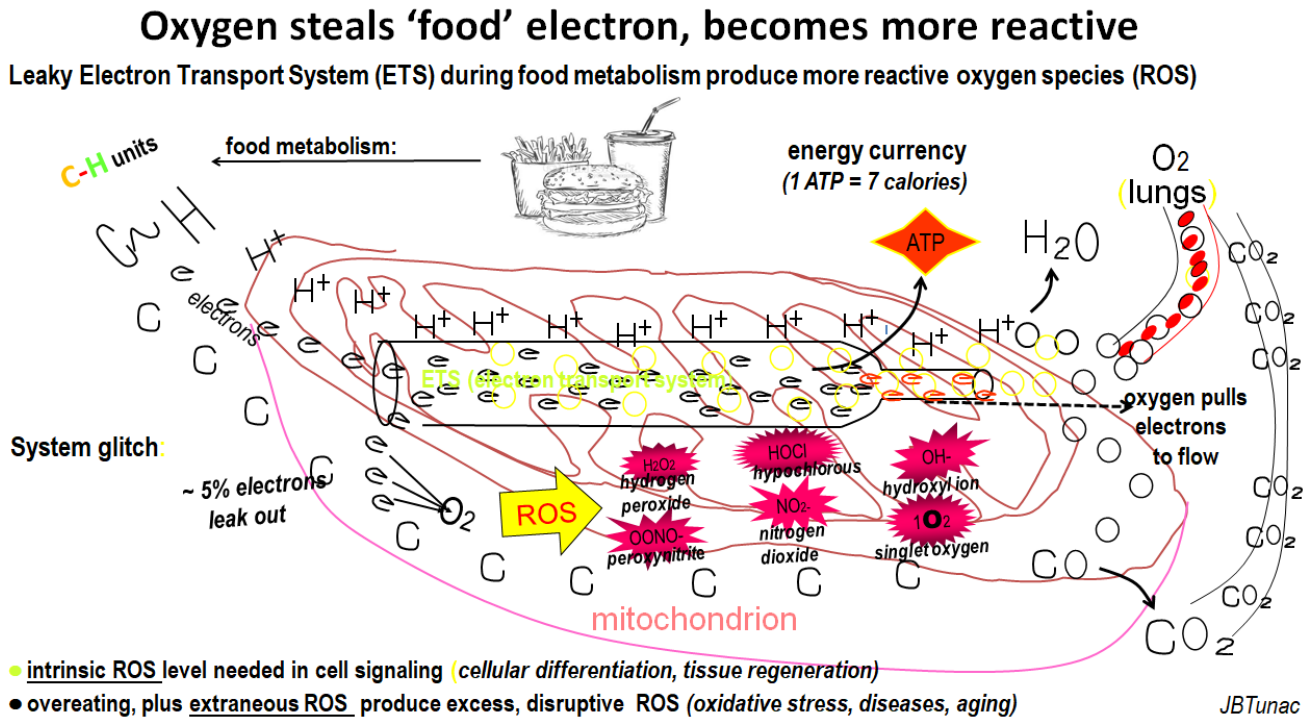


Figure 2. Mitochondria is the power house of every cell that generates electrons to power the activities of the cells

Oxygen that we breathe pulls food electrons in the mitochondria, via the electron transport system (ETS), creating electron flow (current or electricity); consequently generates energy in the form of adenosine triphosphate (ATP). However, ETS has a 'glitch' in that it leaks ~ 3% of food electrons. Leaked electrons over time interact with oxygen and generate more reactive oxygen species (ROS); such ROS steal electrons from molecules in our body (process called oxidation). These are intrinsically produced ROS and beneficial to a certain extent. However, exposure to stressful environmental factors contributes additional ROS, and these extraneous ROS would soon create an excessive level (Fig 3).

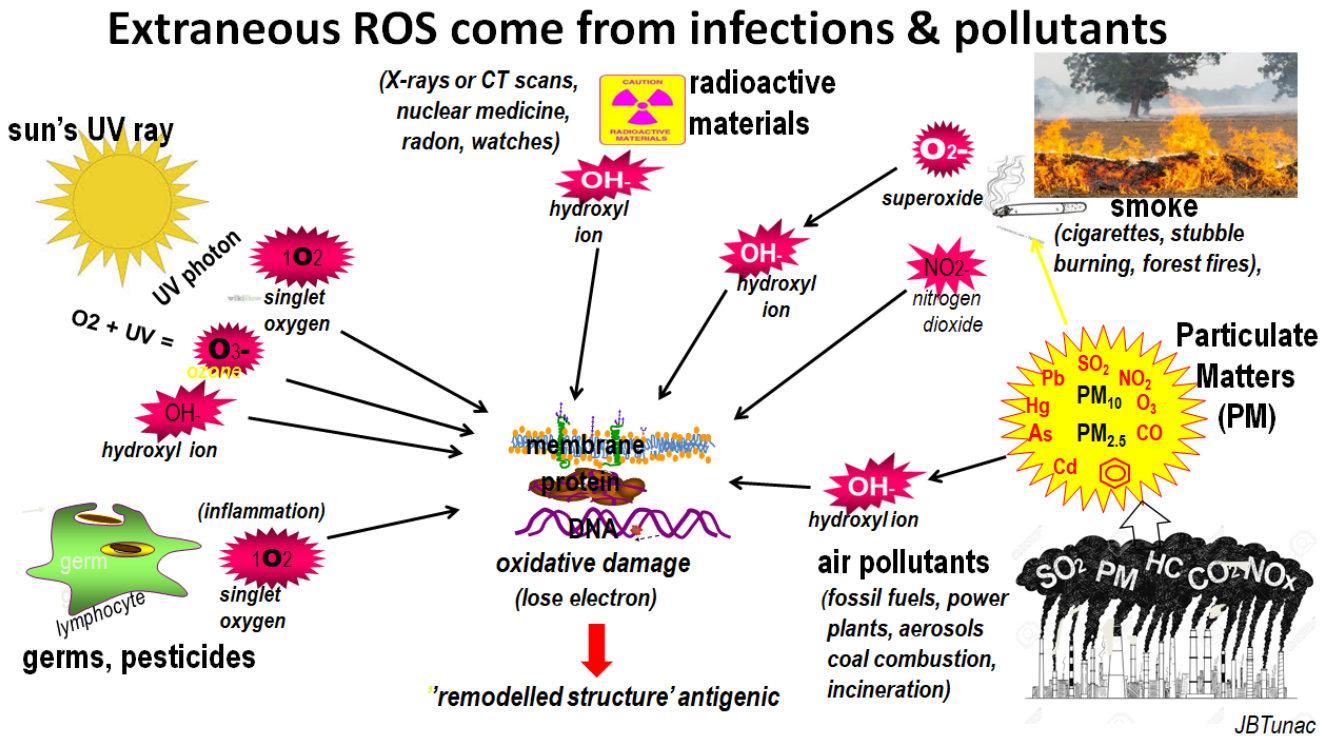


Figure. 3. The environment provides a varied source of chemicals that stress our cells, including infections ozones and chemicals packaged in particulate matters (PMs)

The intrinsically produced ROS are needed as signaling molecules for growth and repair and the right balance is kept in check by antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), peroxiredoxin (PRX) and glutathione reductase (GSR). Excessive ROS level disrupts the ROS-antioxidant balance or homeostasis and results in the various diseases (Fig 4).

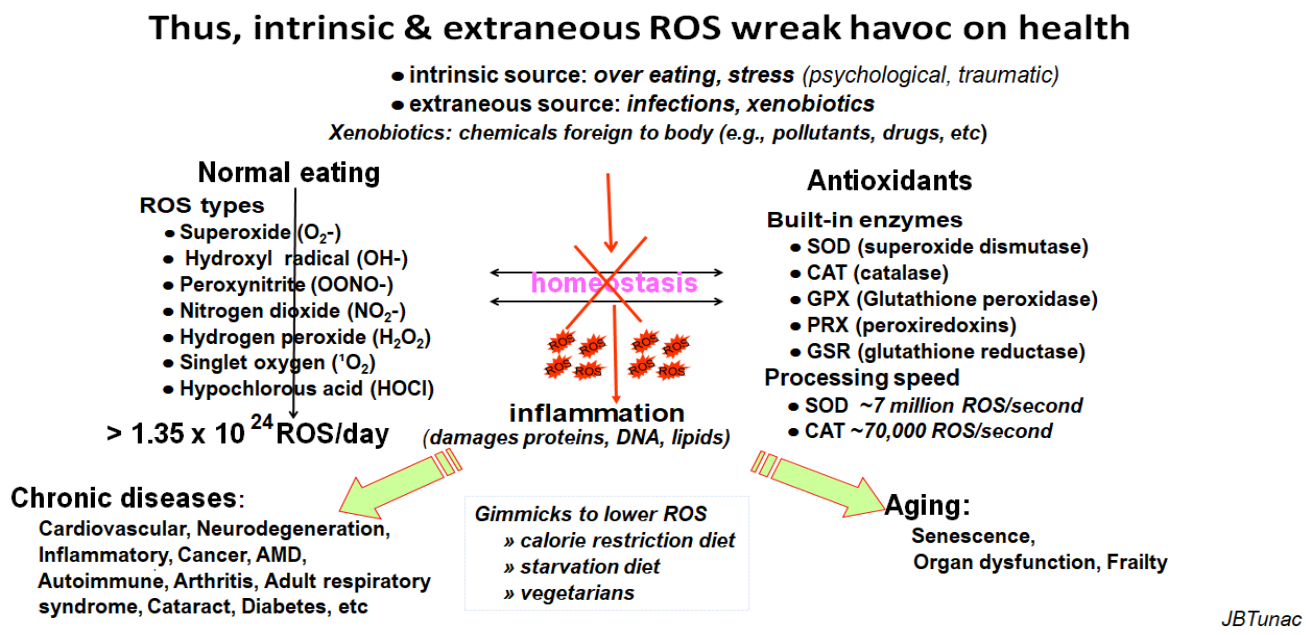


Figure 4. Excess reactive oxygen species (ROS) steal electrons, oxidize cellular molecules and trigger diseases and aging

2. Genesis of diseases:

ROS are more electron negative and reactive than oxygen. Oxygen has two unpaired electrons in separate orbitals in its outer shell. This electronic structure makes oxygen especially susceptible to radical formation. Sequential reduction of molecular oxygen (equivalent to sequential addition of electrons) leads to formation of a group of ROS, which is the bane to all aerobic species. All ROS are extremely harmful to organisms at high concentrations that exceeds the defense mechanism. Excess ROS 'steals' electron from nearby atoms creating a 'foreign or antigenic' molecule and a cell under oxidative stress (Fig.5)

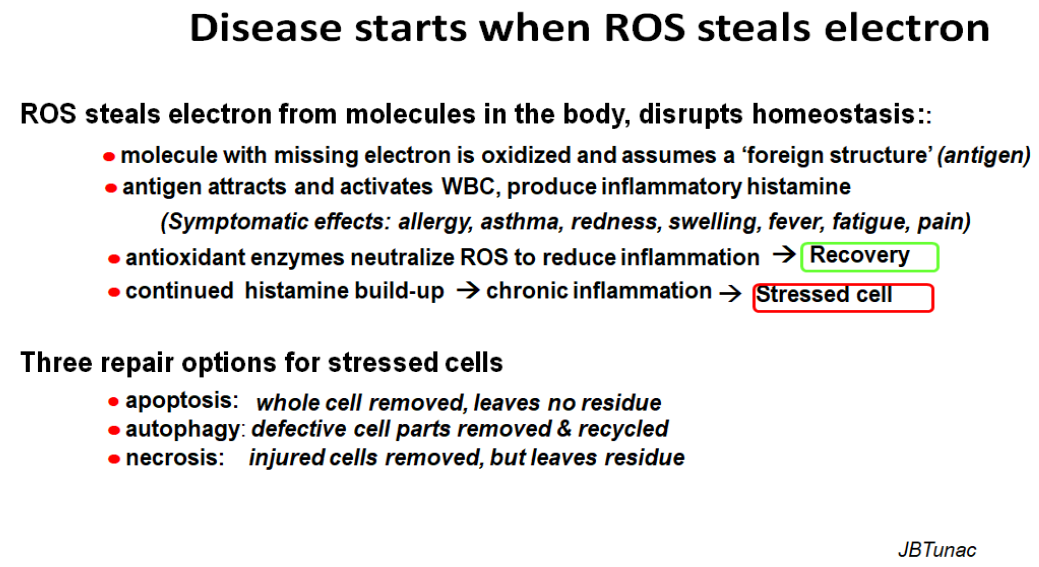


Figure 5. Cells containing molecules with missing electron (oxidized) is under oxidative stress and if not promptly repaired create various diseases.

The antigenic molecule triggers the release of histamine, an organic nitrogenous compound stored in mast cells and basophils, which binds to various cellular receptors and trigger inflammation Histamine increases the permeability of the capillaries to white blood cells and some proteins, to allow them to engage with the antigens (pathogens, xenobiotics) in the distressed tissues.(Fig. 6)

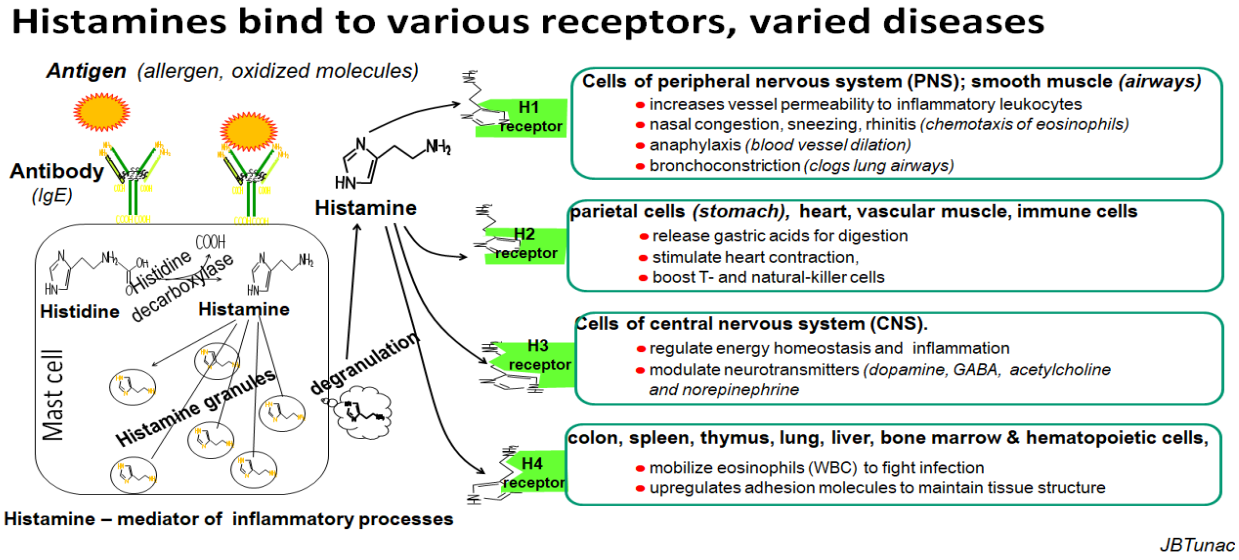


Figure 6. Antigenic molecules triggers the release of histamines and an inflammatory cascade

In distressed tissues, histamine regulates the fate of the cell with three options: apoptosis, autophagy, or necrosis (Fig. 5). Inadequate repair leads to various diseases.

3. Three types of diseases

The World Health Organization (WHO) compiles a listing of diseases called the International Classification of Diseases (ICD). In the 1994 ICD-9 there were 13,000 disease entries, which jumped to 68,000 in the updated ICD-10 in 2015. A jump in the disease entries is accounted for by the increasing exposure to environmental pollutants and chemicals that are artificial to our body (xenobiotics), creating diseases herein we call xenodiseases™. Basically, diseases could be classified into three types (Fig. 7).

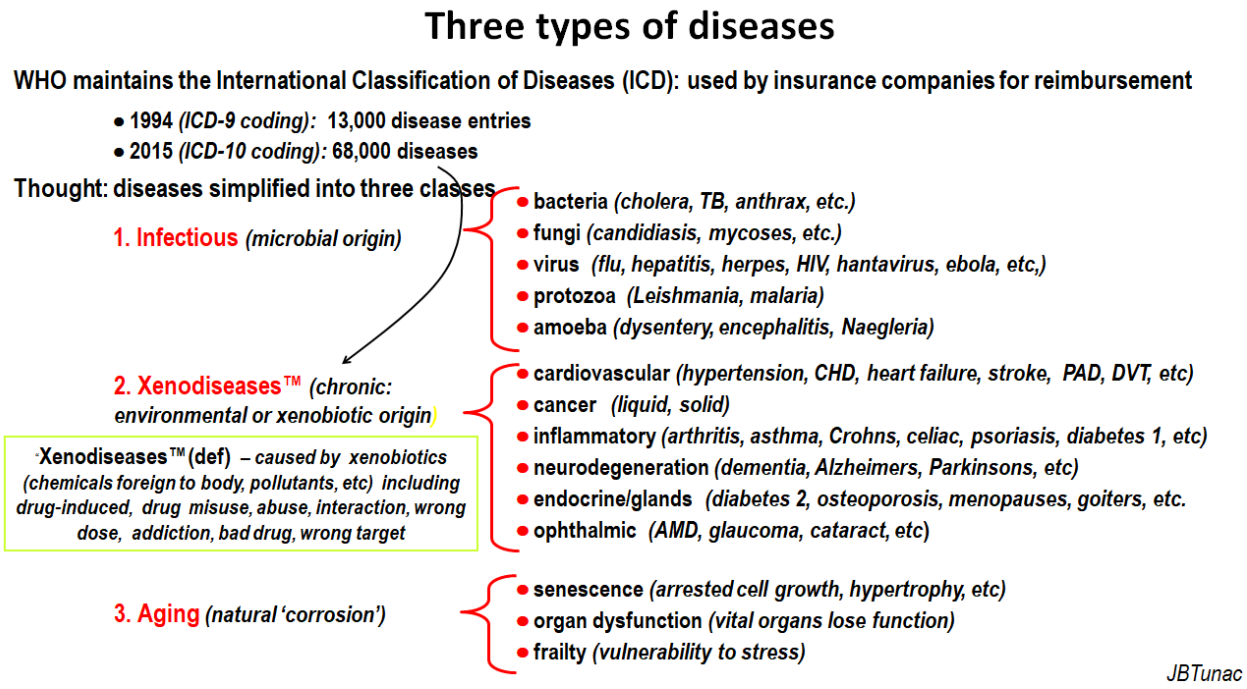


Figure 7. Diseases are basically grouped in 3 types

Indeed, a 25-year study has finally confirmed xenobiotics as the central cause of chronic diseases or xenodiseases™ (Fig 8).

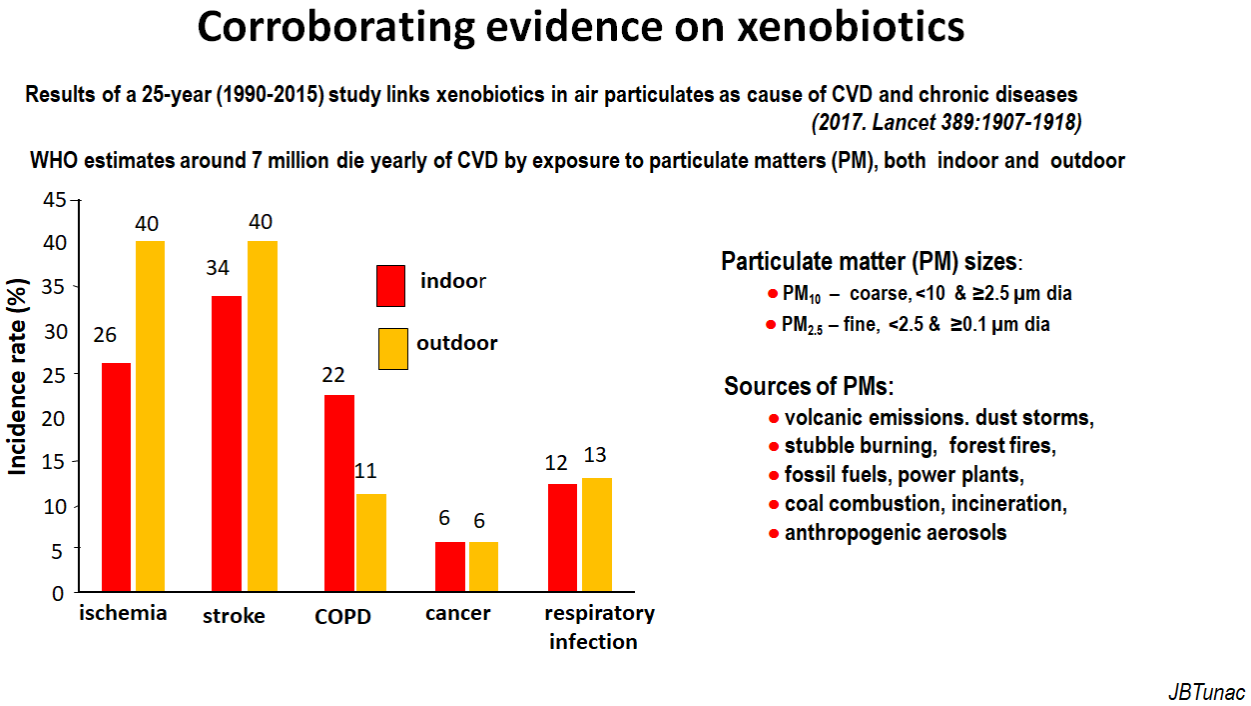


Figure 8. Chronic diseases (xenodiseases™) are now found to be triggered by environmental pollutants (xenobiotics)

PMs effectively package xenobiotics to the blood stream, which cause multifactorial damage to cells including oxidative stress, inflammation, and disruption of the blood vessel lining (Fig 9)

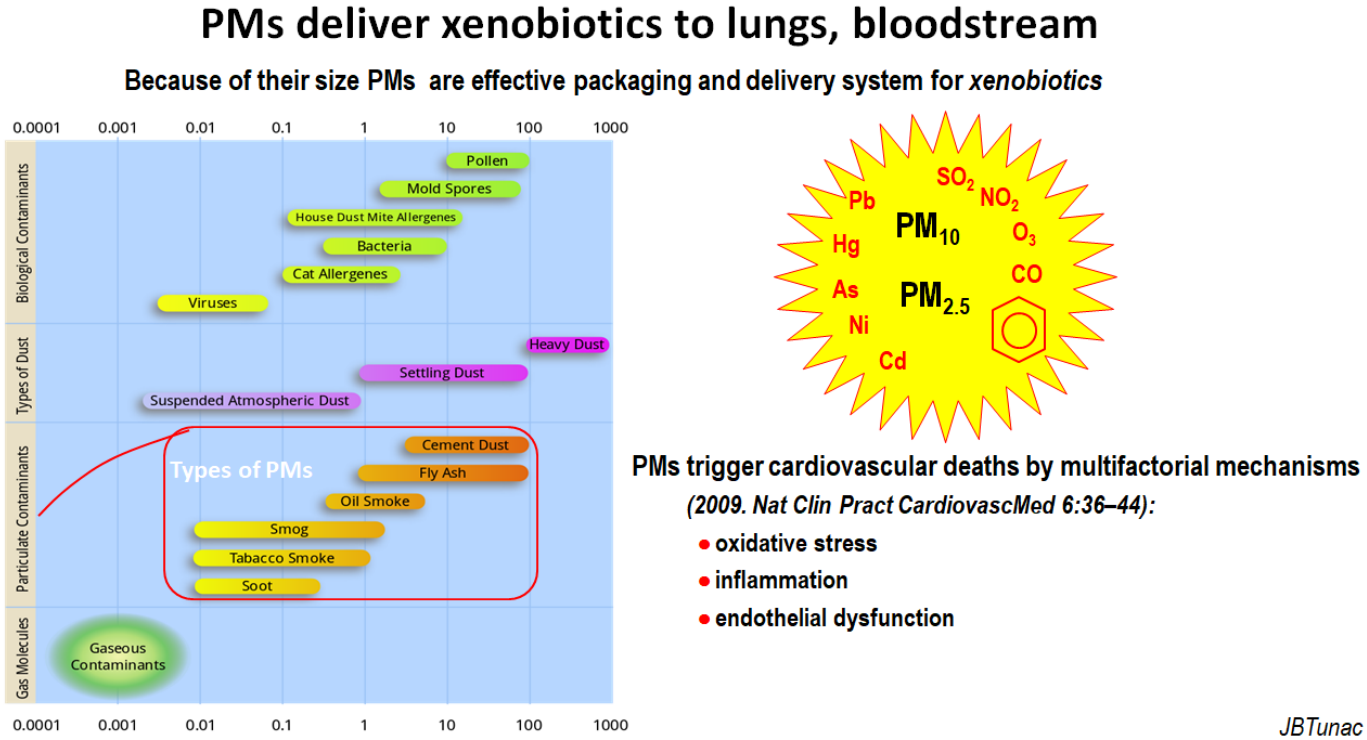


Figure.9. Size of particulate matters (PMs) compared to viruses, pollens, bacteria, etc

4. Cardiovascular disease (CVD) as the top killer and cholesterol-lowering drugs are ineffective:

A review of the top diseases in a 16-year span (2000 – 2016) shows CVD as the top disease with xenodiseases™ generally rising while infections are down. Notably, infections with HIV and preterm birth disappeared from the chart. An infectious disease is invariably caused by a single microorganism and developing a drug (antibiotic or vaccine) against a single causative agent is straightforward and relatively easy. On the other hand, a xenodisease™ is multifactorial in nature with multiple symptoms and traditionally drugs are developed to target a symptom. Symptom targeting drugs are not curative, only palliative at best and worse they contribute to rising incidence of xenodiseases™ (Fig.10).

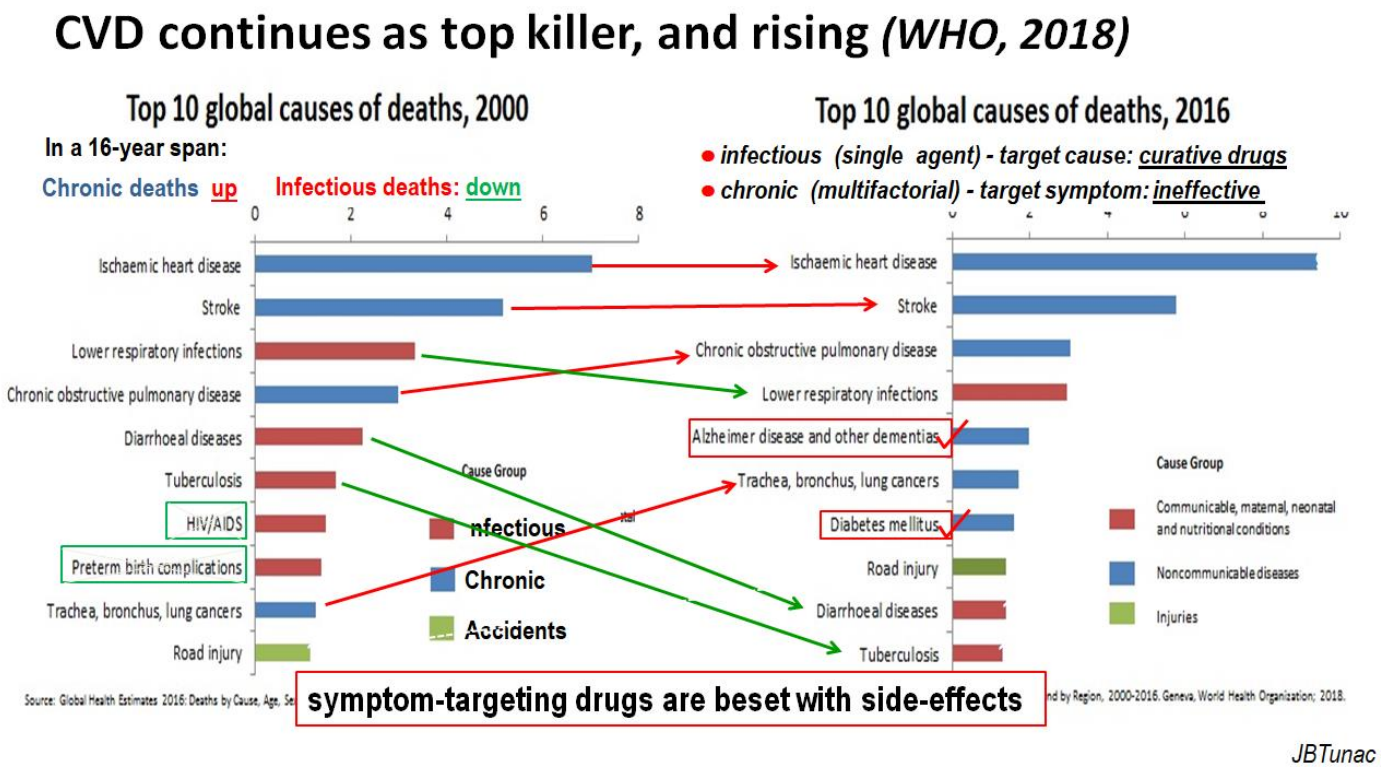


Figure 10. Snap shot of the top 10 global diseases in a 16-year span, 2000-20016

5. Statins contribute to xenodiseases™

Elevated blood cholesterol is singled out as the symptom associated with CVD and that lowering cholesterol became a consensus target for treating CVD. Currently, cholesterol-lowering drugs, particularly statins, are the number one prescription drugs and *Lipitor* the all-time revenue-generating pharmaceutical product (Fig 11)

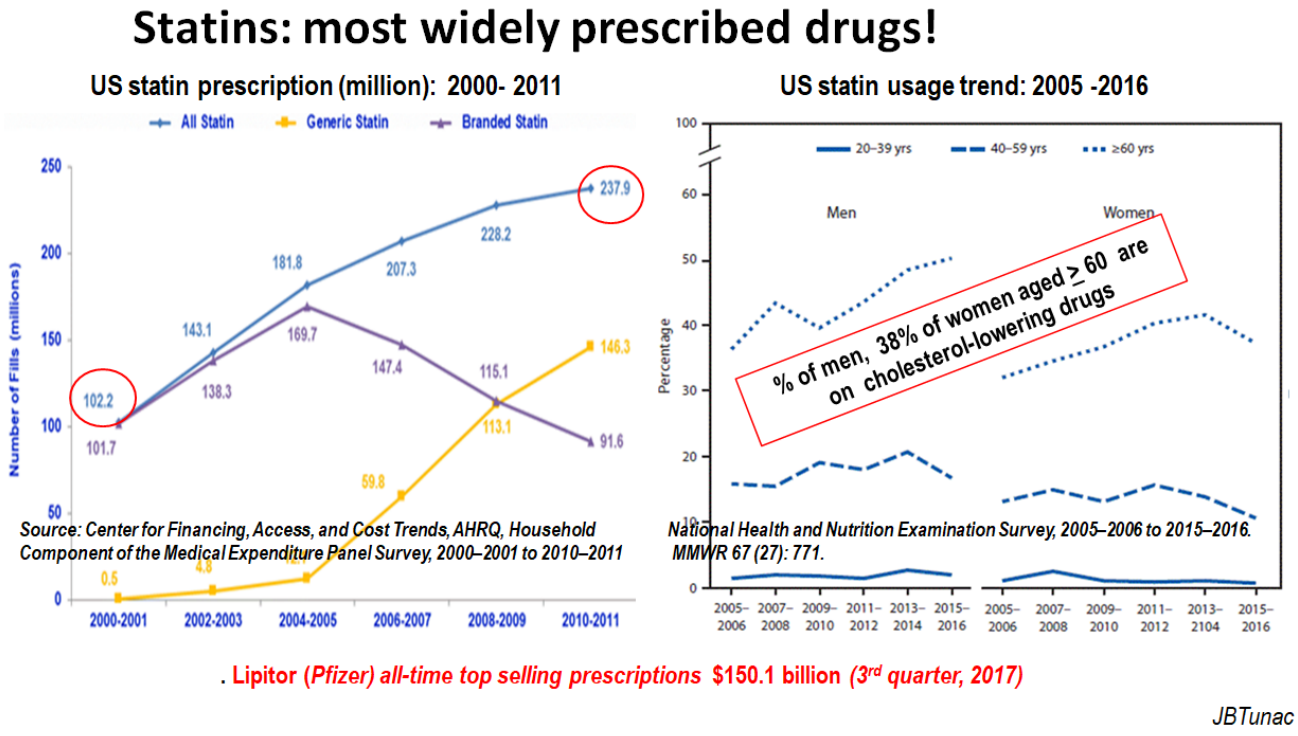


Figure 11. Statins are the most prescribed and best-selling pharmaceuticals to treat CVD

Cholesterol is an integral part of our cells (Fig. 1); it is a key to life and a wrong drug target. For example, statins inhibit the enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG CoA) reductase responsible for cholesterol synthesis and affect subsequent metabolic pathways needed for health. Disruption of cholesterol balance triggers a cascade of diseases including diabetes, fatigue, fibromyalgia, osteoporosis, erectile dysfunction, prostate cancer, and CVD (Fig. 12).

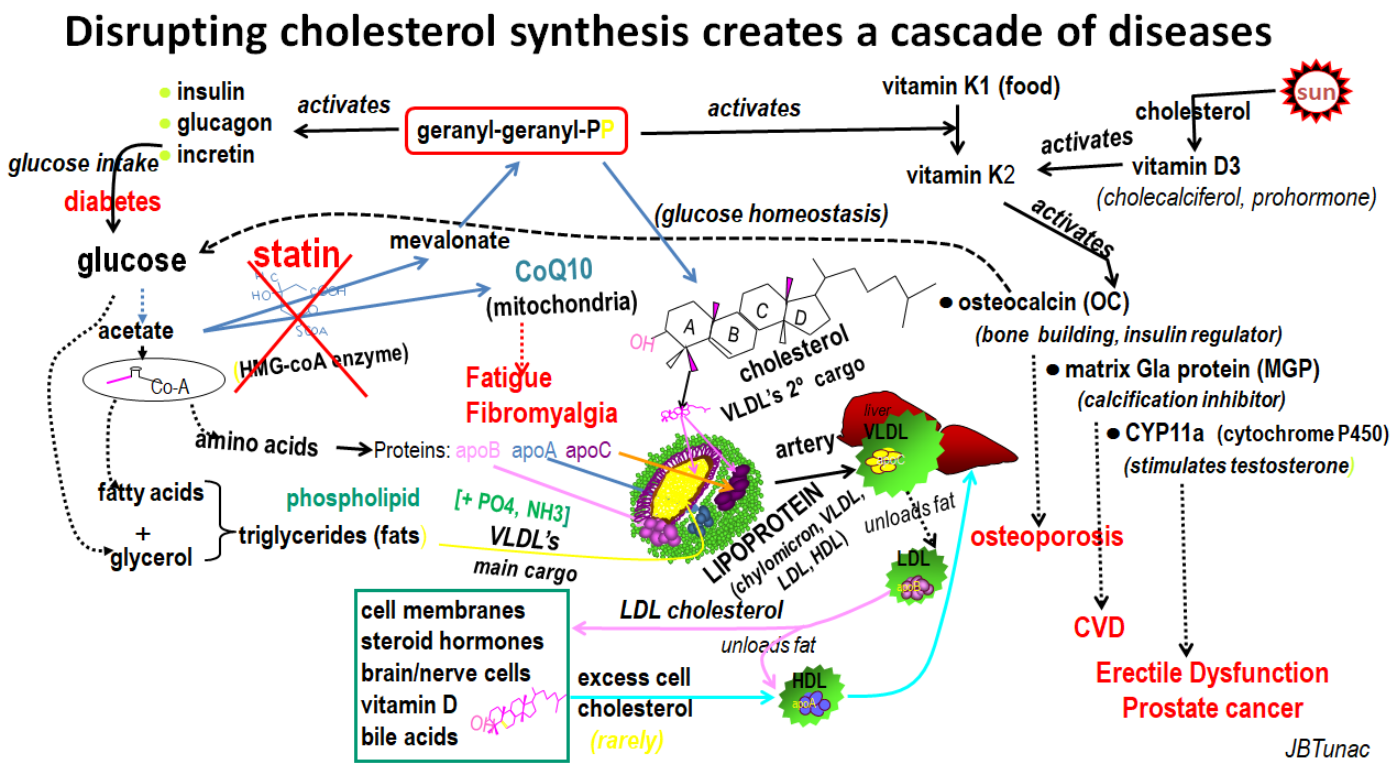


Figure 12. Statins disrupt cholesterol synthesis leading to a host of diseases, contributing to the growing problem of drug-related deaths.

With the growing use of statins, a large clinical trial was finally carried out to assess their value. Results show that statins create drug-induced deaths prompting FDA to issue a "black box warning". However, the latest government cholesterol guideline (Nov 18, 2018) still recommends aggressive statin use and an LDL goal of <70 mg/dL including drafting guidelines to combine statins with the newer cholesterol-lowering drugs, PCSK9 inhibitors (*Repatha, Praluent*) (Fig. 13).

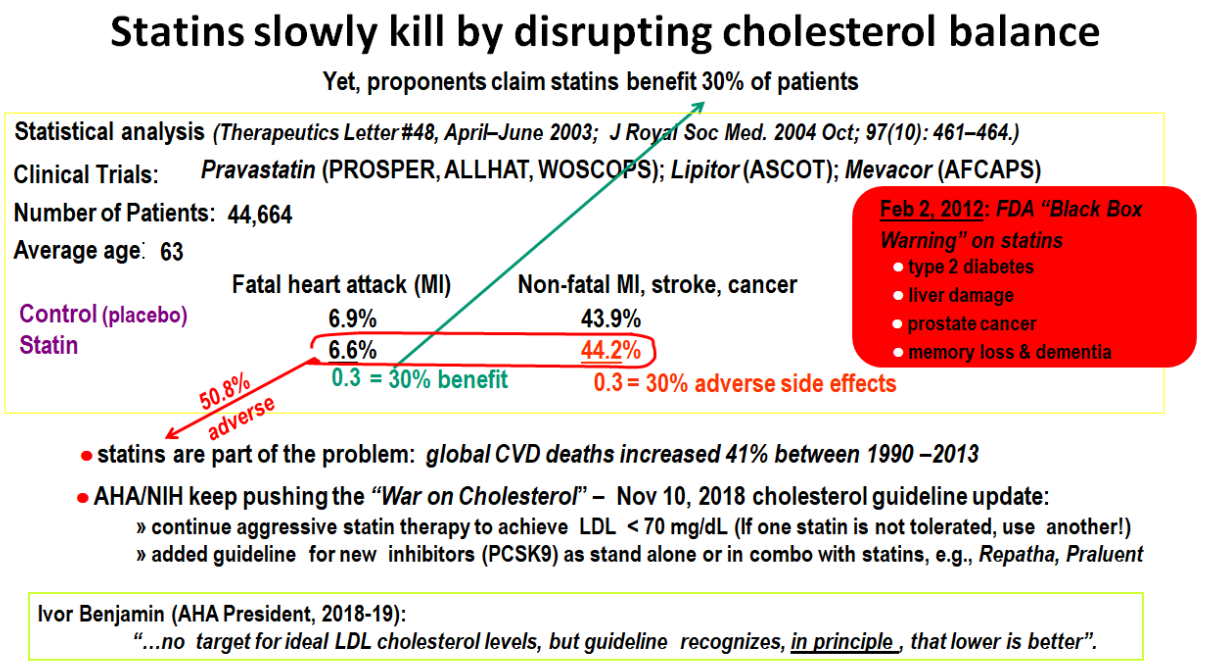


Figure 13. Statistical analysis show statins as risky drugs, but NIH/AHA continue to promote their use

Disruption of cellular cholesterol synthesis by statins or other cholesterol-lowering agents has contributed to the growing list of xenodiseases™. Unbeknownst to most, drug-related complications, are now the leading cause of deaths in humans (Fig 14).

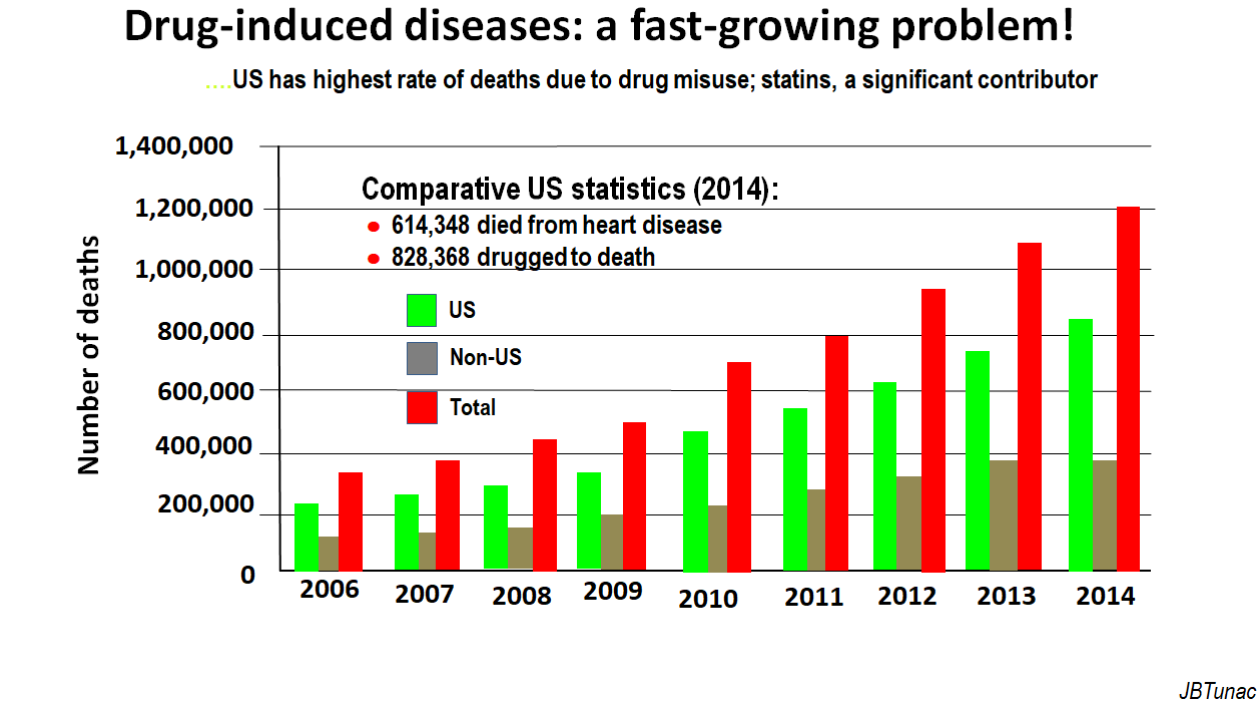


Figure 14 Drug induced diseases are the fastest growing at an alarming rate

6. Cholesterol is beneficial to fight stress:

Actually, high cholesterol is needed in defense to stress:

- cholesterol levels of 300-400 mg/dL range in response to stress *(1999. J. Clin. Endocrinol Metab. 84, 2664–2672; 2002. J. Clin. Endocrinol. Metab. 87, 4872–4878)*
- under chronic stress, synthesis of LDL is increased to generate *cortisol (2015. Afr Health Sci.15: 131–136; 2009. J Ayub Med Coll Abbottabad.21:15 8-61)* high cholesterol essential vs infection, respiratory diseases, depression, hemorrhagic stroke, etc. *(1998. Epidemiol Infect 21:335–47)*
- cholesterol needed for cell growth and repair (2016 J: Biomed Spectroscopy Imaging, 5: S101-S117) statins do not have significant effect in primary and secondary prevention of CVD *(2015. Rev Clin Pharmacol 6: 1-11).*
- statins effectively lower cholesterol but not in treating atherosclerosis (*2015. J Controversies Biomed Res 1:67-92)*

Stress comes in many forms, including psychological, trauma, infections and xenobiotics. Our body combats stress by producing cholesterol, particularly cortisol (hydrocortisone) (Fig. 15).

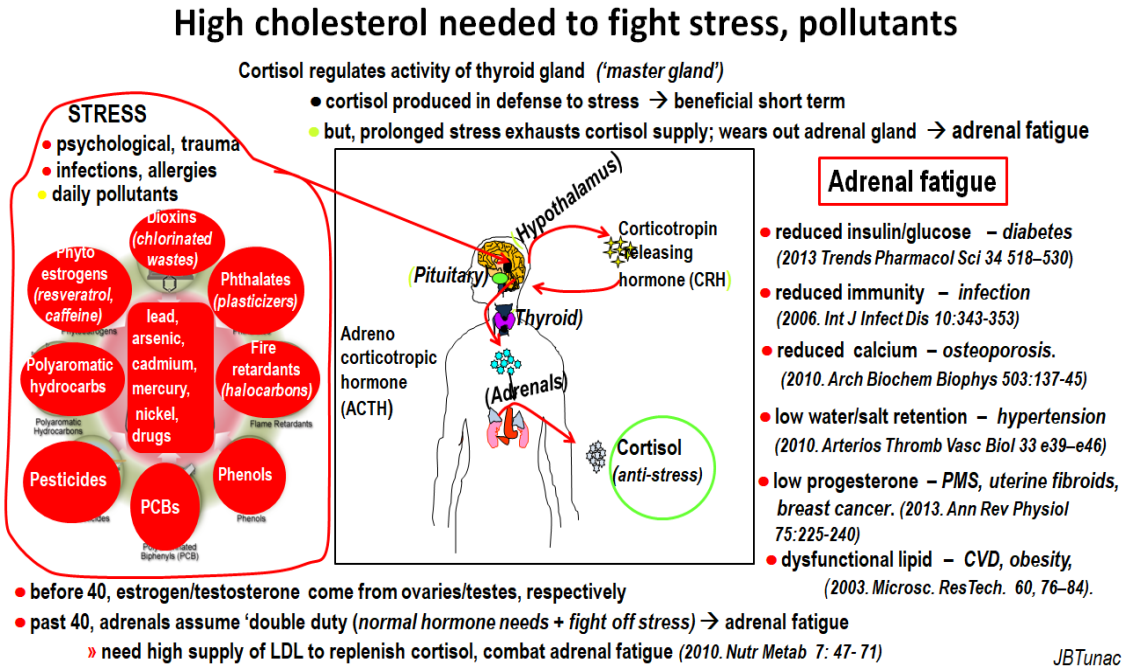


Figure 15. High cholesterol is a symptom of stress, elevated cholesterol is a natural defense to stress and infections

An elevated supply of cholesterol (LDL) is needed to combat stress level. Failure to produce compensatory levels of LDL due chronic exposure to stress results in adrenal fatigue and the following diseases:

- reduced insulin/glucose – diabetes *(2013 Trends Pharmacol Sci 34 518–530)*
- reduced immunity – infection *(2006. Int J Infect Dis 10:343-353)*
- reduced calcium – osteoporosis *(2010. Arch Biochem Biophys 503:137-45)*
- low water/salt retention – hypertension *(2010. Arterios Thromb Vasc Biol 33 e39–e46)*
- low progesterone – PMS, uterine fibroids, breast cancer *(2013. Ann Rev Physiol75:225-240)*
- dysfunctional lipid – CVD, obesity *(2003. Microsc. ResTech. 60, 76–84).*

In women, pregnancy presents added physical and emotional stress manifested as headaches, appetite lose, overeating, etc. A low adrenal hormone cholesterol level is a risk for premature baby (birth before 37 weeks of pregnancy), low-birthweight (less than 5½ pounds), or miscarriage. Diethylstilbestrol (DES) was prescribed in the 40s to supplement low adrenal cholesterol and treat pregnancy-related stress. Retrospectively, DES is a powerful inducer of the adrenal hormone estradiol, elevating its level to a toxic imbalance resulting in drug-induced diseases (Fig.16).

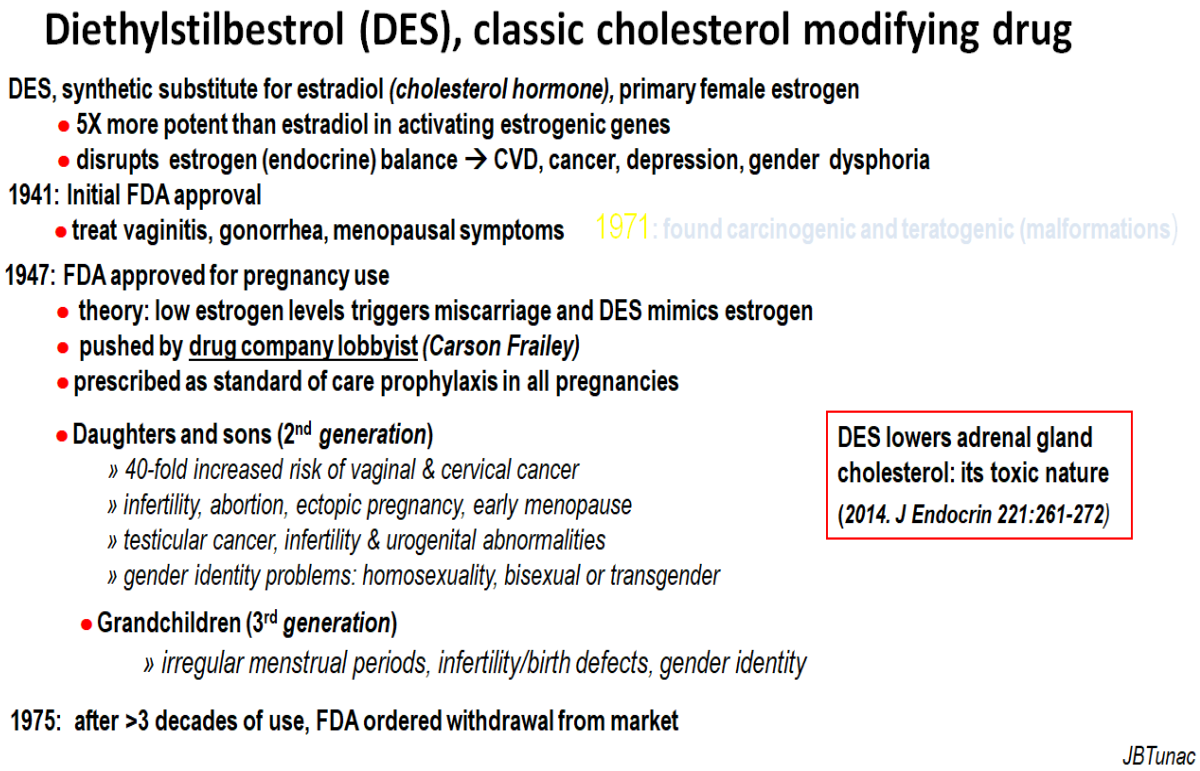


Figure 16. Diethylstilbestrol (DES) supplementation triggesr toxic level of estradiol cholesterol and xenodiseases

Another source of adrenal fatigue is natural sequelae to aging. As we age the capacity of sexual organs (ovaries, testes) to produce cholesterol hormones diminish e.g., 4 types of estrogens (estradiol, estrone, estriol, estetrol) in women, and testosterone for men. After the age of 40, the adrenal glands assume the

double duty of producing stress hormone (cortisol) and estrogens to meet the needs of our body; overworked adrenal glands leads to adrenal fatigue. Hormone replacement therapy (HRT) was designed to alleviate the overworked adrenal gland and to combat adrenal fatigue. However, each woman has varying levels of exposure to daily stress, infection, pollution and chemicals making it difficult to individually optimize HRT dosing, thereby the HRT-related diseases (Fig. 17).

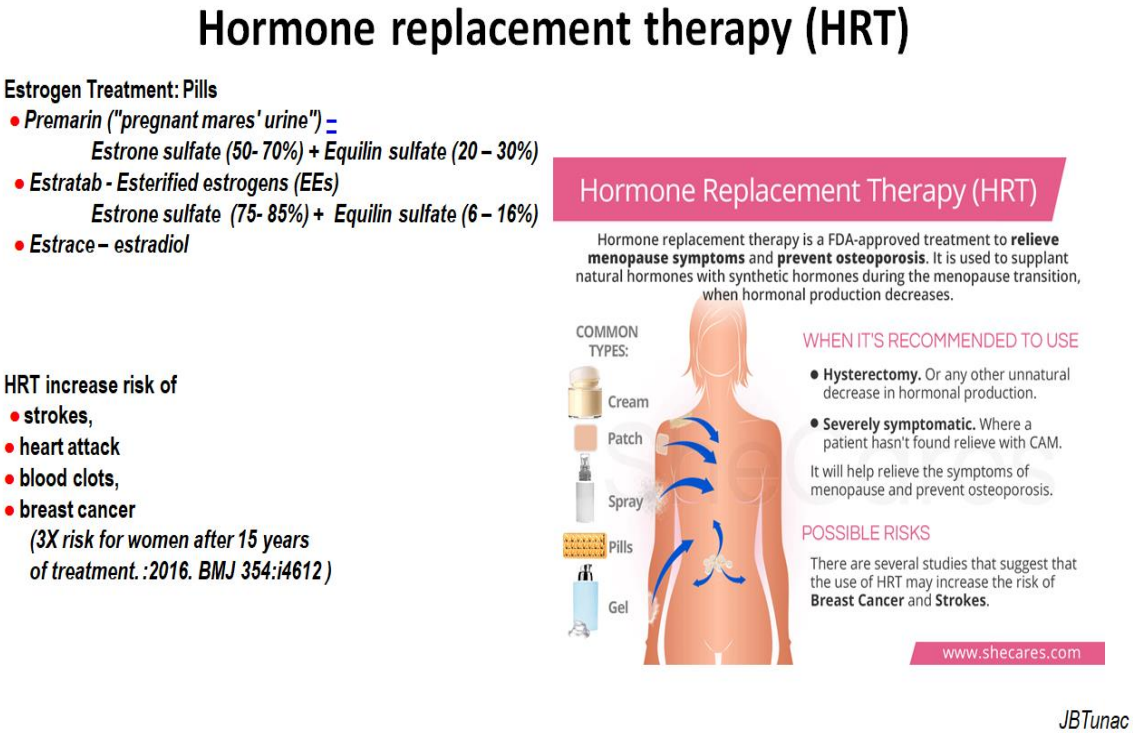


Figure 17. Hormone replacement therapy (HRT) is a strategy to supplement estrogen, but exposure to daily stressors compromises its effectiveness

7. Cholesterol-lowering paradigm to treat CVD is a mistake: based on wrong animal model

Associating elevated level of blood cholesterol to CVD was borne of an experiment by a Russian scientist (Ignatowski) who fed meat, eggs and milk to a rabbit and observed arterial lesions (aka, "fatty streaks", "cholesterol plaque"). Rabbits, being herbivore, do not have the innate ability to metabolize meat products including fat and cholesterol, consequently they accumulate in the circulation and deposited as arterial lesions (Fig 18).

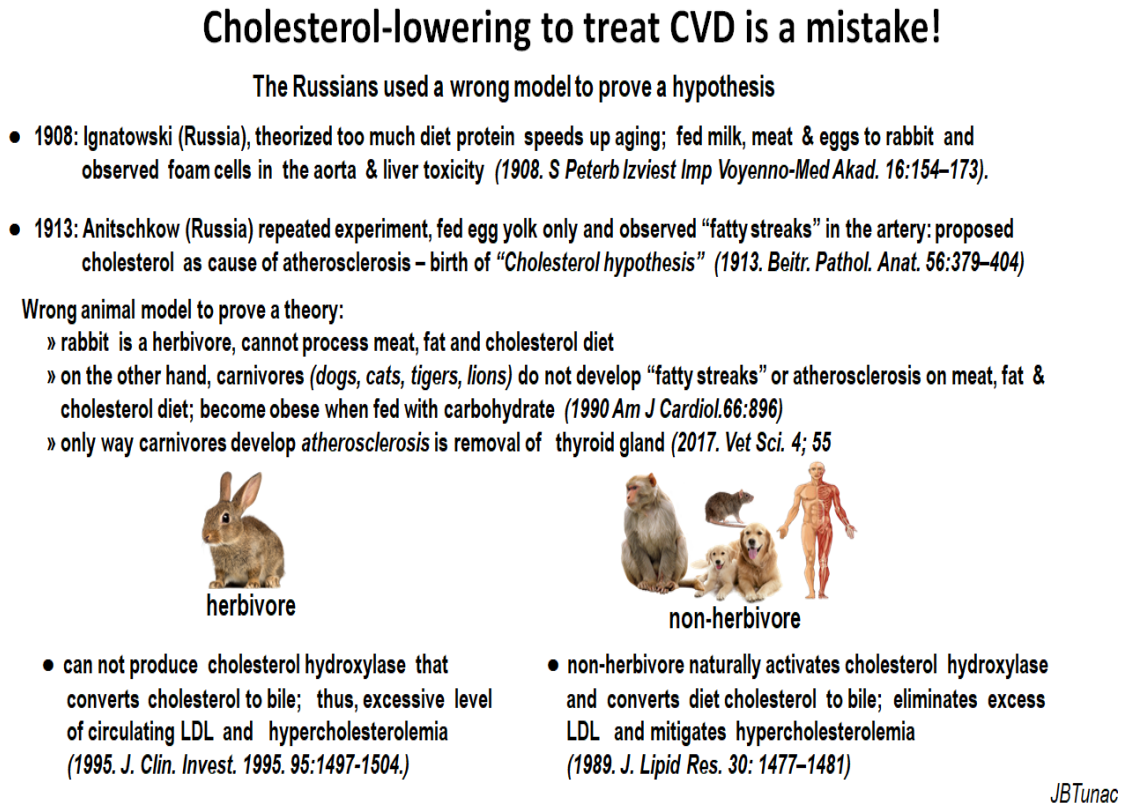


Figure 18. Feeding rabbit with meat allowed 'packaged cholesterol and fat' to pile up in blood artery forming 'foam cells' that matures into plaque

This serendipitous experiment was misconstrued to happen also in humans, which established the consensus that dietary cholesterol leads to the development of atherosclerosis ("Cholesterol hypothesis") in both animals and humans. This is a flawed hypothesis because humans are naturally equipped with an enzyme, cholesterol hydroxylase (CYP7A1), that converts cholesterol to bile and prevent cholesterol accumulation. On the other hand arterial lesions in cholesterol-fed rabbits regressed when switched to low-fat chow (1976. Ann N Y Acad Sci 275(1):363–78.).

Regardless, the rabbit became a de facto model in the study of the pathogenesis and development of human atherosclerosis (Fig 19).

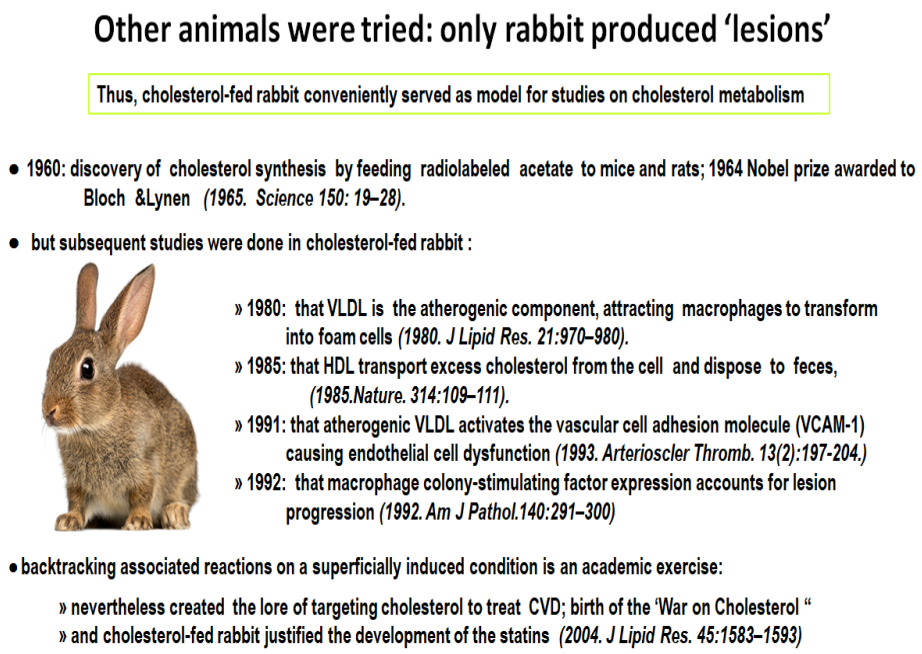


Figure 19. The rabbit as the central animal model in studying cholesterol biosynthesis.

The rising incidence of heart disease in the 20s evoked the Russian "cholesterol hypothesis" (Fig 14), which was subsequently championed by Ancel Keys and the American Heart Association (AHA). The "cholesterol hypothesis' became the foundation of cholesterol-lowering paradigm to treat CVD. The focus on cholesterol-lowering strategies inspired Akira Endo of Japan (Sankyo) to screen for drugs that inhibit cholesterol synthesis, particularly targeting the enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG CoA) reductase. This led to the discovery of citrinin (a potent HMGCoA inhibitor), and then compactin, the first statin that was effective in vitro but failed to lower plasma cholesterol in rats and mice. These two drugs were toxic and dropped from further development (Fig 20).

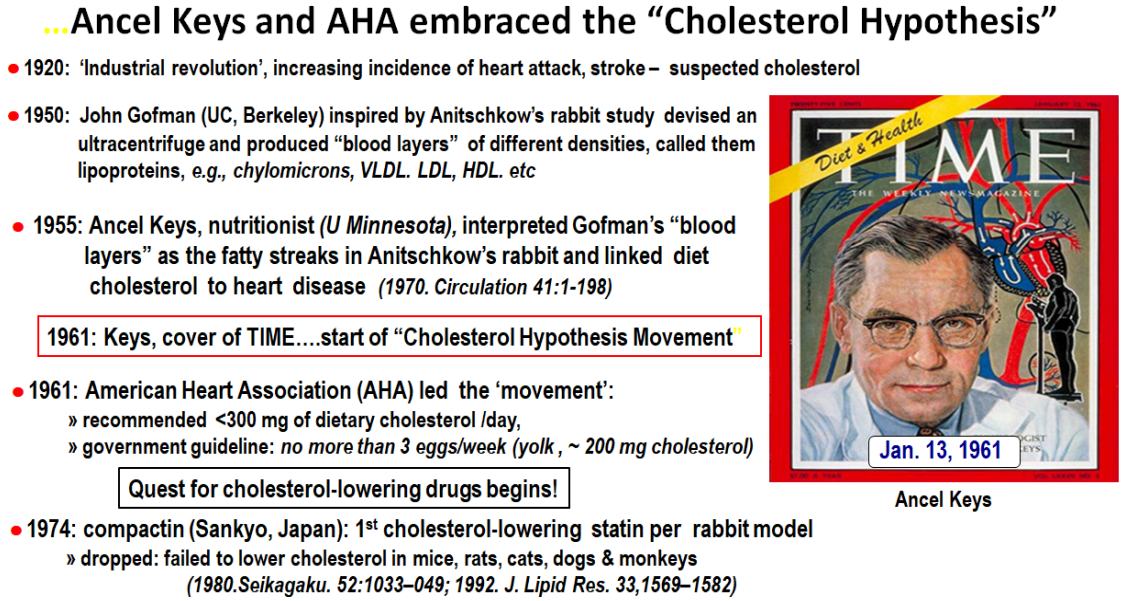


Figure 20. The "cholesterol hypothesis" inspired the cholesterol-lowering paradigm to treat cardiovascular disease (CVD).

In the US, Merck discovered lovastatin (2nd statin, a compactin analog), but dropped early because of anticipated carcinogenicity. However, at this time Brown & Goldstein (Merck consultants) discovered the cause of genetically elevated cholesterol or familial hypercholesterolemia (FH) and urged Merck to reconsider lovastatin for FH patients (Fig 19).

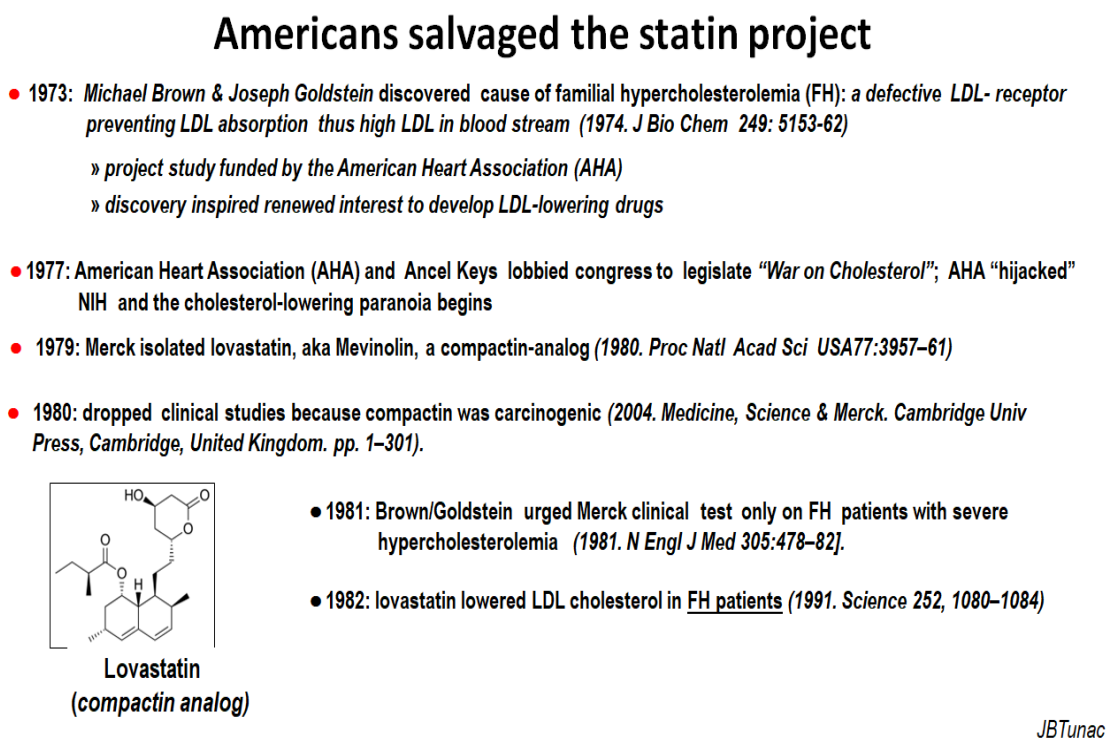


Figure 21. Lovastatin lowered plasma cholesterol in patients with familial hypercholesterolemia (FH).

Lovastatin effectively lowered cholesterol level in FH patients, approved by the FDA and became the first marketed cholesterol-lowering statin. To assess the effect of lovastatin, a cholesterol test (Lipid panel) was introduced and a total cholesterol goal of less than 260 mg/dL Lovastatin was followed by the introduction of other statins to offer a range of effectiveness and toxic tolerance. AHA and NIH offered a blood LDL-cholesterol level goal of 160 mg/dL in 1987 to the current goal of less tha 70 mg/dL. Statins effectively lower plasma cholesterol level with one so-called superstatin (Baycol) effective as low as 0.2 5 mg, but killed people and was withdrawn from the market (Fig. 22).

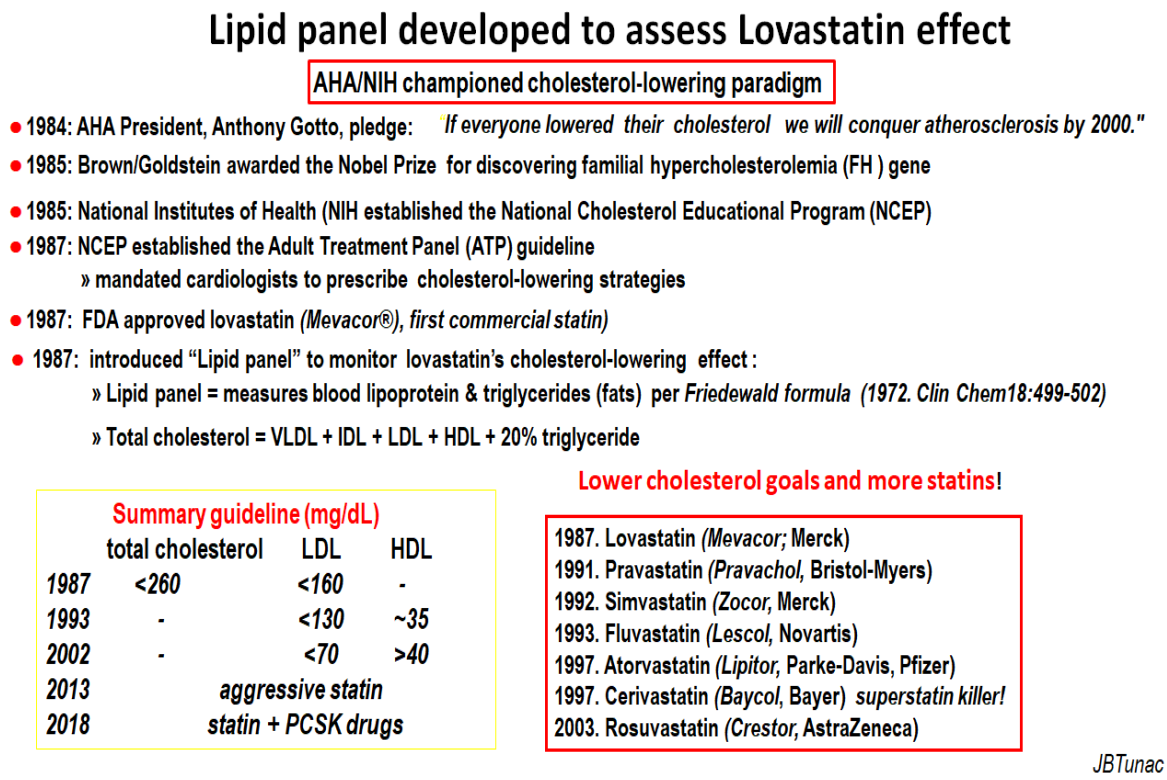


Figure 22. Lovastatin was the first marketed cholesterol-lowering statin approved for familial hypercholesterolemia (FH), but prescribed for the general population.

8. Arterial lesions in animal models are not the same as humans

The rabbit "fatty streak", was the basis for staging atherosclerosis in clinical setting, which subsequently advanced to "cholesterol plaque" (1994.Circulation.89:2462-2478) and the target of statins. Per rabbit model, "cholesterol plaque" triggers arterial stenosis and thus prescribing statins would alleviate arterial narrowing. But mild-to-moderate stenosis still led to embolism and in humans, plaques ruptured regardless of the degree of stenosis; this shifted the paradigm to identifying and targeting "vulnerable plaques" instead of reducing stenosis (Fig. 23)

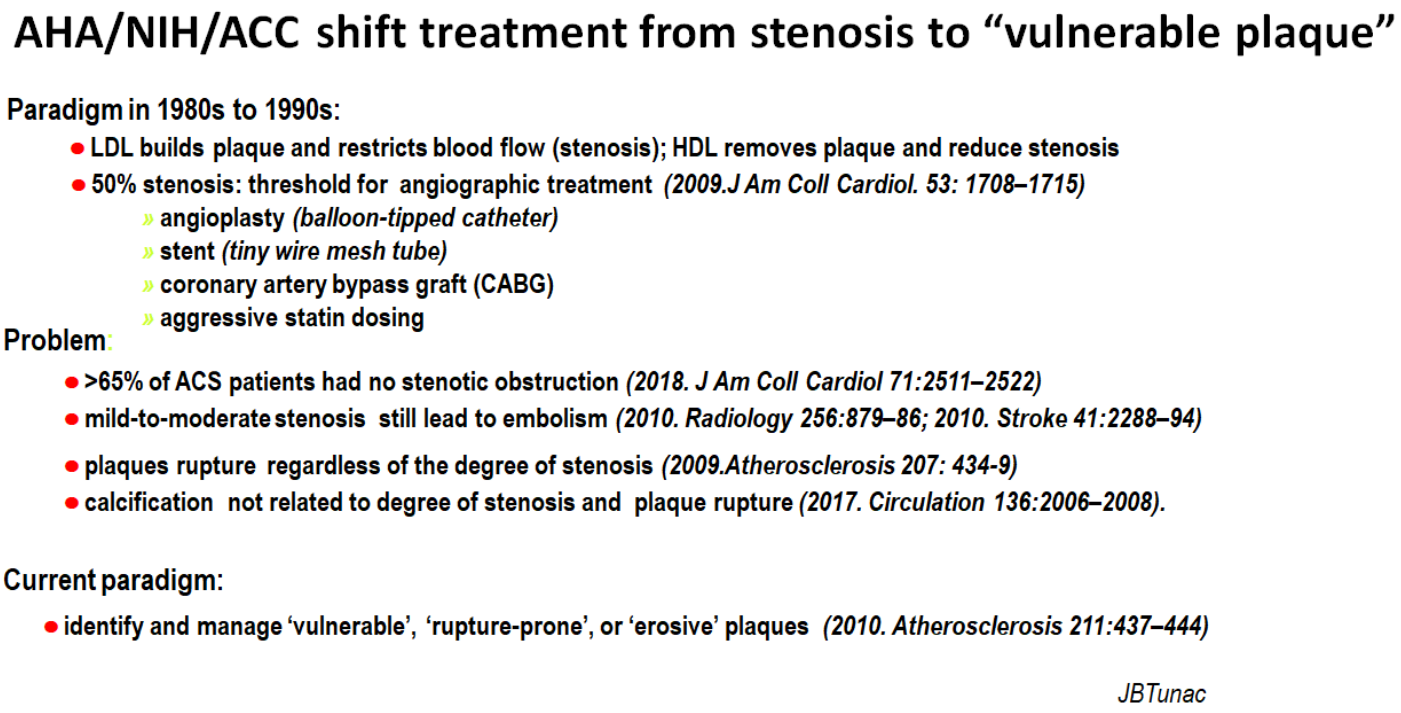


Figure 23. Initial attempt to reconcile animal arterial lesions with clinical setting

To relate the rabbit "fatty streaks'" with clinical data, two types of arterial lesions were initially recognized: *fatty streak* (a thin lipid deposit in thin intima in children) and *fibrous plaque* (a thick fibrolipidic lesion in adults). In the 1950s pathologists opened, flattened, and fixed arteries in formalin and classified development sequentially from *fatty streak* to *fibrous plaque*, and *complicated lesion* (1957. Am J Pathol.1957; 33:875-885). Fatty streaks in infants was described as yellow dots, visible to the unaided eye at the root of the aorta, become more extensive at puberty (fibrous plaque) and in adults, advance to fibrolipid lesions (complicated plaques). The World Health Organization (WHO) added the term *atheroma* to distinguish advanced lesions with a predominantly lipid component (atheroma) from those with a predominantly collagenous component (fibrous plaque) (WHO Techn Rep Ser.1958; 143:1-20). Other terms include *fibroatheroma*, *atheromatous plaque*, *fibrolipid plaque*, or *fibrofatty plaque* to mean atherosclerosis (Davies MJ. Colour Atlas of Cardiovascular Pathology. London, England: Harvey Miller Publishers; 1986:73.)

Meanwhile, AHA organized a committee to define plaque with the aim of identifying vulnerable plaques. Initially, plaques were classified into 3 types; the assumption was for type I to progress through type III (Fig 24).

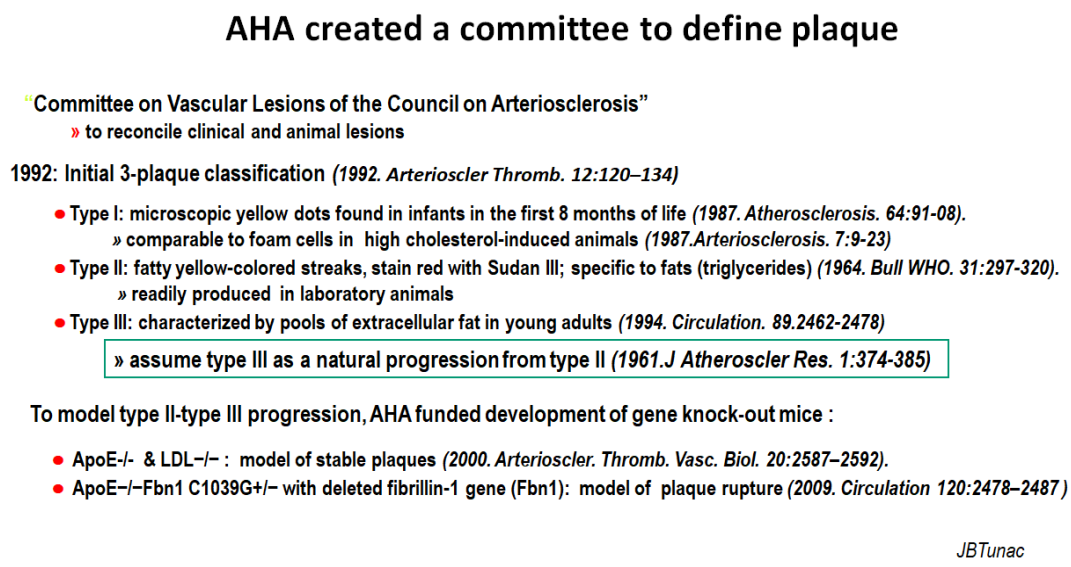


Figure 24. Three types of plaques in an attempt to unify rabbit lesions with clinical data.

However, the transition from type II to type III was not well defined, which prompted the development of artificial mouse model. As a species the mouse, like humans are omnivores (2008. Nat Clin Pract Cardiovasc Med 5(2):91–102), which is highly resistant to atherosclerosis (1996. Science. 272(5262):685-8). To create a hypercholesterolemic mouse, the apoE gene was deleted (ApoE−/− mouse, apoE knock-out mouse) so dietary cholesterol accumulates as cholesterol lesions. In both rabbit and ApoE−/− mouse, dietary cholesterol are not absorbed and linger in the arterial circulation to eventually produce atherogenic lesions. Because of its rapid reproduction, ease of handling, and its ability to monitor atherogenesis in a reasonable time frame, the apo-E mouse eventually replaced the rabbit as a model for atherosclerosis Thus, the ApoE mouse fed with high cholesterol diet, was extensively used to model plaque development and classification (Fig 25).

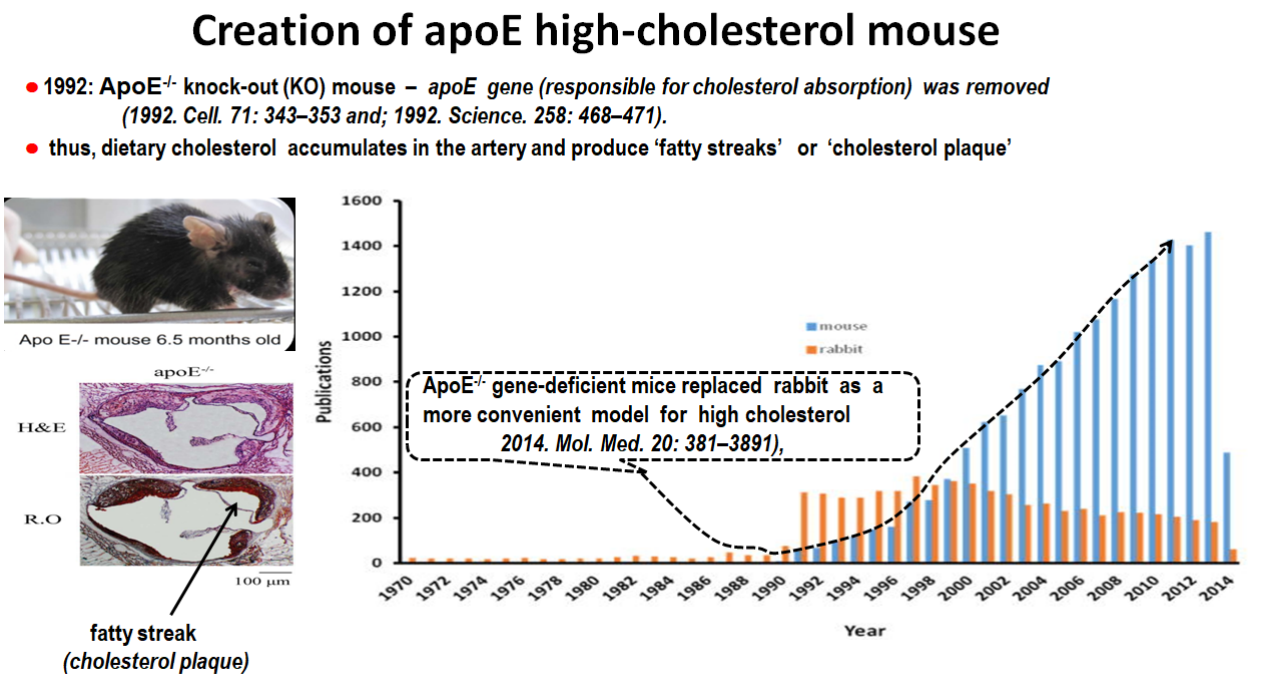


Figure 25. Historical use of rabbit and ApoE$^{-/-}$ mouse as models of atherosclerosis.

Lipoproteins are special packaging system for fats and cholesterol for delivery to the blood stream, e.g., chylomicron, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). The protein portion of the lipoprotein is responsible for binding to specific receptors in the cells for absorption and delivery (endocytosis) of the packaged fat or cholesterol to the cells. Once VLDL unloads its fat cargo, it becomes IDL; subsequently, IDL unloads its fat cargo to become LDL, and unloading of fat from LDL becomes HDL. *(Note: cholesterol is always packaged in lipoproteins; there is no free-floating cholesterol in the blood stream or free-cholesterol sticking onto arterial wall, and no such thing as "good- or bad-cholesterol").* The protein responsible for binding these lipoproteins to unload their cargo was initially designated as the arginine-rich peptide, but later renamed apoE. In an ApoE$^{-/-}$ mouse, the apoE gene was deleted (apoE knockout or apolipoprotein E-deficient), which prevents absorption of VLDL, IDL, HDL .

In an ApoE−/− mouse, the absence of the apoE binding protein allows the lipoproteins to circulate in the blood stream and overtime "stick" on the arterial wall resulting in hypercholesterolemia. Currently, the ApoE−/− mouse is the model of choice in the study of human atherosclerosis. (Fig 26)

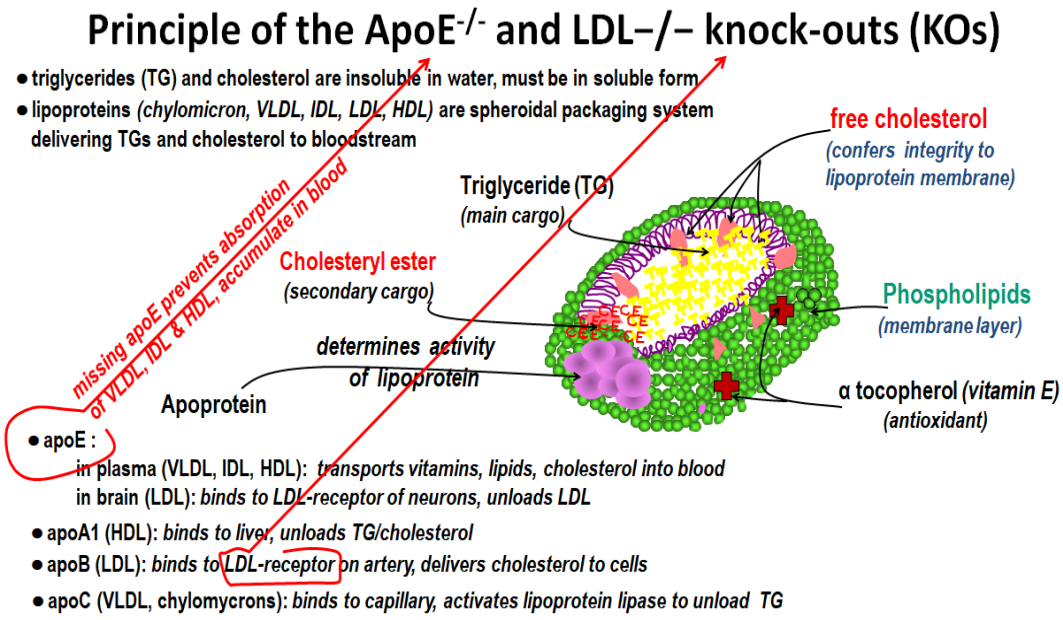


Figure 26 Make up of lipoproteins and their function in delivering fats and cholesterol

In summary, dietary fats and cholesterol are packaged in lipoproteins for transport into the blood stream. Rabbits, being herbivores, do not have a functional cholesterol hydroxylase enzyme to dispose lipoprotein-cholesterol, which eventually accumulate as arterial 'lesions'. On the other hand, mice and humans have adequate cholesterol hydroxylase, which converts lipoprotein-cholesterol into bile and prevent arterial lesions. Knocking-out or deleting genes involved in cholesterol metabolism allow the artificial accumulation of lipoprotein-cholesterol in artery; such genes include apolipoprotein E (APOE) knockout (1992. Cell 71(2):343–53; 1992. Science 258(5081):468–71) and the LDL receptor (LDLR) (1993. J Clin Invest 92(2):883–93).

The ApoE−/− mouse was extensively used to model plaque formation and morphology and the data were incorporated in the updated AHA plaque classification system. In the updated classification, the AHA Committee recommended extension of the numerical nomenclature to precisely define\d lesion types (Fig. 27).

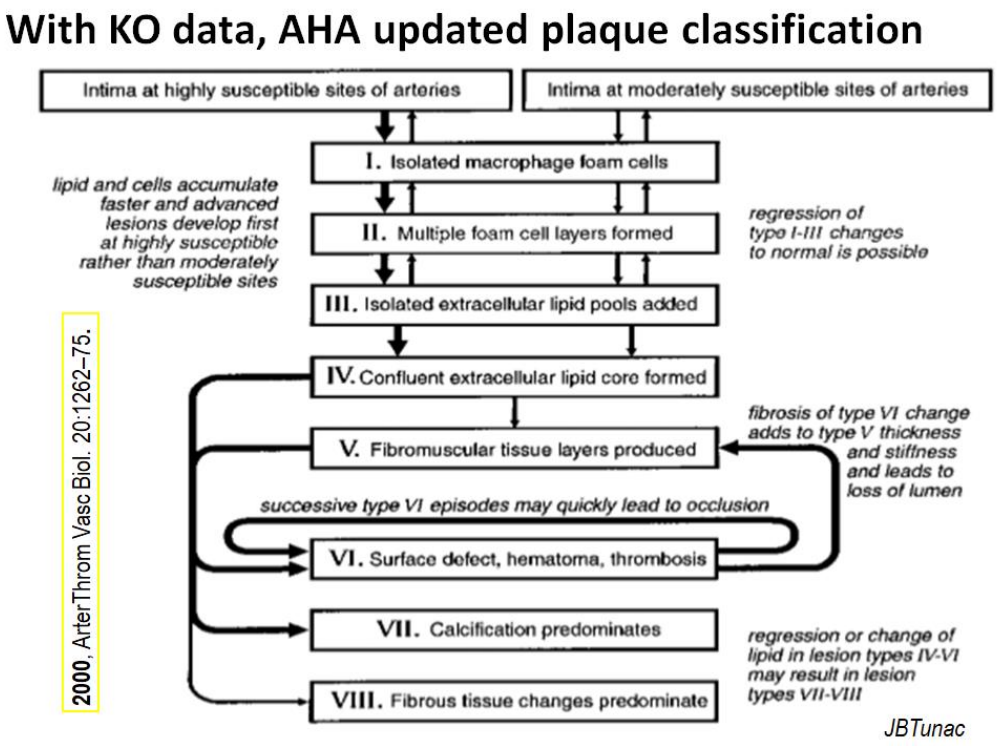


Figure 27..Outline of the sequence in the evolution of atherosclerotic lesions from type I to type IV and of the various possible subsequent pathways of progression to lesion types beyond type IV.

Major understanding of the development of atherosclerosis has been achieved predominantly by the use of genetic mouse models such as ApoE−/− and LDLR−/− mice (2011. Nature. 473: 317–3251) including the role of the innate immune system, particularly inflammation as central drivers and therapeutic targets of atherosclerosis (2015. Nat. Rev. Cardiol. 12: 199–2112). However, the accelerated form of atherosclerosis achieved by high cholesterol levels in these mouse models is typically far beyond that seen in patients and do not reflect human atherosclerosis in many aspects (2011. Nature. 473: 317–3251). (Fig. 28).

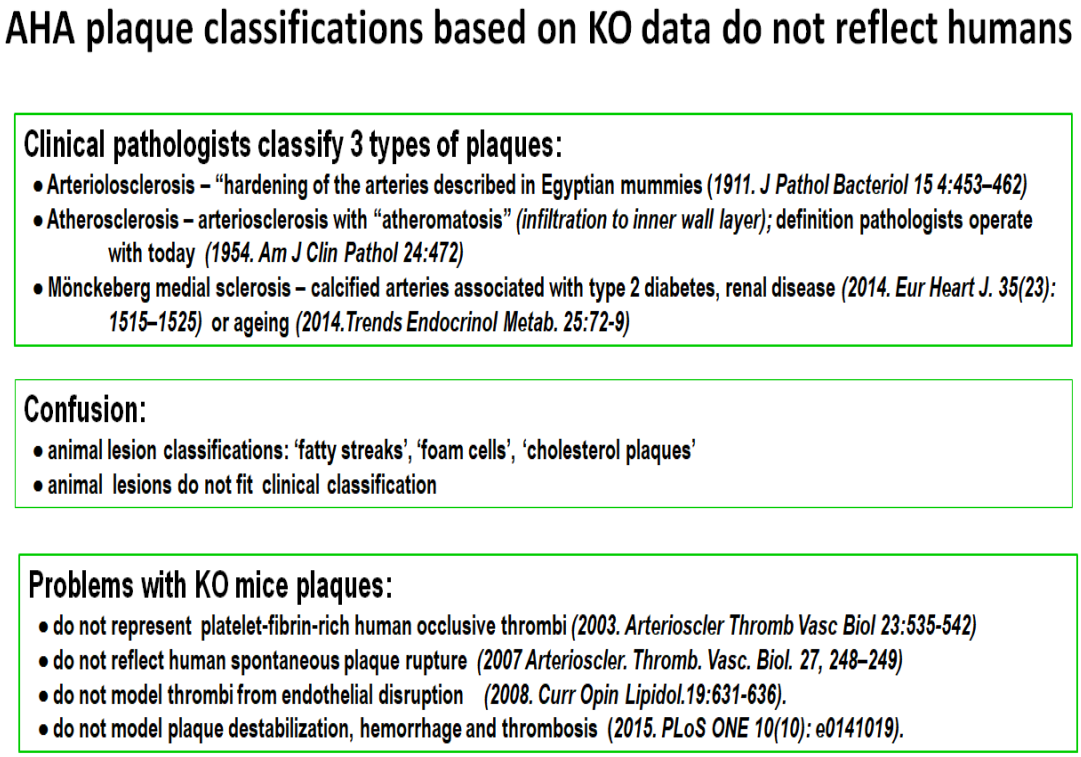


Figure 28. Clinical definitions of plaques by pathologists do not match those observed in animal models

9. Animal plaques are superficial, humans are sub endothelial.

Acute myocardial infarction has been associated with plaque rupture or erosion (2013. N Engl J Med. 2013;368:2004–2013), particularly those with thin-cap fibroatheroma (TCFA), with large necrotic core (2006. J Am Coll Cardiol. 47:C13–C18.). Thus, identification of TCFAs and other high-risk plaque features in humans have been the focus of a number of studies , e.g., > 400 NIH research awards (> $150 M/yr) and almost 2,000 research papers in the National Library of Medicine database (2014. NIH Research Portfolio Online Reporting Tools (RePORT: http://report.nih.gov). Also, industry been keenly interested with numerous preclinical or clinical stages to identify vulnerable plaques (2012 J Am Coll Cardiol. 60:569–580).

Atherosclerotic plaques in mice develop in specific sites such as the aortic root, the lesser curvature of the aortic arch and the branch points of the brachiocephalic, the left carotid and the subclavian arteries. However, mice show only minor plaque development in the coronary and carotid arteries, which are the main sites of atherosclerotic plaque development in humans (2004. Arterioscler. Thromb. Vasc. Biol., 24:12-22).

Although KO mice are useful in understanding the concepts of plaque rupture, none of them exhibit the full combination of the characteristics seen in human vulnerable/ruptured plaques; .plaque rupture with occlusive thrombus, the most common complication of human atherosclerosis, is rarely observed (2010. Curr. Opin. Lipidol., 21: 434-440. Consequently, clinical events such as MI or ischemic stroke are almost never seen in these models (2011. Thromb. Haemost., 106: 1-19). The use of KO mice as model are generally flawed because animal plaques are superficial while human plaques are subendothelial (Fig.29)

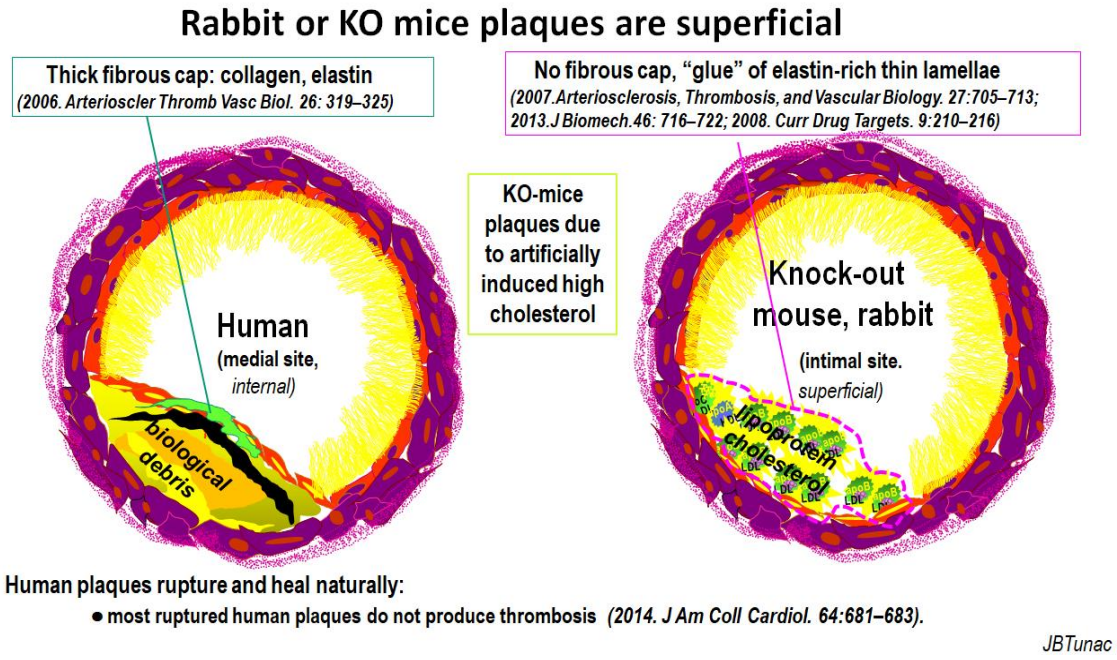


Figure 29. Plaques produced in animal models are superficial, while human plaques are subendothelial

In humans atherosclerosis causes clinical disease through luminal narrowing or by precipitating thrombi that obstruct blood flow to the heart (coronary heart disease), brain (ischemic stroke), or lower extremities (peripheral vascular disease). The most common of these manifestations is coronary heart disease, including stable angina pectoris and the acute coronary syndromes. Atherosclerosis is an arterial injury driven disease that leads to plaque formation. In humans, the plaque is located in the subendothelial layer (Fig. 30)

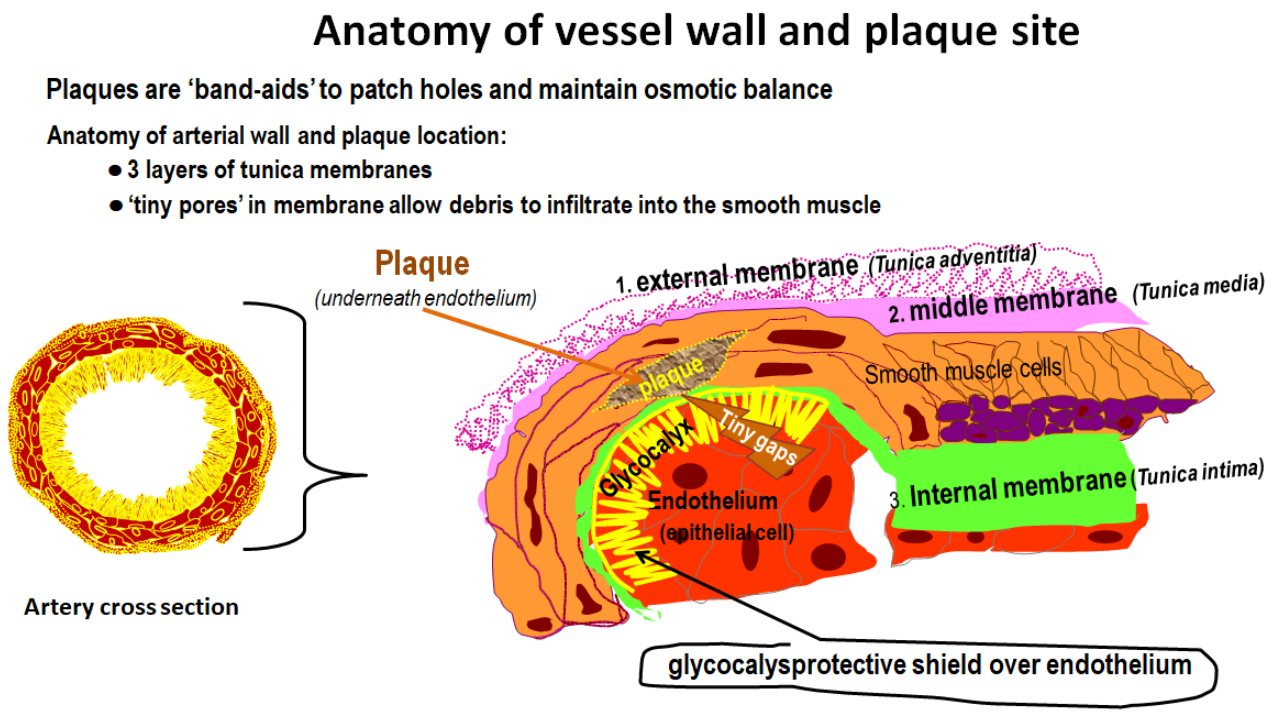


Figure 30. Clinical plaque site is subendothelial.

10. Human plaques rupture and heal naturally

In humns, plaques start forming at age 6 and they rupture and heal naturally. KO mice, do not show 'spontaneous' plaque ruptures (2011.Thromb. Haemost., 106: 1-19). In humans, about 0.15% of ruptured plaques produce fatal clot or embolus. (Fig. 31).

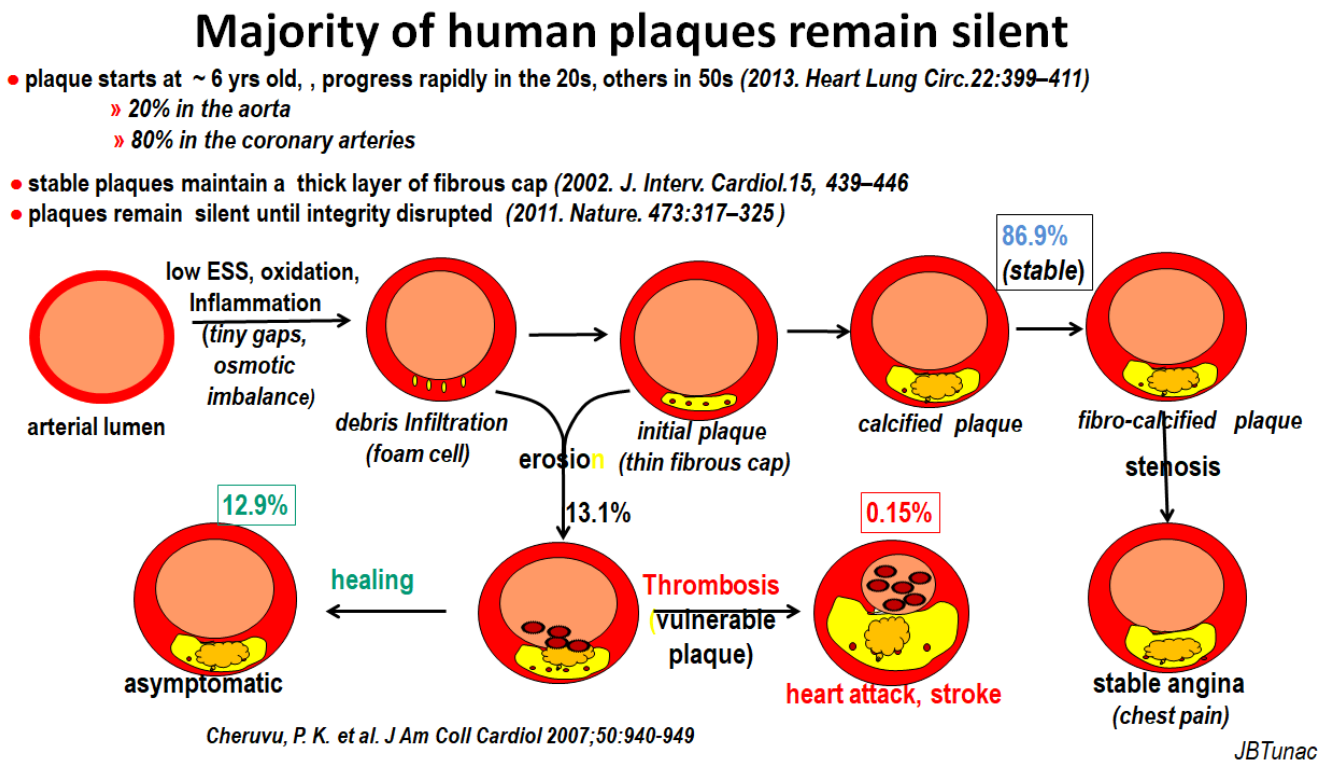


Figure 31.Most human plaques rupture and heal without any adverse symptoms

Plaque rupture produces a clot as a defense mechanism to contain the injury. In most cases the clot is not big enough and does not cause any damage, however, the clot is amplified in certain circumstances. The amplification is determined by a cascade of events. For example, ruptured plaque releases materials, which contain high levels of tissue factor (TF) (2015. J Atheroscler Thromb. 22:543–549). TF is a transmembrane protein essential for normal hemostasis (2004. Arterios Thromb Vasc Biol, 24: 1015–1022). Exposure of subendothelial TF triggers a cascade of events including the formation of a TF- VIIa complex (1993. Blood, 81:734–744), which initiates blood coagulation by activating factor IX and factor X phosphatidylserine proteases zymogens (proenzymes) to factor IXa and factor Xa, respectively, amplify thrombin production (2011..J Biol Chem.286:23247–23253); TF-FVIIa-FXa complex activate FVIII providing additional levels of FVIIIa during the initiation phase (2017. Blood. 130:1661–1670) net result: generate large amounts of thrombin, which cleaves fibrinogen to fibrin monomers that are cross-linked by the transglutaminase FXIIIa forming insoluble fibrin clot (2009. Hamostaseologie 29: 7–16).

Microbial infection can cause plaque disruption and a phenomenon called ‘immunothrombosis’, which activate the immune system to confine and prevent dissemination of pathogens by coordinating platelets to form intravascular coagulation .(2013 Nat Rev Immunol 13:34–45) Platelets, carry the transcripts for all pathogen responsive toll-like receptors (2015. Arterioscler Thromb Vasc Biol 35:1030–1037) and induce prothrombotic events, secrete cytokines, chemokines, and antimicrobial peptides, leading to sequestration and destruction of bacteria (2011.Nat Rev Immunol 11:264–274) and viruses (2014 Blood 124:791–802). Components of the activated immune system are monocytes (macrophages) and neutrophils; monocytes carry the pro-coagulant TF and microvesicles during infection (2016. Crit Care

Med 44:e574–e578), while neutrophils release highly prothrombotic neutrophil extracellular traps (NETs) in a process called netosis release of decondensed chromatin to cause cell death). Netosis is beneficial but highly prothrombotic; NETs increase thrombin levels, activate platelets and coagulation (2014. Blood 123:2768–2776.). Thus, immunothrombosis is an efficient way of enabling the immune system to fight diverse infections, but contributes to clot amplification and the overall cardiovascular burden. Clot amplification involves 3 interconnected cascades: coagulation, cellular, and component (Fig. 32).

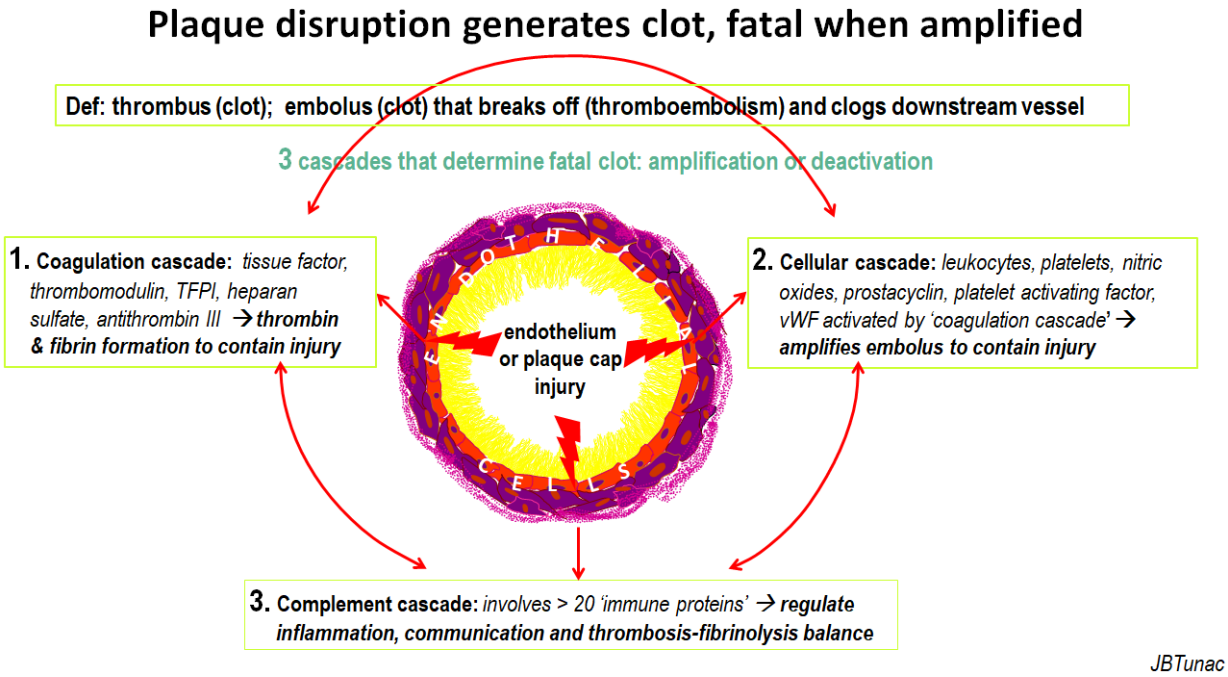


Figure 32 Cellular components of the blood are activated by the coagulation cascade and once activated promote amplification of thrombosis

11. The cardiovascular system:

The cardiovascular system consists of the heart, with its intricate conduits of branching elastic pipes, the arteries and arterioles; the tissue beds supplied by the capillaries; and finally a system of converging pipes, the venules and veins. The system traverses the whole human body carrying blood; arteries are blood vessels that transport blood away from the heart, and veins transport the blood back to the heart. Capillaries carry blood to tissue cells and are the exchange sites of nutrients, gases, wastes, etc. The blood contains oxygen, nutrients, wastes, and immune and other functional cells that help provide for homeostasis and basic functions of human cells and organs.

The pumping action of the heart usually maintains a balance between cardiac output and venous return. Cardiac output is the amount of blood pumped out by each ventricle in one minute. The normal adult

blood volume is 5 liters and passes through the heart once a minute, but cardiac output varies with the demands of the body.

The pulsations generated by the pumping action of the heart radiate out along the arteries and at discontinuities such as vessel bifurcations, the pulsations are partially reflected, back toward the heart, and partially transmitted along the vessel, becoming damped as they approach the distal arterioles. The arterioles regulate the distribution of blood flow to the various capillary beds before the blood is returned in a relatively steady stream to the heart. The changing velocity profiles across the various vessels are reflections of the hemodynamic characteristics of the cardiovascular system. (Fig 33)

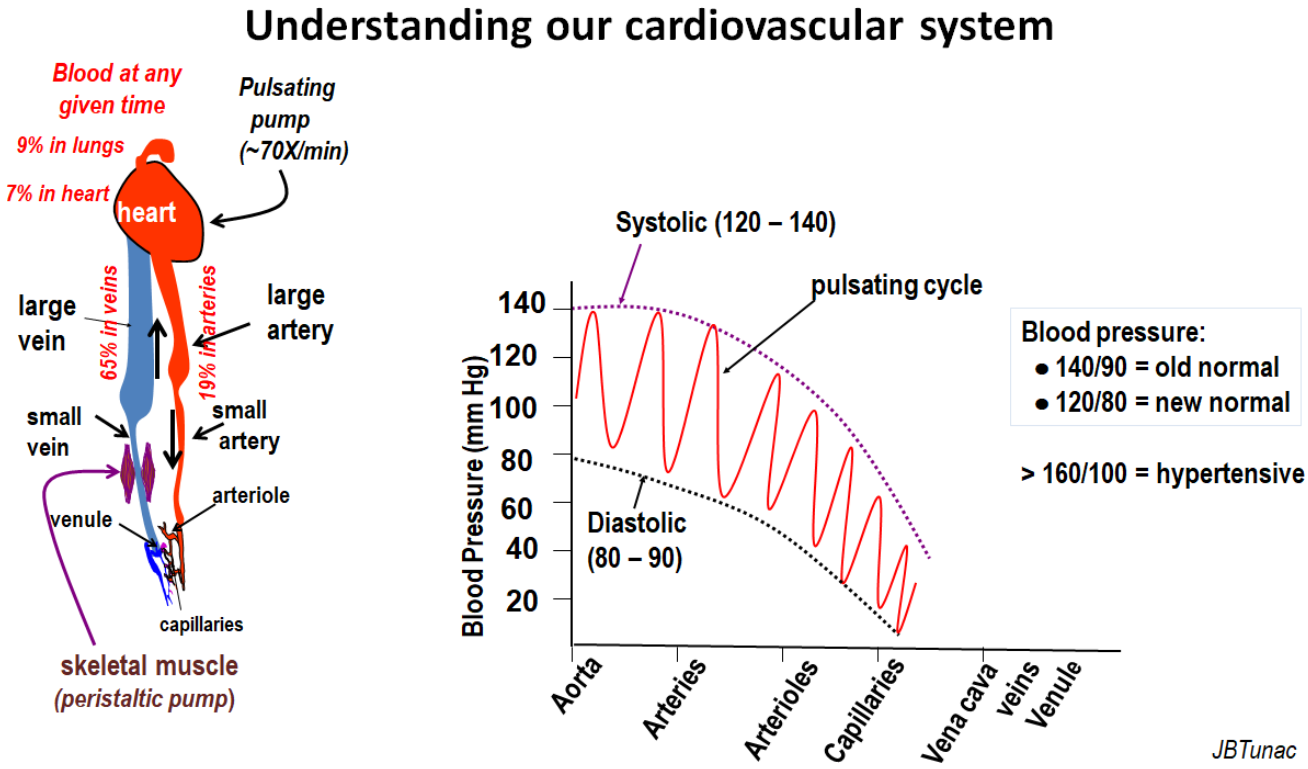


Figure 33 Our cardiovascular system circulate and transport nutrients (amino acids and electrolytes), oxygen, carbon dioxide, hormones, and blood cells to and from the cells in the body.

As blood flows through peripheral tissues, blood pressure forces water and solutes out of the plasma, across capillary walls, which involve. diffusion, filtration, and reabsorption . Ions and small organic molecules enter or leave the bloodstream by diffusion between adjacent endothelial cells. On the other hand, large water–soluble compounds including plasma proteins are normally unable to cross the endothelial lining except at fenestrated capillaries, such as those of the hypothalamus, the kidneys, many endocrine organs, and the intestinal tract. Lipids, such as fatty acids and steroids, and lipid–soluble materials, including soluble gases such as oxygen and carbon dioxide, can cross capillary walls by diffusion through the endothelial cell membranes

12. Glycocalyx (GCX) controls vascular permeability and diseases.

Body cells are organized in 4 tissues including epithelium nervous, muscle and connective tissue.. Epithelium covers body surfaces, lines internal closed cavities including glands, body tubes and the vascular system. Epithelial tissues: protect underlying tissues from radiation, desiccation, toxins, pathogens, and physical trauma; regulate exchange of chemicals between tissues and a body cavity; secrete hormones into the blood vascular system, provide sensation. Endothelial cells line the internal surface of the circulatory system including the lumen of the arteries, veins, lymphatic vessels, blood capillaries and cavities of the heart. Yet another layer on top of the endothelium is glycocalyx, which provides the first line of protection from physical, chemical, and biological wear and tear (Fig. 34).

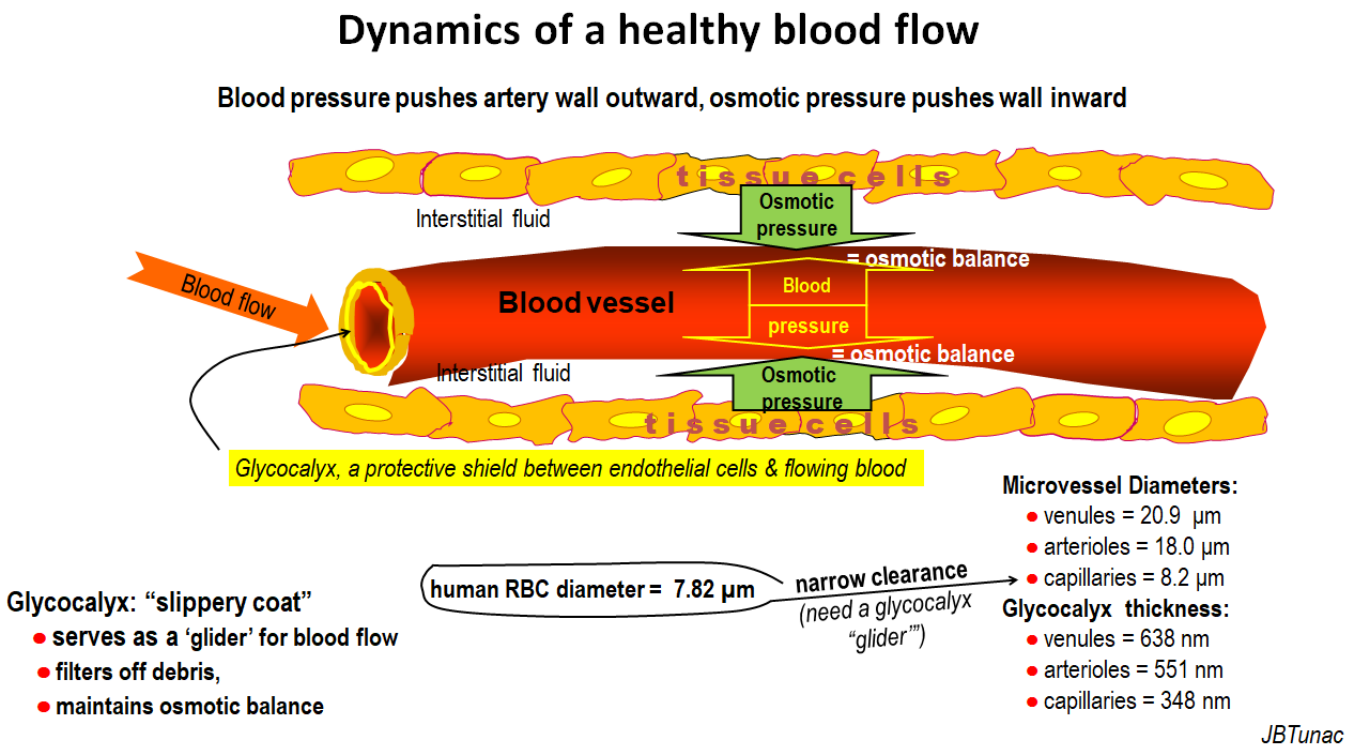


Figure 34. Blood vessel is lined with a 'slippery coat' called glycocalyx that promote healthy blood flow dynamics.

Glycocalyx is a fuzz-like carbohydrate-rich coat that projects out and covers the membrane of endothelial cells, which filters off cell debris and prevents adhesion of coagulatory and inflammatory cells to the vascular endothelial lining. Other critical function of the glycocalyx include: 1) transmits fluid shearing forces to the cytoskeleton of endothelial cells and stimulates the production of nitric oxide, which is vital in controlling blood flow and blood pressure; 2) regulate the supply of nutrients and oxygen, and the removal of waste and carbon dioxide; and, 3) maintains capillary integrity, and prevents loss of fluid through leakage (Fig. 35)

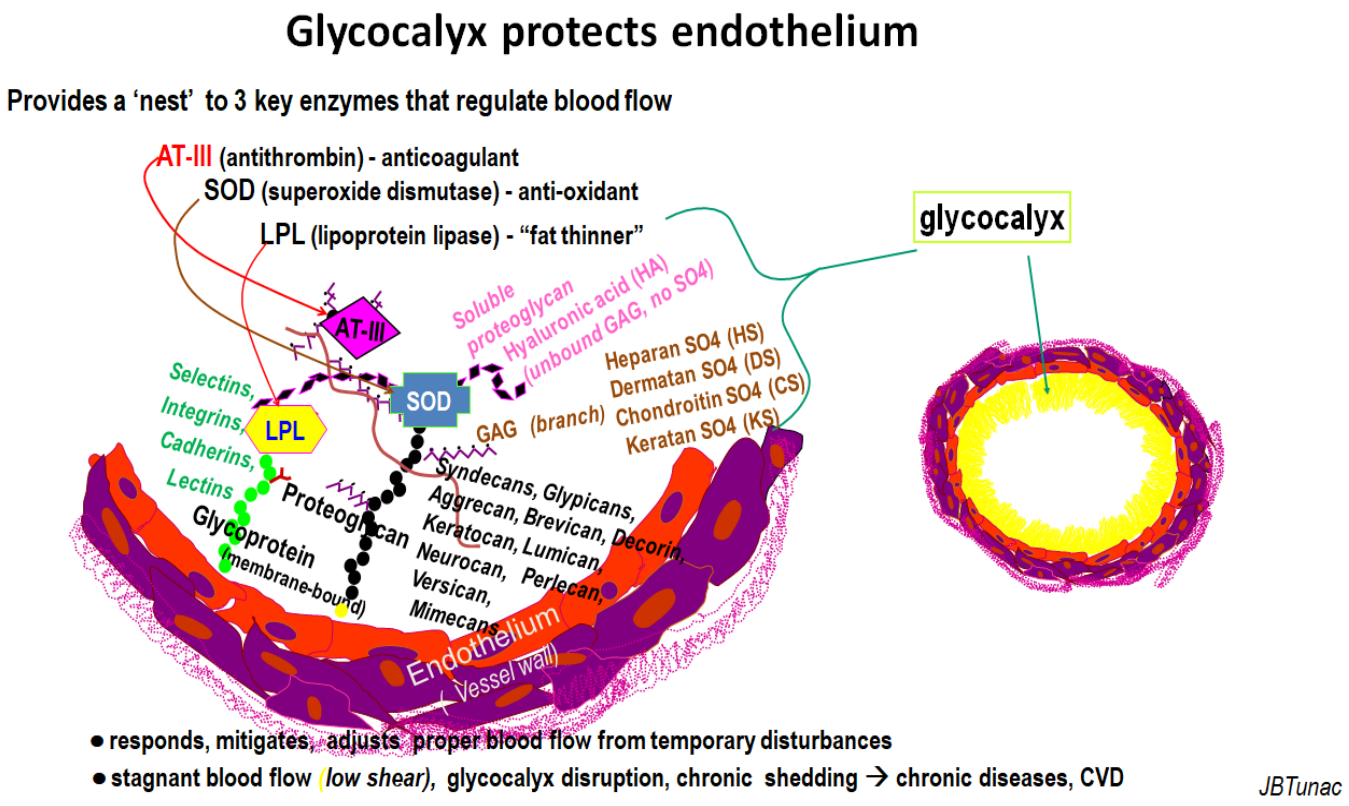


Figure 35. Anatomy of the protective endothelial glycocalyx showing glycoprotein and proteoglycan components including nested proteins (AT-III, SOD, LPL)

Glycocalyx is connected to the endothelial cell via several glycoprotein and proteoglycan backbone molecules [2007.Pflugers Arch. 454(3):345–359.]. The glycoproteins are protein-glycan conjugates (2006. J Intern Med. 259(4):339–350), which are adhesion molecules that contribute to pathological state (2014. Anaesthesia. 69(7):777–784). The three families of adhesive molecules include the selectin family, the integrin family, and immunoglobulin superfamily (2006. J Intern Med. 259(4):339–350].

13. Disruption of glycocalyx triggers epithelial and vascular diseases including CVD

The GCX is an extracellular matrix that covers the luminal surface of the vascular system. This structure is not just a barrier for vascular permeability but contributes to various functions including signal sensing and transmission to the endothelium. Thus, pathological changes to this structure are involved in the development of various diseases. (Fig. 36)

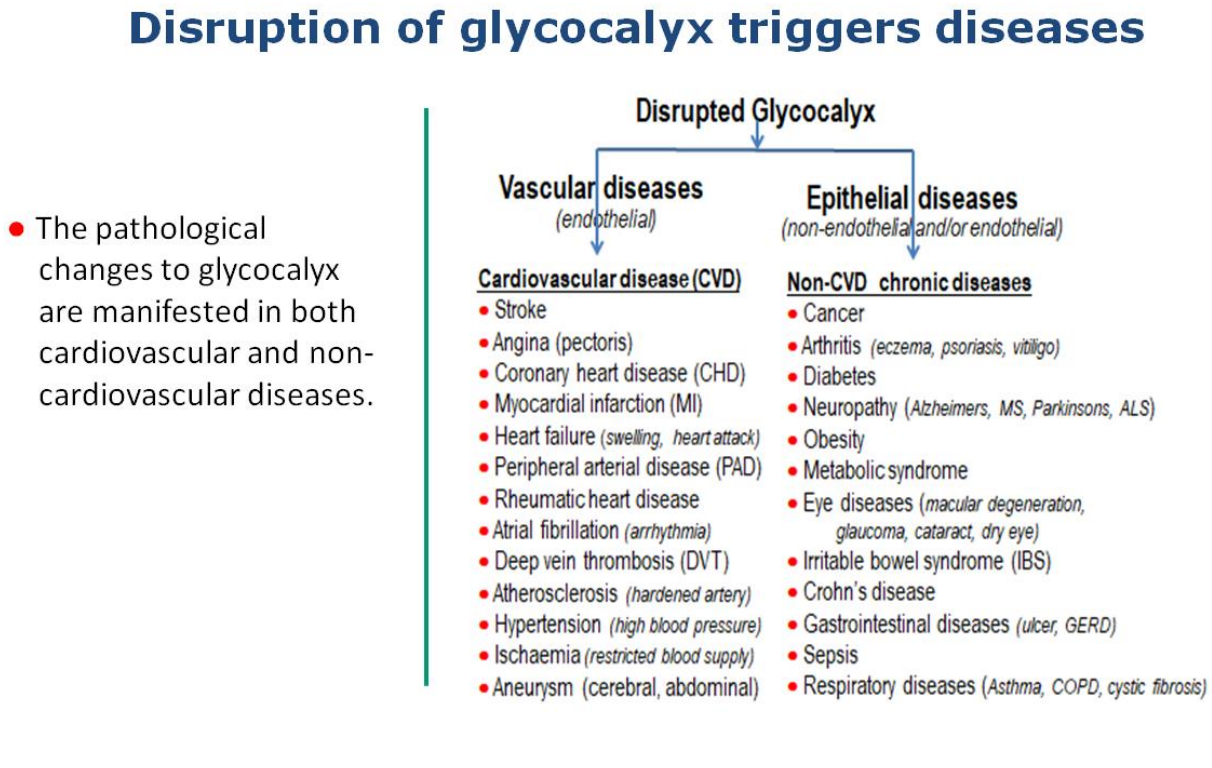


Figure 36.. Disruption of the glycocalyx accounts for a number of vascular-related pathophysiologies including CVD

.The arterial endothelium glycocalyx (AEG) plays a crucial role in the initiation and progression stages of CVD because it is the barrier between blood flow and arterial layers. Normal AEG senses changes in the microenvironment and regulate functions, such as vascular tone, circulating cell adhesion, coagulation, fibrinolysis, and vessel wall inflammation, in response to hemodynamic changes. Dysfunction of the vasculoprotective AEG is a first step in the atherosclerosis process, which may lead to the disruption of the endothelium (2016.therosclerosis. 252:136–146), Endothelium disruption allows blood debris and lipoproteins to infiltrate the subendothelial space (1995.Arterioscler Thromb Vasc Biol. 15(5):551–561), which subsequently become oxidized or enzymatically cleaved and trigger inflammation. Inflammation involves the recruitment of monocytes across the endothelial monolayer into the intima where they proliferate and differentiate into macrophages (2000. Nature. 407(6801):233–241). The macrophages ingest oxidized debris and lipoproteins, developing into foam cells then mature into plaque (1993. Nature. 362(6423):801–809),

The straight arterial segment with unimpeded blood flow has high shear stress (SS), which maintains a thick protective glycocalyx layer. On the other hand, segments of bends or bifurcations with oscillating blood flow have low shear stress (1993. Arterioscler Thromb 13:310); the glycocalyx lining at these sites are typically thinner and prone to injury, which causes endothelial dysfunction and atherosclerosis (2004. Arterioscler Thromb Vasc Biol 24: 12–22). Such bends are found in various parts of the vasculature including the aortic arch, carotid sinus, brain, heart and limbs (2011. Physiol Rev. 91:327–387). (Fig 37)

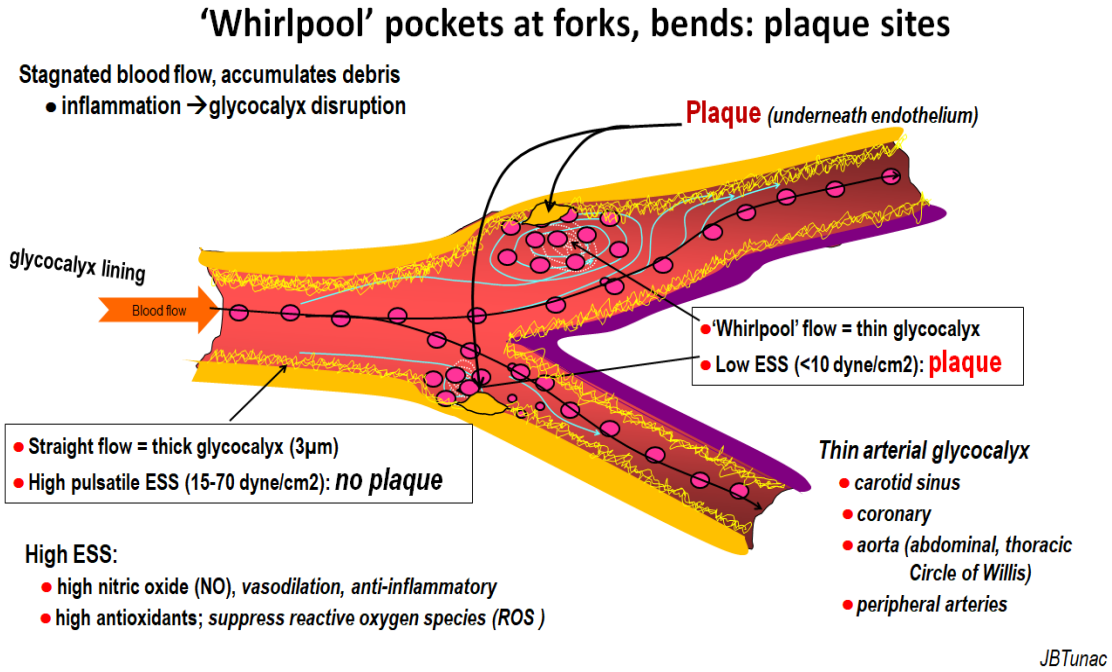


Figure 37, Blood flow slows down at arterial forks and bends, creating a "whirlpool pocket" and plaque site.

Thus, the coronary arteries are plaque prone because they have the most bends and bifurcations. On the other hand, the descending thoracic aorta, where blood flow is uniform and unidirectional does not form plaque. (1993 J Biomech Eng.115 (4B):588–594). Normal and high SS is atheroprotective and is involved in compensatory remodeling (2005. Nat Clin Pract Cardiovasc Med 2: 401–407), but when compensatory remodeling fails, the plaque intrude into the lumen (2005. Nat Clin Pract Cardiovasc Med 2: 401–407)

Acute inflammation due to infection or injury predisposes the plaque to rupture. Ruptured plaque triggers clot (embolus) formation, causing stroke (clogged artery to the brain), heart attack (clogged artery to the heart), or PAD (clogged artery to the arms or legs). Arterial bends and plaques are found throughout the vasculature with CHD representing the most death in CVD due to thromboembolism (Fig 38).

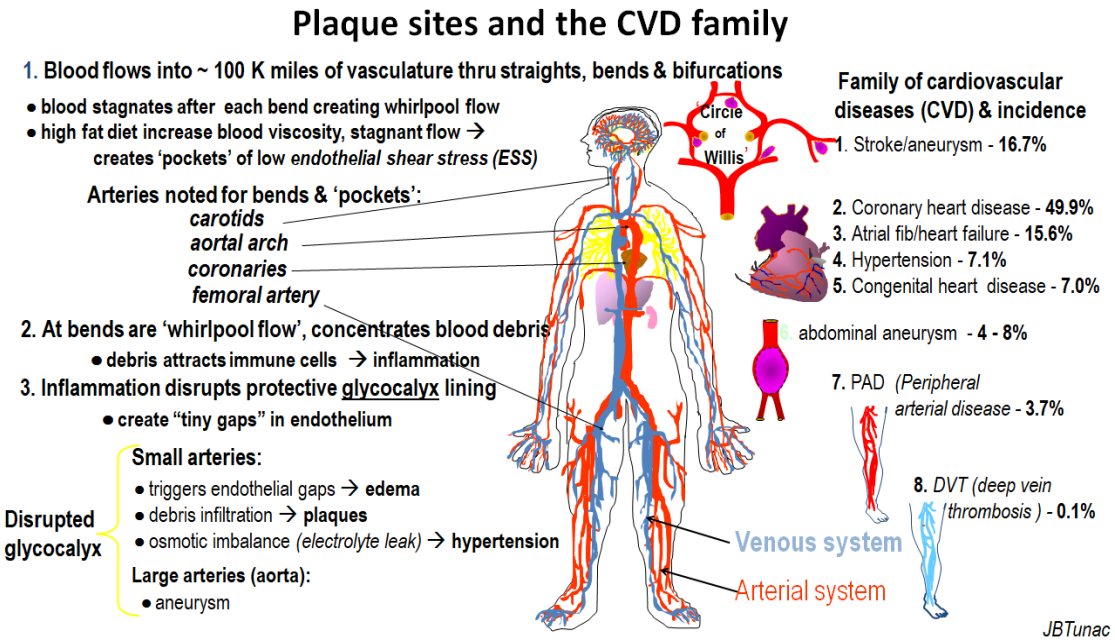


Figure 38. Family of CVD as manifested in different parts of the vascular system.

Of the coronary arteries, the left anterior descending (LAD) has at least 4 bends and as many plaques accounting for coronary heart disease (CHD) as the most prominent (~50%) of CVD cases (1993. Arterioscler Thromb 13:310). (Fig 39).

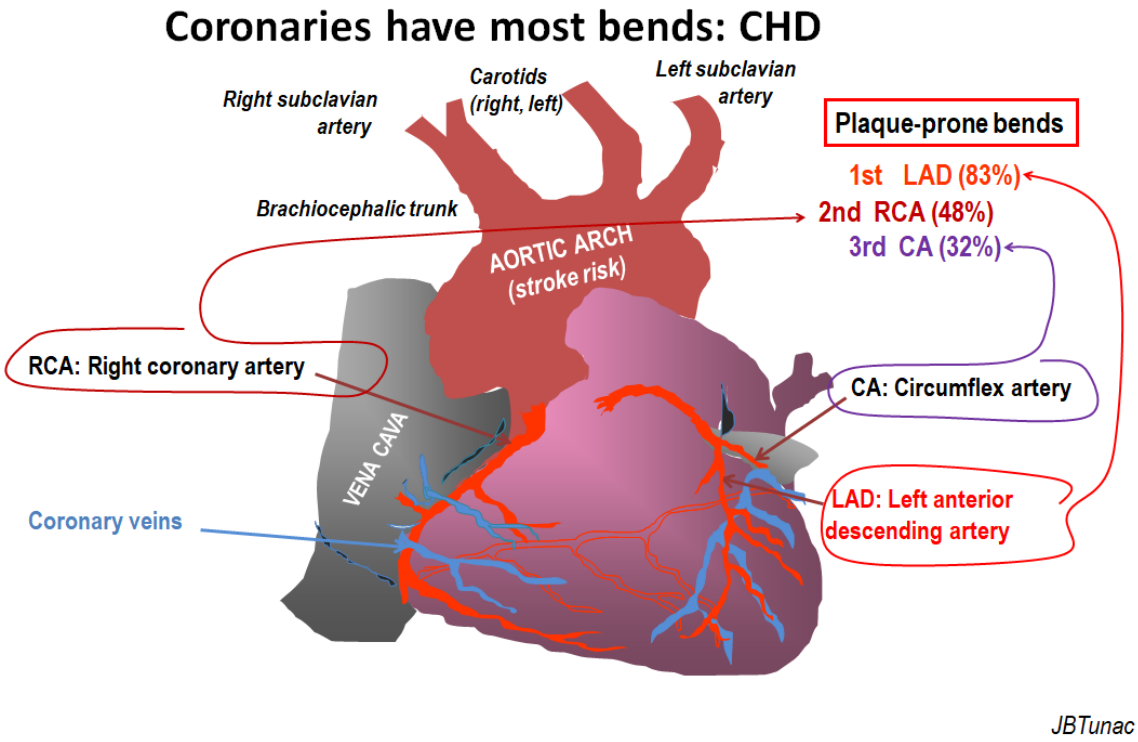


Figure 39. Coronary arteries are noted for most bends and plaques

14. High blood viscosity exacerbate plaque formation

Elevated blood viscosity is a predictor of cardiovascular disease, which is exacerbated by very low density lipoprotein (VLDL). Fats or triglycerides are packaged in VLDL, The biogenesis of VLDL and their secretion into the circulatory system by the liver plays an important role in overall lipid homeostasis. Enhanced production of VLDLs and their eventual secretion into the circulatory system constitute one of the major risk factors for the development of atherosclerosis (2002.Circulation. 106: 2137–2142). VLDL is converted in the bloodstream to low-density lipoprotein (LDL) and intermediate-density lipoprotein (IDL). While dietary fats or triglycerides are repackaged in VLDL, dietary cholesterol is converted by cholesterol hydroxylase into bile; about 1.2 grams of cholesterol is needed by an average human and most of it is synthesized by the liver (Fig 40)

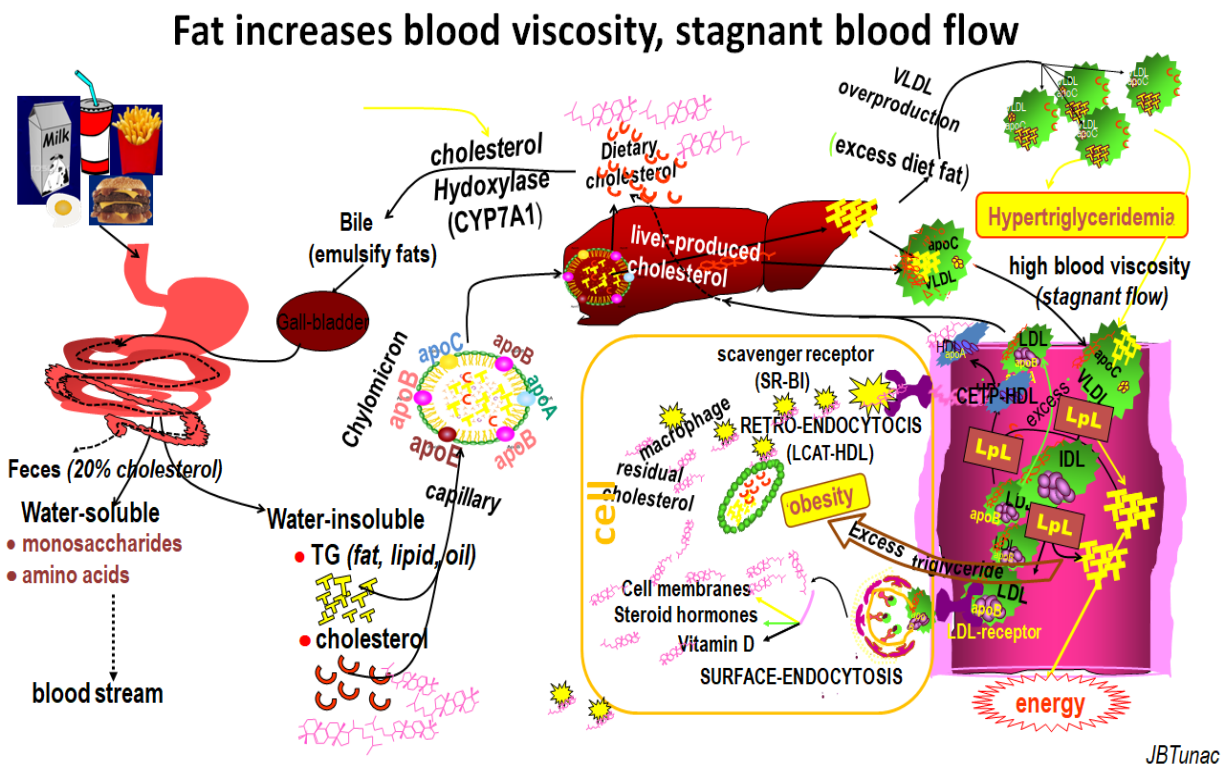


Figure 40. Fat diet is packaged in very low density lipoprotein (VLDL) for delivery in the blood stream; high fat diet means high VLDL packaging, which makes blood viscous.

High-fat meals cause endothelial dysfunction and mild inflammation in the vessel walls (2014. Nutr J 13: 12); even just one fat meal load in healthy young men would show reduced coronary blood flow (2002. Ann Intern Med 136: 523-528).

A substantial number of epidemiologic studies show risk of a major cardiovascular event (death, acute myocardial infarction, or acute need for cardiovascular surgery) increases with increased blood viscosity (2000. Eur Heart J 21:515). VLDL is the most atherogenic triglyceride remnant lipoprotein (TGRL) particles (2002. Clin Chem 48: 217-219) and VLDLs are the major component and atherogenic particles of TGRL in the circulation. (2014 Atherosclerosis 236: 244-250]. VLDL remnants rapidly penetrate the arterial wall, increase endothelial inflammation and facilitate the infiltration of monocytes, which results in foam cell formation and atherosclerosis (2005. Atherosclerosis 181: 321-327).

Thus, a diet of saturated fatty acid was associated with a higher CHD risk (2016. Arterios Thromb Vasc Biol. 36:2011–2018). On the other hand, diet of low fat reduced CVD (2014. J Human Hypertension 28: 170–175), which is consistent with the classic Mediterranean diet (2017.Diabetes Spectrum 30:72-76). Every animal based food contains both cholesterol and fats; while the cholesterol content is constant (except egg yolk and cheeses), the fat content varies with beef averaging 9.6% fat. The Western type diet averages 21% fat and 0.15% cholesterol (Fig 41)

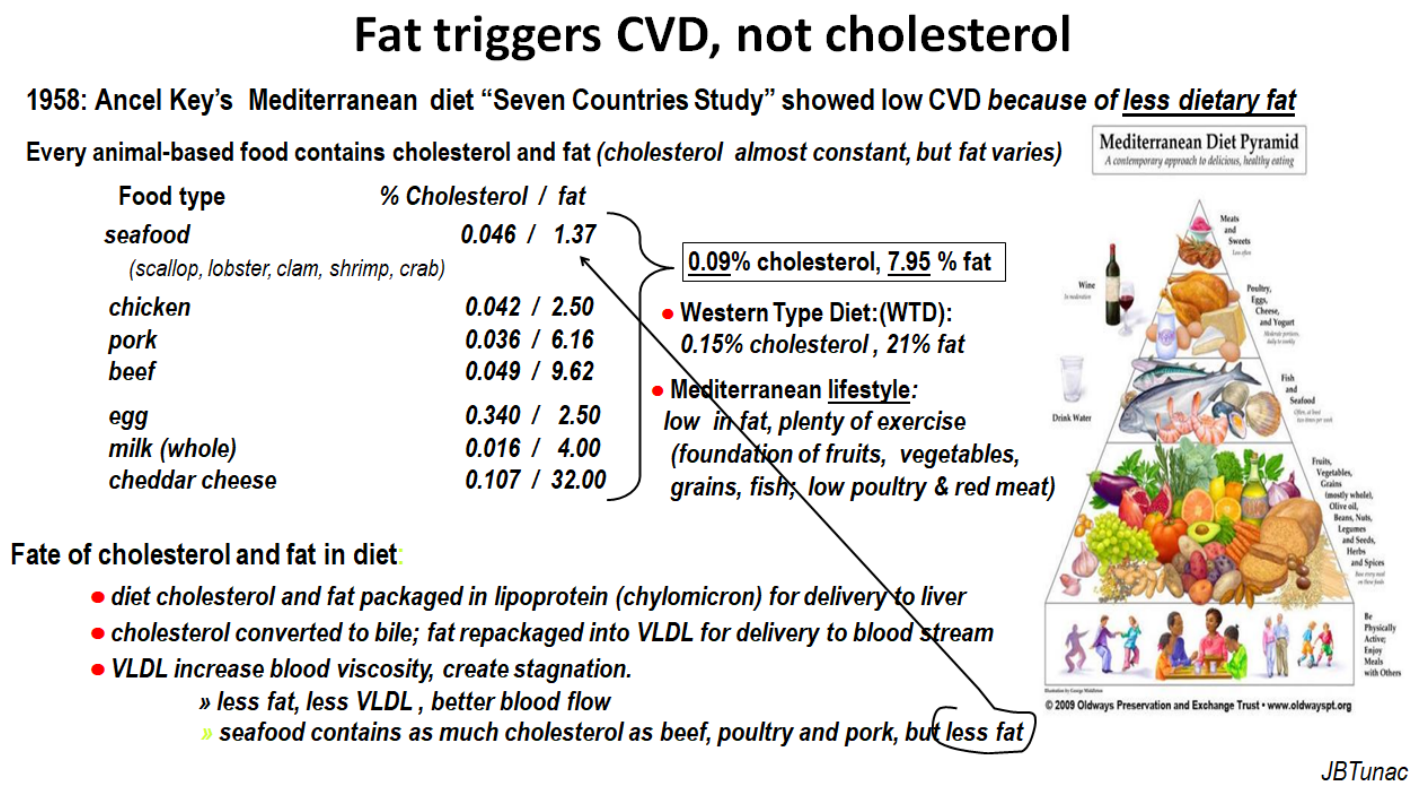


Figure 41. Animal-based diet contains both cholesterol and fat, but fat content varies with cheese and beef as highest. The Western type diet notoriously associated with for heart disease contains 21% fat.

The processes leading to plaque build-up (atherosclerosis) generally begin in the early years of life, as young as 5 years old, but the symptoms generally do not become apparent until after the age of 40 years. A natural sequence to the 'tiny gaps' is edema (fluid buildup) and osmotic imbalance (concentration of solution). Thus, electrolytes that normally reside outside the cell (extracellular) like sodium (Na), potassium (K), calcium (Ca), chloride (Cl), and bicarbonate (HCO_3), diffuse inside through the 'gap'. On the other hand, electrolytes normally found inside the cell, such as potassium (K), magnesium (Mg), and phosphate (PO_4) diffuse out. These electrolyte imbalance create various circulatory abnormalities most notably hypertension, heart failure, and venous blood clots.

15. Stagnant pockets trap debris and pollutants and cause inflammation

In summary, arterial blood flow especially at bends and bifurcations slows down and further stagnates when blood viscosity increases due to fat diet packaged in VLDL. Stagnant blood create a low shear stress and "whirlpool flow" where 'blood debris' gravitates (e.g., dead cells, 'microbial contaminants', etc) and xenobiotics (chemicals, smoke, pollutants, particulate matters). These attract monocytes, which produce oxidative (ROS) and inflammatory (cytokines) factors; chronic inflammation leads to destruction of the protective glycocalyx lining.

Destruction of the glycoclyx exposes the endothelium to injury creating 'tiny gaps' in the endothelial wall, subsequently blood debris and macrophage infiltration to form plaques. Plaques rupture and heal naturally, but certain plaques generate clots that become amplified and degenerate into thromboembolism causing heart attack or stroke. Biological pollutants (dead cells, microorganisms) and chemical pollutants (xenobiotics) are key risk factors in the pathophysiology of CVD (Fig 42.)

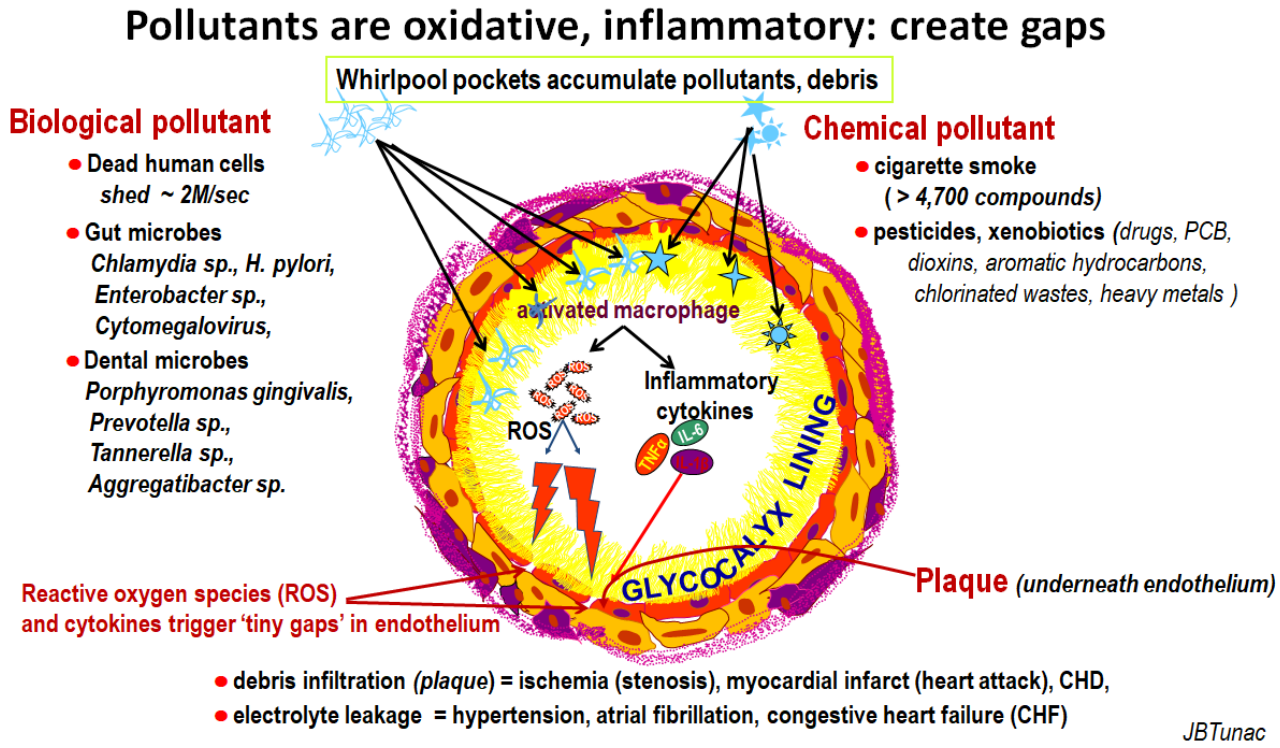


Figure 42. Biological and chemical pollutants in the arterial bends triggers inflammation, tiny endothelial gaps creating electrolyte leakage (hypertension) and debris infiltration (plaque)

The processes leading to plaque build-up (atherosclerosis) generally begin in the early years of life, as young as 5 years old, but the symptoms generally do not become apparent until after the age of 40 years. A natural sequence to the 'tiny gaps' is edema (fluid buildup) and osmotic imbalance (concentration of solution). Thus, electrolytes that normally reside outside the cell (extracellular) like sodium (Na), potassium (K), calcium (Ca), chloride (Cl), and bicarbonate (HCO_3), diffuse inside through the 'gap'. On the other hand, electrolytes normally found inside the cell, such as potassium (K), magnesium (Mg), and phosphate (PO4) diffuse out. These electrolyte imbalance create various circulatory abnormalities most notably hypertension, heart failure, and venous blood clots (Fig. 43).

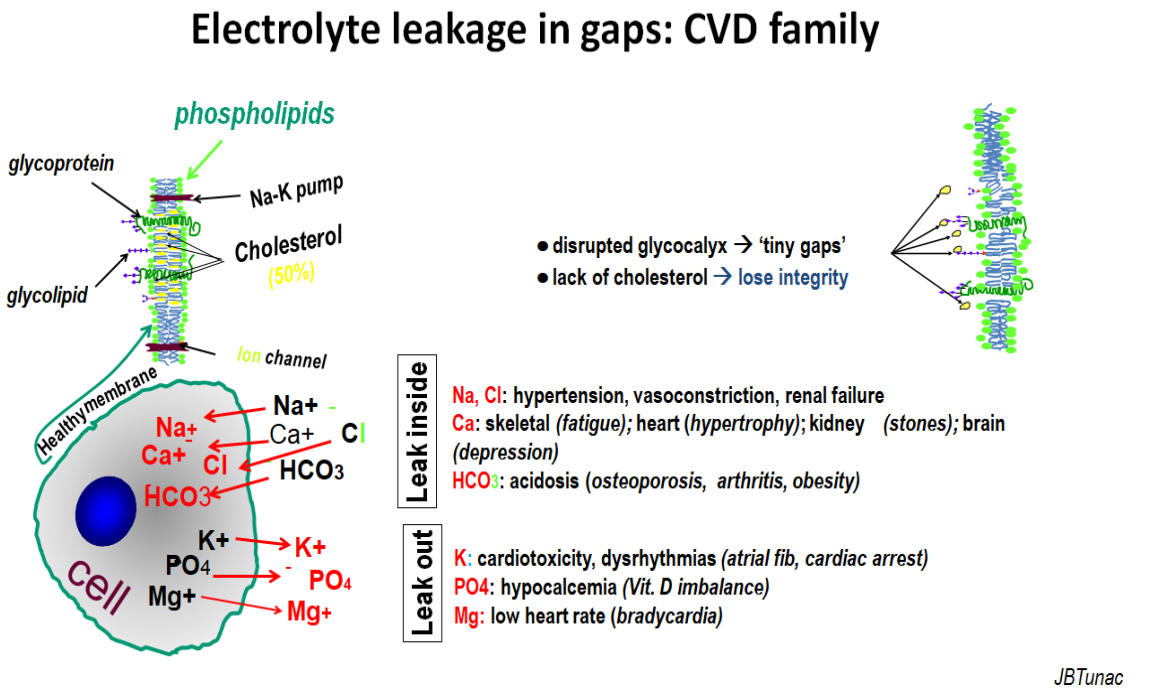


Figure 43. Tiny gaps create osmotic imbalance allowing diffusion of electrolytes and a family of cardiovascular diseases (CVD)

Thromboembolism is the breakage of amplified (large) clot to become an embolus and clog downstream vessel that is too small to let it pass causing stroke (clogged artery to the brain), heart attack (clogged artery to the heart), or PAD (clogged artery to the arms or legs). (Fig 44)

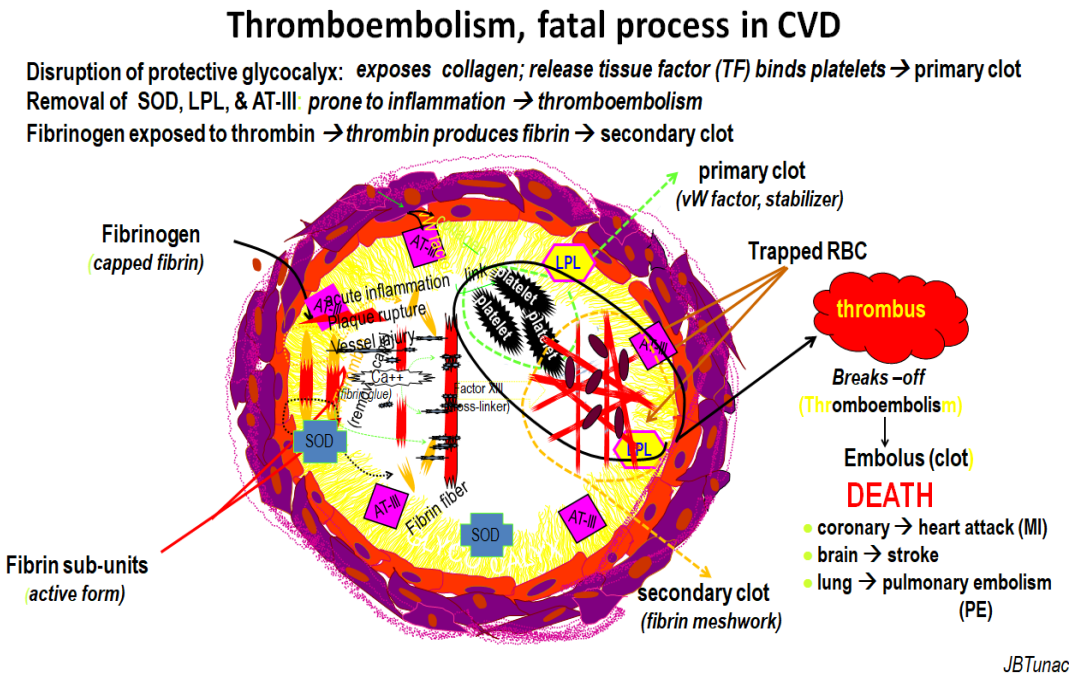


Figure 44. Formation of clot starts with a disrupted glycocalyx (primary clot) and progresses into a secondary clot (embolus), this is the fatal component of CVD

Experimental Results

Biomarker development

The primary prevention of CVD is dependent upon the ability to identify high-risk individuals long before the development of overt events. This highlights the need for biomarkers and accurate diagnosis. Despite an overwhelming number of individual biomarkers reported on cardiovascular disease, no one biomarker is found to be reliable in predicting or diagnosing a cardiovascular event.

The glycocalyx is an endothelial surface layer which lines the epithelial cells of the vascular system, protecting epithelial cells from vascular flow shear stress, coagulants and platelets, and leukocyte-cell interactions. Glycoproteins component of glycocalyx function as receptors on the cell surface, such as selectins, integrins, and members of the immunoglobulin superfamily; these are weaved into the net of the endothelial glycocalyx. Also, the endothelial surface layer consists of secreted proteoglycans (eg, versican and perlecan) and their adsorbed plasma proteins (eg, orosomucoid and albumin). Together with glycosaminoglycans (GAGs) and plasma proteins, the endothelial surface layer as a whole forms a dynamic barrier to circulating cells and soluble biologic macromolecules. When damaged, the glycocalyx sheds three key components: syndecans, heparan sulfate, and hyaluronan (hyaluronic acid). Coincident to shedding is the release of plasminogen activator inhibitor–1 and signal pathologic conditions (2017. Crit Care 21:26).

Thus, disturbance of the endothelial glycocalyx marks early stages of various clinical pathophysiologies and monitoring glycocalyx disruption promises to be an important diagnostic biomarker and therapeutic target in clinical settings (2019. Chinese Med J.132:963–975). Currently. there is no feasible or practical diagnostic tool to measure glycocalyx disruption (Fig. 45)

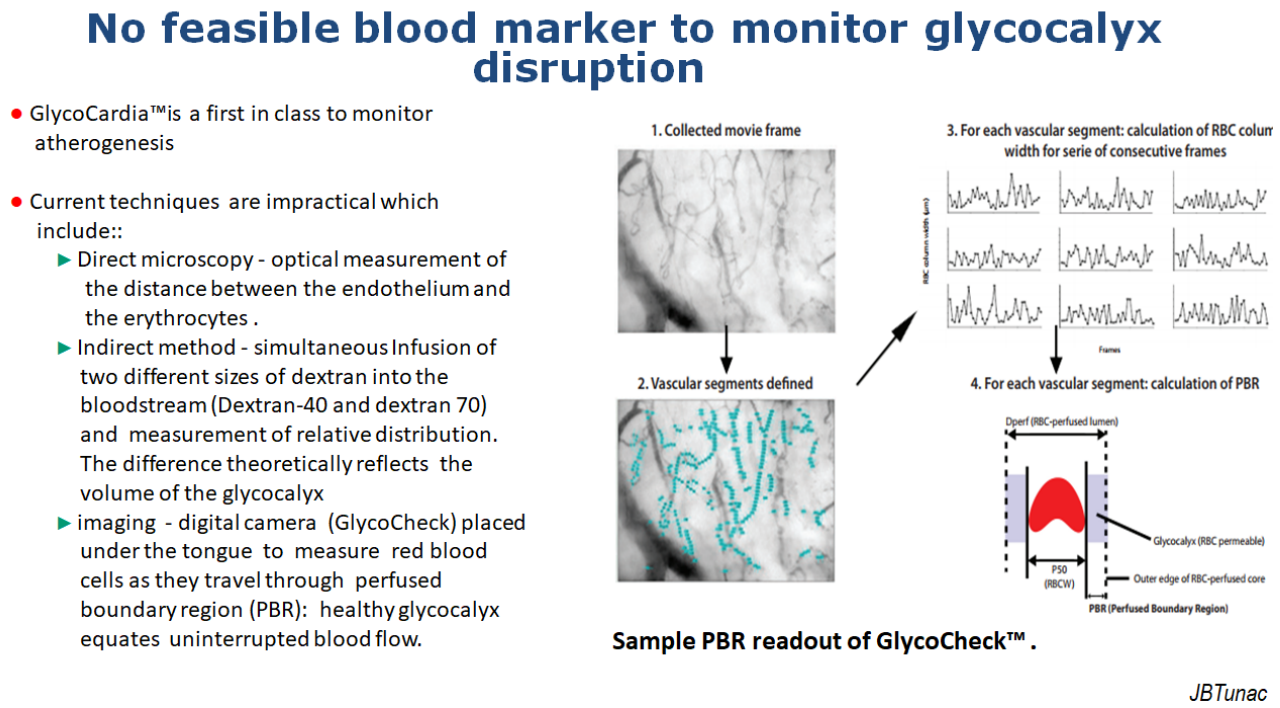
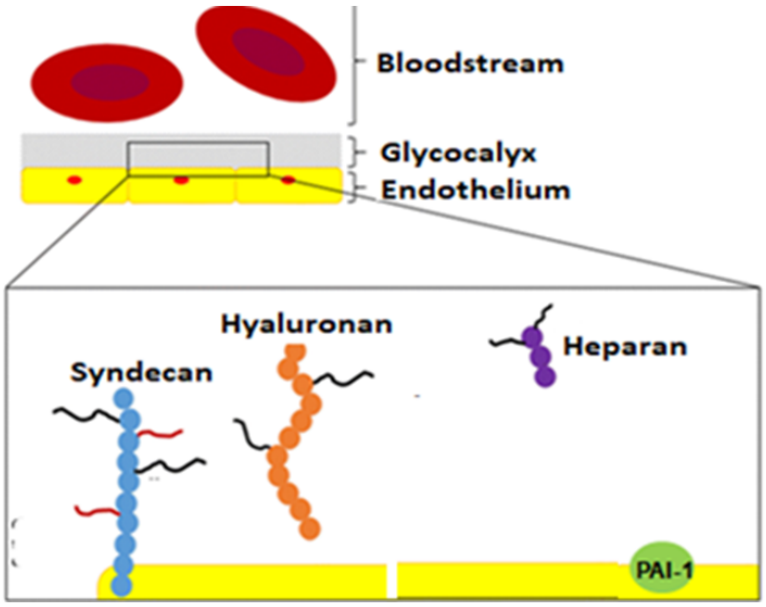


Figure 45. Current techniques for measuring glycocalyx disruption involve microscopy and imaging

There is no one single biomarker adequate as predictive of a multifactorial disease as CVD, thus a need for a panel. We evaluated a number of biomarkers associated with glycocalyx disruption and selected 4: hyaluronan, heparan, syndecan-1, and plasminogen activator inhibitor-1. (Fig. 46)

Four glycocalyx components chosen as biomarkers

- A number of biomarkers were evaluated including soluble fibrin (SF), thrombin-antithrombin complexes (TAT), antithrombin III, plasminogen activator inhibitor-1 (PAI-1) and glycocalyx remnants (hyaluronan, heparan, syndecan).
- Of these biomarkers, 4 were found to be highly correlative to plaque formation *(GlycoCardia™):*
  1. syndecan-1 (SDC-1)
  2. hyaluronan (HAS-1)
  3. Heparan (HS)
  4. plasminogen activator inhibitor (PAI-1)



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.Figure 46. Glycocalyx components chosen for the 4-panel GlycoCardia™

These components have been individually proven in the clinic as associated with various diseases including CVD.*(Fig 47, 48, 49, 50)*

Hyaluronan in clinical use as a diagnostic marker

- Range of diseases clinically diagnosed by hyaluronan

Disease category	Biomarker Level		Reference
	Control	Diseased	
	Hyaluronan (hyaluronic acid)		
Arthritis	29.1 ng/ml	37.4 ng/ml	2004. Ann Rheum Dis 63(9):1166-8.
Stroke	170.4 ng/ml	219.7 ng/ml	2014. J Neuroinflammation 11: 101
Hypertension	26.2 ng/ml	73.0 ng/ml	2013. Tohoku J Exp Med 230(1):7-11
Dengue fever	100.1 ng/ml	935.9 ng/ml	2017. Scientific Reports 7, No. 46191
Malignant mesothelioma	≤49 µg/ml	>100 µg/ml	2013. Respir Investig 51(2):92-7
Septicemia	11 ug/L	344 ug/L:	2012. Clin Biochem 45(2):82-7
Hepatitis	0.03 ng/ml	0.19 ng/ml	2016.Clin Exp Med 16(4): 523–528
Liver cirrhosis	30-40 ug/L	100 – 300 ug/L	2000. Eur J Gastro Hepatol.12(10):1121-7
Hepatocarcinoma	117.86 ng/ml	426.36 ng/ml	2003. Asian Pac J Allergy Immunol 21(2):115-20
Fibrosis	16 µg/l	160 µg/l	2005. Comp Hepatol 4: 6

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Figure 47. Clinical blood levels of hyaluronan in various diseases

Syndecan-1 in clinical use as a diagnostic marker

- Range of diseases clinically diagnosed by Syndecan-1 (SDC-1)

Disease category	Biomarker Level Control	Diseased	Reference
	Syndecan-1		
Heart failure	1.19 ng/ml	4.14 ng/ml	2015. Scientific Reports 5, Article # 8916
Acute decompensated HF (ADHF)	91.4 ng/ml	133.7ng/ml	2015. Circulation 79(7):1511-1519
Chronic heart failure (CHF)	5.7 ng/mL	22.5 ng/mL	2001. J. Cardiology 57(3):325-332
Acute coronary syndrome (ACS)	42 ng/ml	77 ng/ml	2016. Atherosclerosis 247: 184–188)
Ischemia	77 ng/ml	92 ng/ml	2013. Eur J Anaesthesiology 30:196–197
Endotheliopathy of trauma" (EoT)	13 ng/ml	108 ng/ml	2017. J Am Coll Surgeons 225 (3):419-427)
Global and regional ischemia	1.2 ug/dL	50.4 1.2 ug/dL	2007. Circulation 116: 1896-1906
Preeclampsia	28 ng/mL	218 ng/mL	2016. PLOS One
CVD (Hemodialysis Patients)	5.04 ng/ml	21.9 ng/ml	2016. Plos One
CVD (HIV patients)	36.5 ng/ml,	71.8 ng/ml	2017. AIDS Res.Human Retroviruses.33(7
Renal failure (Dialysis pati	27.5 ng/ml	111.0 ng/ml	2012. JASN 23 (11): 1900-1908)
ischemia	1.2 µg/dL	78.0 µg/dL	2007. Circulation 116:1896-1906
Hemorrhagic Shock	27.0 ng/ml	554.0 ng/ml	2011. PLOS One
Lung cancer	16 ng/ml	40 ng/ml	2002. Cancer Res 62: 5210–5
Inflammatory bowel disease (IBD)	21.2 ng/mL	29.5 ng/mL	2013. Gastroenterology 144(5: S-423

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Figure 48. Clinical blood levels of syndecan-1 in various diseases

Heparan sulfate in clinical use as diagnostic marker

- Range of diseases clinically diagnosed by heparan sulfate (HS)

Disease category	Biomarker Level Control	Diseased	Reference
	Heparan sulfate		
Ischemia	590 ug/dL	5,900 ug/dL	2007. Circulation 116:1896-1906
Pre-eclampsia (hypertension)	60.5 mg/l	123.1 mg/l	2002. Acta Obstet Gynecol Scand 81(4):308
Mucopolysaccharidoses (MPS),	12.4 ug/ml	24.4 ug/ml	2005. Inherit. Metab. Dis 28: 743–757
Dengue fever (hemorrhagic)	16.78 ng/mL	108.55 ng/mL	2017. Scientific Reports 7, No. 46191

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Figure 49. Clinical blood levels of heparan sulfate in various diseases

Plasminogen activator inhibitor-1 in clinical use

- Range of diseases clinically diagnosed by Plasminogen activator inhibitor-1 (PAI-1)

Disease Category	Biomarker Level Control	Diseased	Reference
Plasminogen activator inhibitor-1 (PAI-1)			
Ischemic stroke	11.8 IU	17.2 IU	2000. Acta Neurochir Suppl 76:277-8
Acute Stroke (Thrombolysis Failure)	23.6 ng/ml	45.2 ng/ml	2005. J Clin Neurol 2:142-147.
Chronic angina pectoris	9.6 U/ml	17.5 U/ml	1990. Thrombosis Haemostasis 63(3):336-3
Acute myocardial infarction (AMI)	10.1 ng/ml	16.3 ng/ml	2014. Int J Clin Exp Med 7(4):1059–1063
Angina (unstable plaque)	40.0 ng/mL	95.4 ng/mL	2004. Am Heart J 147(1):158-64
Coronary arterial disease (CAD)	2.97 ng/ml	5.26 ng/ml	1991. Blood Coagul Fibrinolysis 1:41-5
AMI (unstable angina)	19.35 ug/dl	44.02 ug/dl	2012. J Pak Med Assoc 62 (7): 681-684
Hypertension	32.1 ng/mL	39.8 ng/mL	2002. Am J Hypertension 15 (8):683–690
Coronary Artery Disease	2.4 ng/ml	8.8 ng/ml	2014. Addict Health 6(3-4): 119–126).
Glioma	2.5 ng/ml	3.54 ng/ml	2008. Anticancer Res 28: 415-418
Type 2 diabetes	12.9 u/ml	23.4 u/ml	2005. Pediatric Research 58:483–487
Acute Myocardial Infarction	10.0 µg/L	15.1 µg/L	1998. Circulation 98:2241-2247

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Figure 50. Clinical blood levels of plasminogen activator inhibitor-1 in various diseases

The 4-panel, herein called *GlycoCardia* ^{CHD}, was tested and found to correlate with plaque formation in our animal model. (Fig. 51)

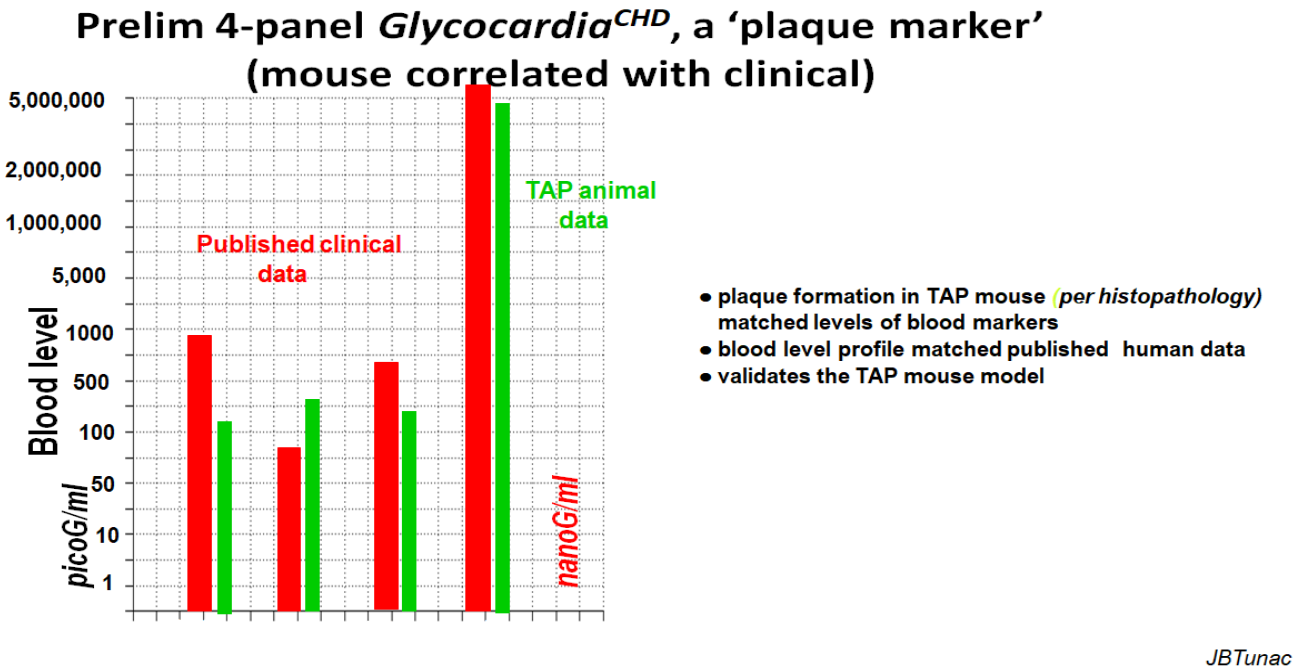

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Figure 51. Blood level profile of Glycocardia^{CHD} animal (TAP™ mouse) model matches that of published clinical data

In addition to the above, 3 other biomarkers associated with cell disruption, are added to complement the diagnosis of a wider range of chronic diseases: gamma (γ′) fibrinogen, growth differentiation factor-15) (GDF-15), and pregnancy associated plasma protein-A (PAPP-A). This 3-panel herein designated *GlycoCardia^{HF}*; the clinical utility of each biomarker is described below:

1. Gamma (γ') fibrinogen:

Plasma fibrinogen is a coagulation factor and an acute-phase inflammatory marker that has been implicated in the pathophysiology of cardiovascular disease (CVD) (2005. JAMA. 294:1799–1809). Fibrinogen is a key component of the hemostatic system, playing a role in both primary and secondary response. Thrombin-catalyzed cleavage of fibrinopeptides (Fp) A and B converts fibrinogen into fibrin, which spontaneously polymerizes and forms double-stranded protofibrils that assemble into branched fibrin fibers, forming the fibrin clot (2008. Cardiovasc Hematol Agents Med Chem 6:181-189). Biomarker for early clotting, significantly associated with coronary artery disease and myocardial infarction in the Stockholm Coronary Artery Risk Factor study and the Framingham Heart Study. (2007.J Thromb Haemost 5:766 – 73); significantly associated with stroke in the Erasmus Stroke Study and others (2012. Thrombosis Researc 129: 807-809); increases during inflammation and differentially regulated from total fibrinogen under pathologic conditions, as demonstrated in the Periodontitis and Vascular Events study (2011. Thromb Haemost 105:605 – 9).

Human levels of γ' fibrinogen

- study of 133 patients diagnosed with coronary artery disease (CAD): diseased, 0.413 g/L vs 0.299 g/L in the controls (1996. J Biol Chem 271(38):23121-23125..
- epidemiologic study on myocardial infarction (MI) in the Stockholm Coronary Artery Risk Factor cohort: diseased 0.28 g/L higher than controls (2007. J Thromb Haemost 5:766 – 73). \
- patients with a history of CVD and periodontal disease: highly elevated compared to controls, 0.622 g/L (2010 Clin Chem 2010; 56:781 – 8).
- independent predictor of CAD: hypertensive participants 433.36 vs 405.70 mg/dL in controls (2017.Rev Esp Cardiol. 70:34-41)
- positively associated with deaths due to peripheral artery disease (PAD), heart failure (HF) and CVD deaths: lowest quartile 8.0 - 24.34 mg/dl; highest quartile, ≥ 35.19 mg/dl (2015. Arterioscler Thromb Vasc Biol. 35(12): 2700–2706).
- study on 3,042 participants of the Framingham Heart Study Offspring Cohort. Individuals with prevalent CVD, 0.278 mg/ml vs. 0.258 mg/ml without (2011.Arterioscler Thromb Vasc Biol. Oct; 31(10): 2345–2352).
- Physicians Health Study of 14,916 subjects: levels of 343 mg/dL, twofold increase in the risk of a myocardial infarction (2013. University Heart Journal 9:40-46).
- significantly higher in patients with ischemic stroke, 0.37 g/L, vs 0.32 g/L in controls (2011.Thromb Haemost, 105:430-4

2. Growth differentiation factor-15 (GDF-15)

GDF-15 (growth differentiation factor-15) is a protein belonging to the transforming growth factor beta

superfamily, which functions in regulating inflammatory pathways, apoptosis, and cell repair and cell growth associated in cardiovascular and neoplastic disorders (2000. Molecular and Cellular Biology. 20 (10): 3742–51) GDF-15 serves as a prognostic protein in patients with different diseases such as heart diseases and cancer expressed in low concentrations in most organs and upregulated because of injury of organs such as such as liver, kidney, heart and lung (2005. Shock. 23 (6): 543–8). Growth differentiation factor-15 (GDF-15) is a stress responsive cytokine, which increases during tissue injury and inflammatory states and is associated with cardiometabolic risk. Increased GDF-15 levels are associated with cardiovascular diseases such as hypertrophy, heart failure, atherosclerosis, endothelial dysfunction, obesity, insulin resistance, diabetes, and chronic kidney diseases in diabetes. GDF-15 is an inflammatory marker associated with increased cardiovascular and noncardiovascular mortality; plays pivotal role in development and progression of cardiovascular diseases such as heart failure, coronary artery diseases, atrial fibrillation, diabetes, cancer, and cognitive impairment (2013. Clinical Chemistry 59: 1550–1552 2014. Circulation 130:1847–1858). Increased GDF-15 level is linked with the progression and prognosis of the disease condition.

- stratified the blood GDF-15 levels into three categories, that is, normal (<1200 pg/mL), moderately elevated (1200–1800 pg/mL), and highly elevated (>1800 pg/mL). (2010. Aging Cell, 9: 1057–1064)
- elevated levels of GDF-15 of >1800 ng/L, high risk for mortality within one year (2008.BMC Public Health, 8:148)
- GDF-15 concentrations ≥1800 ng/L, increased risk for all-cause and cardiovascular death compared to those with <1200 ng/L. (2012. Clinical Chemistry 58:172–182)
- associated with reduced endothelium-dependent vasodilation in resistance vessels from <948 ng/L (1st quartile) - >1390 ng/L 4th quartile) (2009.Eur Heart J. 30:2346–2353).
- median concentration of 1253 ng/L at baseline: hazard ratio (HR) for the highest compared to the lowest quartile for CV death, 2.63; for sudden death, 3.06; for heart failure (HF) death, 4.3; for cancer death, 2.5; for hospitalization for HF, 5.8 (3.2–10); for MI 1.4; and for stroke, 1.8 (2017.Clinical Chemistry 63:1 140–151) (2017).

3. Pregnancy associated plasma protein-A (PAPP-A)

Pregnancy-associated plasma protein-A (PAPP-A) level is an independent predictor of acute cardiovascular event occurrence. PAPP-A is associated with thin cap plaque leakage and a higher burden of coronary thin-cap fibroatheroma (TCFA); elevated in patients with acute coronary syndromes and patients with risk factors, such as obesity, hypertension, and/or diabetes relative to healthy subjects (2015. Biomark Med. 9:731–741); highly expressed in vulnerable atheromatous plaques (2016. Medicine (Baltimore) 95:e2563; 2004. Circulation 109:1724–1728; 2005. Clin Chem 52:1096–1103).

- Patients with ≥3 VH-TCFAs had a higher PAPP-A level than patients with 1 to 3 VH-TCFAs or without any VH-TCFA (13.3±11.8 versus 7.8±4.7 versus 7.4±4.7 mIU/L, $P<0.001$, respectively). (2016. Medicine (Baltimore).95(3): e2563).
- Pregnancy Associated Plasma Protein-A (PAPP-A) Levels in Acute Coronary Syndrome: PAPP-A levels were significantly elevated in patients with acute myocardial infarction (AMI) and in patients with unstable angina (UA) mean levels 64.26 and 36.23 ng/ml respectively; Mean PAPP-A levels in controls were 10.68 ± 1.04 ng/ml. (2015. Indian J Clin Biochem. 30(2): 150–154).
- Pregnancy-associated plasma protein A associates with cardiovascular events in diabetic

hemodialysis patients. median PAPP-A concentration of 17 mIU/ patients in the 4th PAPP-A quartile 4th quartile (≤20.9 mIU/L) had an adjusted 2.6 fold increased risk of sudden death and 2.8 fold increased risk of stroke as compared to the patients in the 1st quartile (≤13.4 mIU/L) (2014. Atherosclerosis.236:263-269

An example of an algorithm or fingerprint combining *GlycoCardia*^{CHD} and *GlycoCardia*^{HF} is shown (Fig.52)

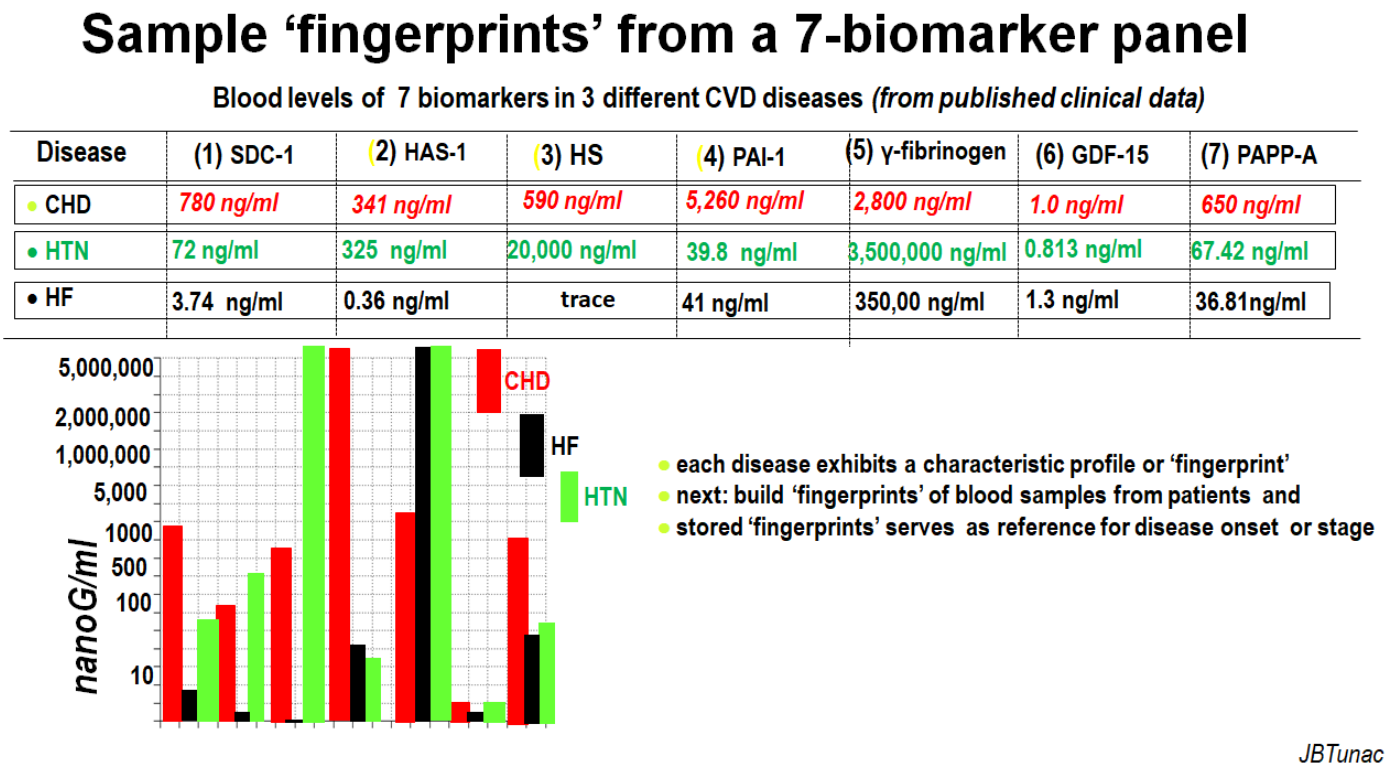

Figure 52. blood level profile of the 7 biomarkers including data from TAP™ mouse and published clinical data

These 7 biomarkers address the multifactorial pathophysiology of CVD, whereas the widely used lipid panel measures cholesterol and triglycerides (Fig 53).

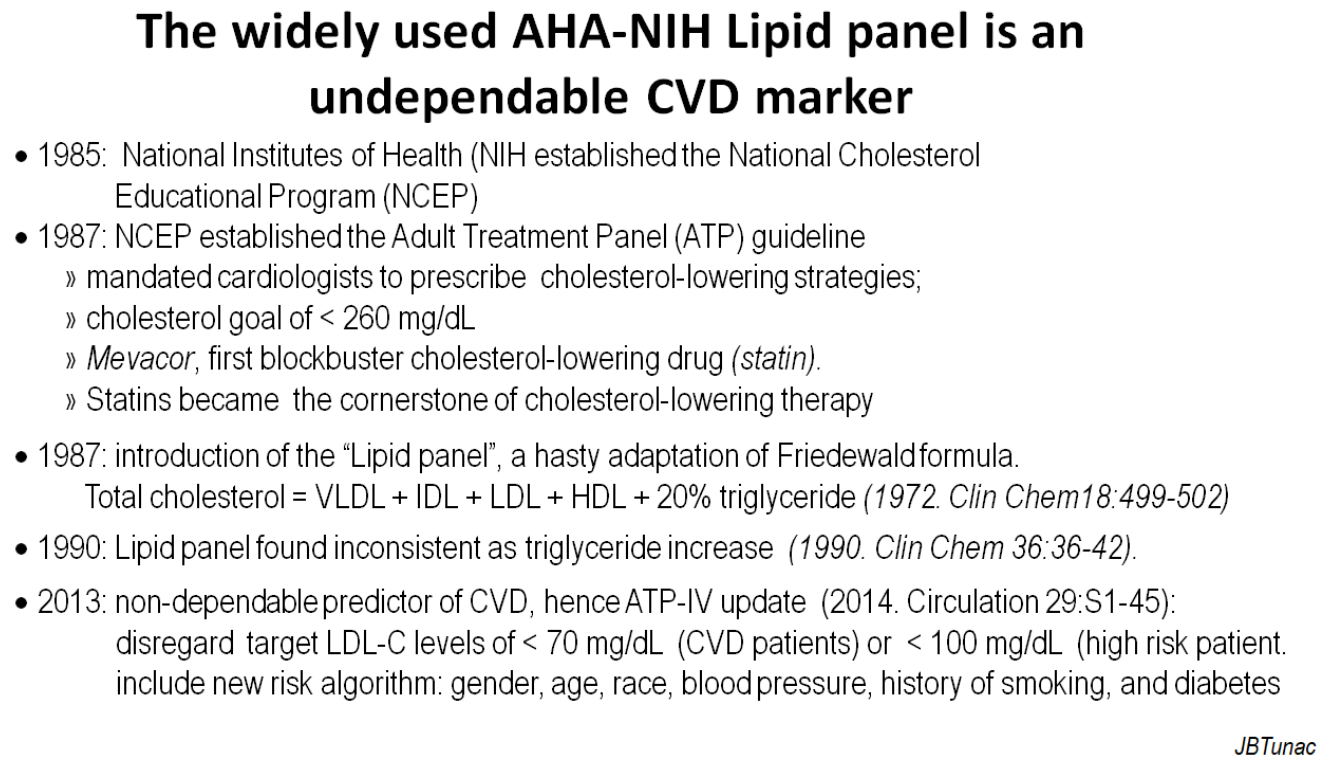

Figure 53. Currently used lipid panel for diagnosing CVD

Development of Glycocardia as diagnostic kit

The development pathway of a GlycoCardia™ kit involves testing fingerprinting of clinical samples that have been identified and sorted by a physician. An algorithm or fingerprint is created per statistical analysis to identify or diagnose new cases and serve as a guide for treatment programs (Fig. 54)

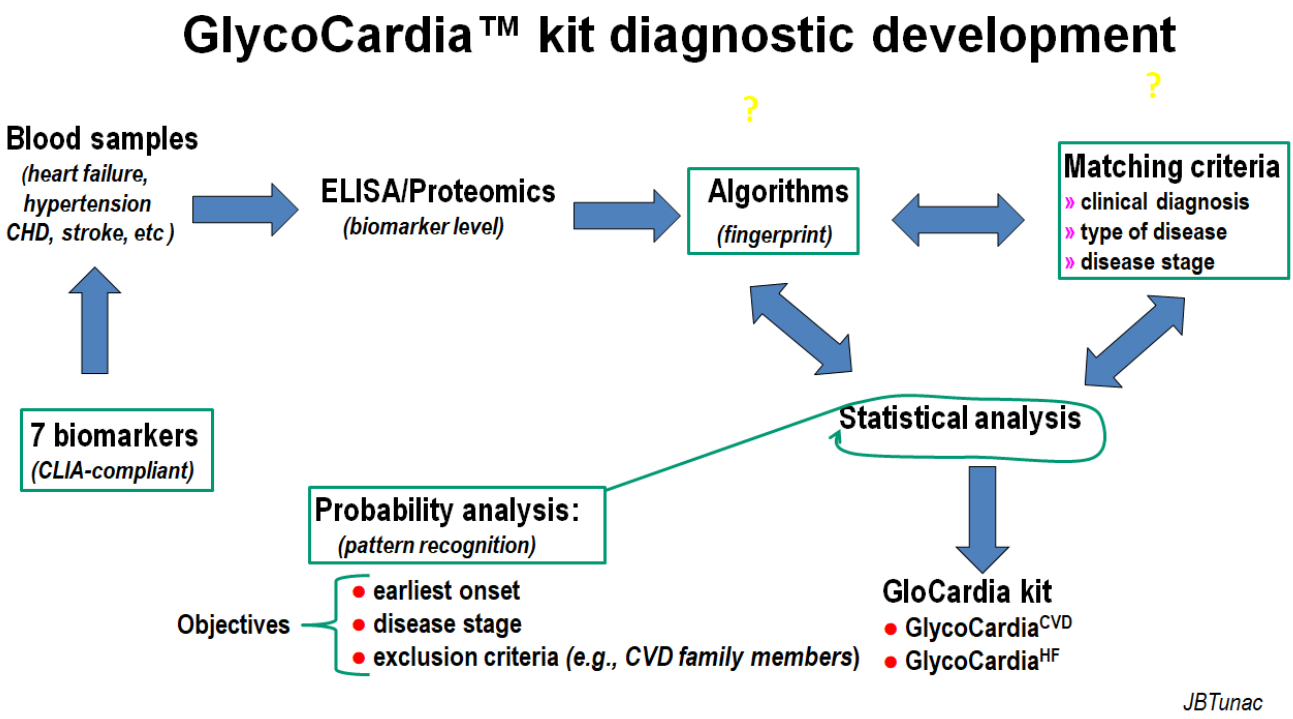

Figure 54. Development pathway and the making of a GlycoCardia "fingerprint"

GlycoCardia as a companion and stand-alone diagnostic

GlycoCardia™ can be used twofold: 1) as a companion diagnostic for Embotricin™, or 2) 'stand-alone' diagnostic to monitor or evaluate the traditional symptom-targeted therapies for their ability to restore glycocalyx (hyaluronan, heparan, syndecan), clotting potential (PAI-1), mitigate hypertension, heart failure, stroke, and CHD. The latter presents an immediate market for GlycoCardia™; thus, a patient consults a clinician and evaluated for drug therapy (Fig 55).

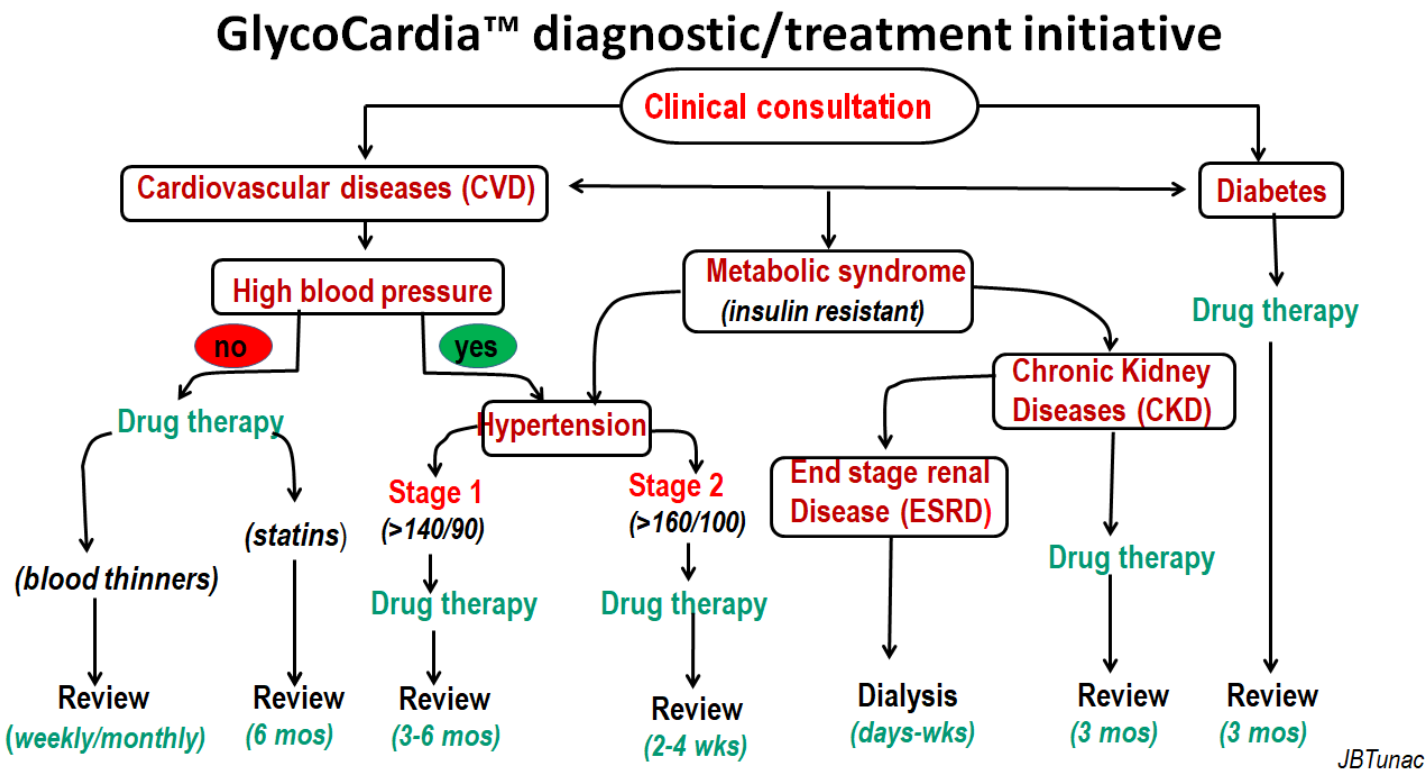

Figure 55. Schedulede use of GlycoCardia™ to monitor Embotricin™ or traditional treatment

Therapeutic development:

CVD is a multifactorial disease involving: blood flow (glycocalyx) disruption, oxidation, inflammation and thromboembolism. The integrity of the endothelial glycocalyx is determined by the balance between shedding and synthesis, but under pathologic conditions the balance is disrupted resulting in the shedding of one or more of its components (eg, heparan sulfate, syndecan-1, or hyaluronic acid) into the blood. However, the balance can be restored or rebuild by self-assembly, to its native hydrodynamical thickness within 5 to 7 days (2009. Circul Res 2009; 104:1318–1325.) In another report, heparan sulfate (HS) can be restored on the surface of endothelial cells in 20 h in vitro (2013. Cell Mol Bioeng 6:160–174). In this regard, we constructed a flow chart to identify every possible site in the vasculature as drug targets (Fig. 56)

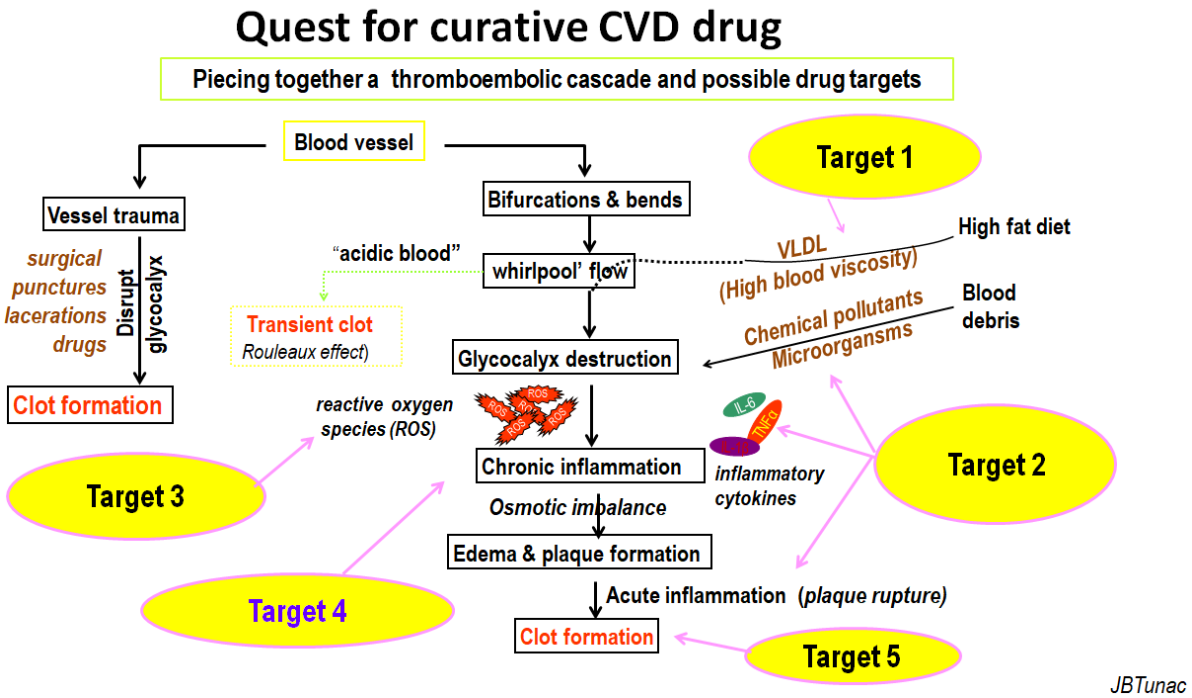

Figure 56. The thromboembolic cascade and identified 'druggable' targets

Based on the targets and experience in active drug ingredients, we rationally synthesized drugs incorporating ingredients known to possess antioxidant, anti-inflammatory, antimicrobial, and anticoagulant properties::

- Indole - Antioxidant , directly detoxifies ROS/reactive nitrogen species (RNS) , increases the activity of antioxidative enzymes while suppressing pro-oxidant enzymes in mitochondria, stabilizes the mitochondrial inner membrane
- Xylose - serves as a primer for the formation of heparin/heparin sulfate and chondroitin/dermatan sulfate chains, which is initiated by the attachment of α-D-N-acetylglucosamine (GlcNAc) or β-D-N-acetylgalactosamine (GalNAc), respectively. The glucosaminoglycan (heparin/heparan sulfate) and the galactosaminoglycan (chondroitin/dermatan sulfate) chains then assemble by the alternating addition of GlcUA and GlcNAc or GlcUA and GalNAc, respectively.
- Lipoate – Inactivates the nuclear factor kapp B (NF-κB that plays a crucial role in immune response, inflammation, cell growth and survival, and development, acts as powerful antioxidants
- Choline – acts in the synthesis of membrane phospholipids, specifically phosphatidyl choline (PC), which is the predominant phospholipid (>50%) in mammalian membranes. PC is important in maintaining cellular integrity, and signaling functions.
- Piperine- the pungent compound contained in black pepper (Piper nigrum L.), Piperine exhibits anti-oxidant, anti-inflammatory and anti-degenerative properties, as well as enhancement of drug absorption
- Nicotic acid - water-soluble vitamin and nicotinamide is a derivative of niacin that forms the coenzymes nicotinamide adenine dinucleotide (NAD) and its phosphorylated form, nicotinamide adenine dinucleotide phosphate (NADP). NAD is a key molecule used in the production of energy. .
- Isothiocyanates (ITC) - sulfur-containing compounds broadly distributed among cruciferous

vegetables, antioxidant and anti-inflammatory properties.

- myxin - phenazine di-N-oxide which causes enzymatic one-electron reduction, which both donate and accept electrons to other electron transfer molecules for their biological activities.
- Cysteine - an important source of sulfur, which forms the very reactive sulfhydryl (SH or thiol) group for the stabilization of and function of protein and enzymes

Using the above ingredients, we synthesized nine (9) proprietary lead drugs designed to restore a healthy glycocalyx layer and prevent thromboembolism; these include drugs with antioxidant, anti-inflammatory, antimicrobial, and anticoagulant properties (Fig 57)

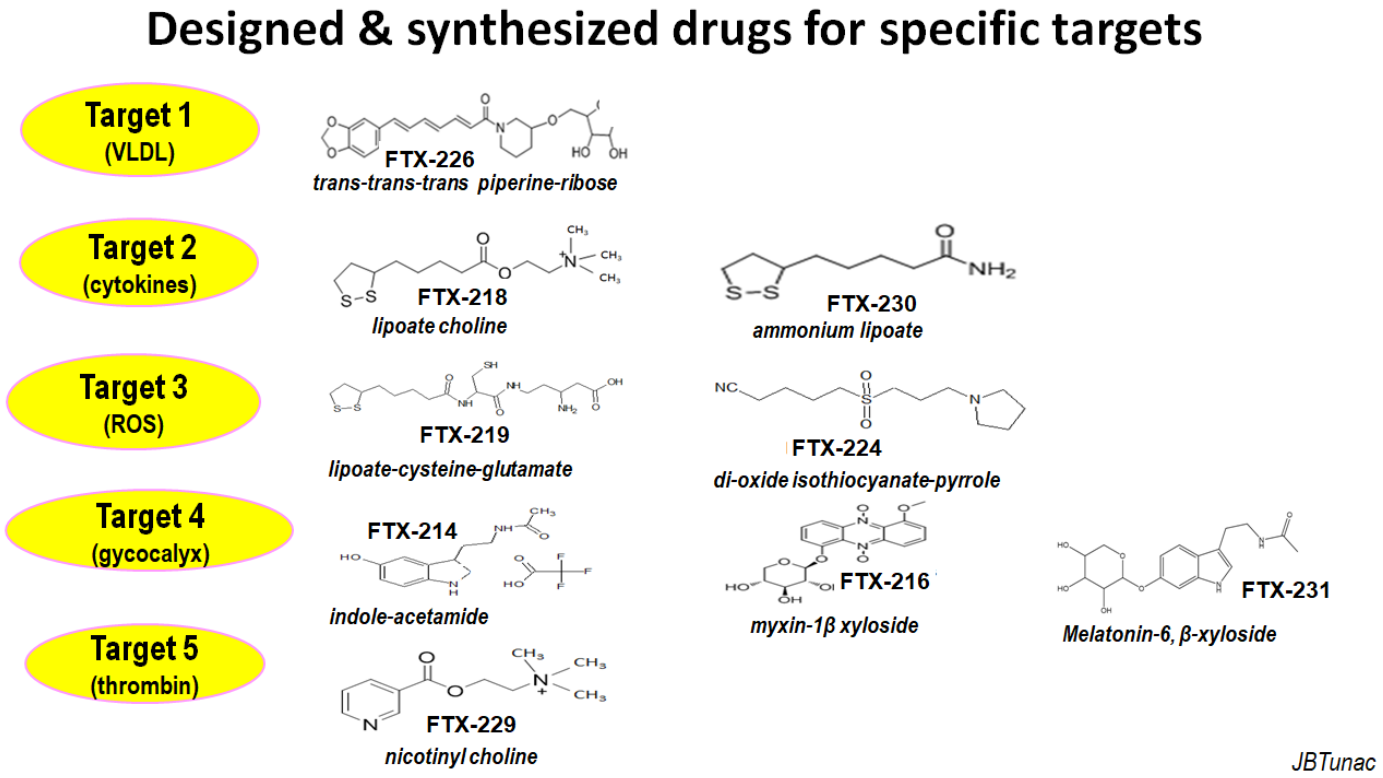

Figure 57. Structures of 9 proprietary FTX compounds synthesized for specific targets

The proposed mode of action (MOA) of the different FTX drugs are described in a separate sheet (see attached: FTX synthesisMOA))

Animal model development

The knockout ApoE mice (apoE*3-Leiden, apoE−/−) are the standard model for atherosclerosis but do not represent clinical settings:

- no studies yet showing plaques that become disrupted spontaneously (2005. Circ Res. 96: 667–674);
- fatal human plaques are fibrous lesions without necrotic cores.(1996. Circulation. 93: 1354–

1363);but no appropriate animal model available (2001. J Pathol. 195: 257–263; 2001.Arterioscler Thromb Vasc Biol.21: 1470–1476).

Most importantly, there is no animal model that represents glycocalyx disruption in relation to CVD.Incidental rat models have been used to assess glycocalyx disruption (shedding of syndecan-1 and heparan sulfate) in hyperglycemia (2011.Anesth Analg. 112(6): 1289–1295). haemorrhagic shock (2013. J Trauma Acute Care Surg. 75(5):759–66); inflammation and ischaemia-reperfusion injury (2004. Am J Physiol Heart Circ Physiol. 286(5):H1672–80) and coagulation function after hemorrhagic shock (2013.J Trauma Acute Care Surg.75(5):759–66).

To evaluate the FTX drug leads, we developed a mouse model that mimics CVD and the thromboembolic cascade, called the Tunac Arterial Plaque (TAP) mouse™ model, *(2017. J Clin Exp Cardiolog Suppl 8:1).* This involves feeding mice with high fat diet and exposure to biological and chemical agent (PCB), which resulted in a mouse that produced well-formed subendothelial plaques (Fig 58).

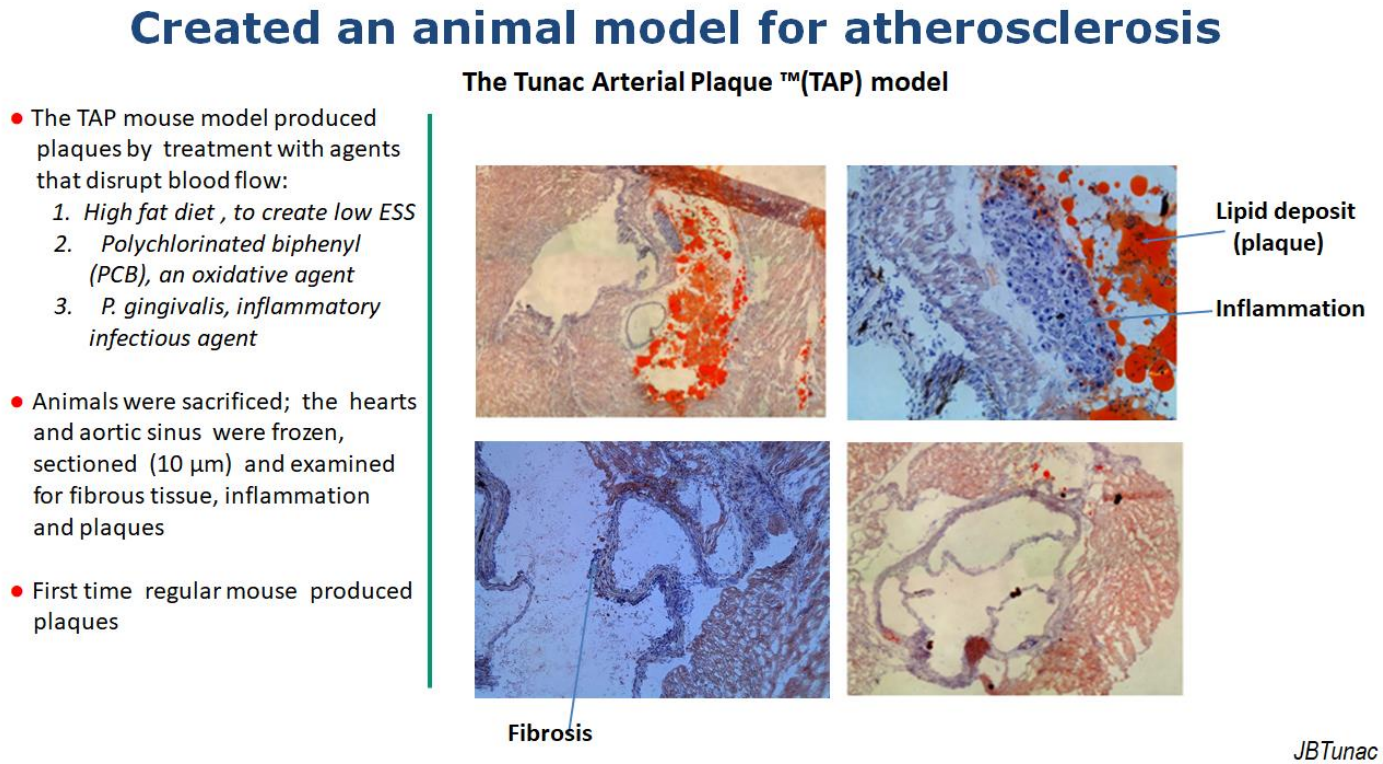

Figure 58. The Tunac arterial plaque (TAP) mouse™ model, first time plaques were formed in a natural mouse

Drug evaluation and *GlycoCardia* ^{CHD} as companion diagnostic:

The complex nature of thromboembolism does not lend to a one-drug treatment. Thus, our proprietary approach was not to administer the FTX-drugs as monotherapies, but as combo drugs formulated as one pill. The traditional drug development paradigm is one drug-one disease. Thus, in an abbreviated factorial design, we rationally formulated drug combinations (3-drug combos) and tested them in the TAP animal model (Fig 59)

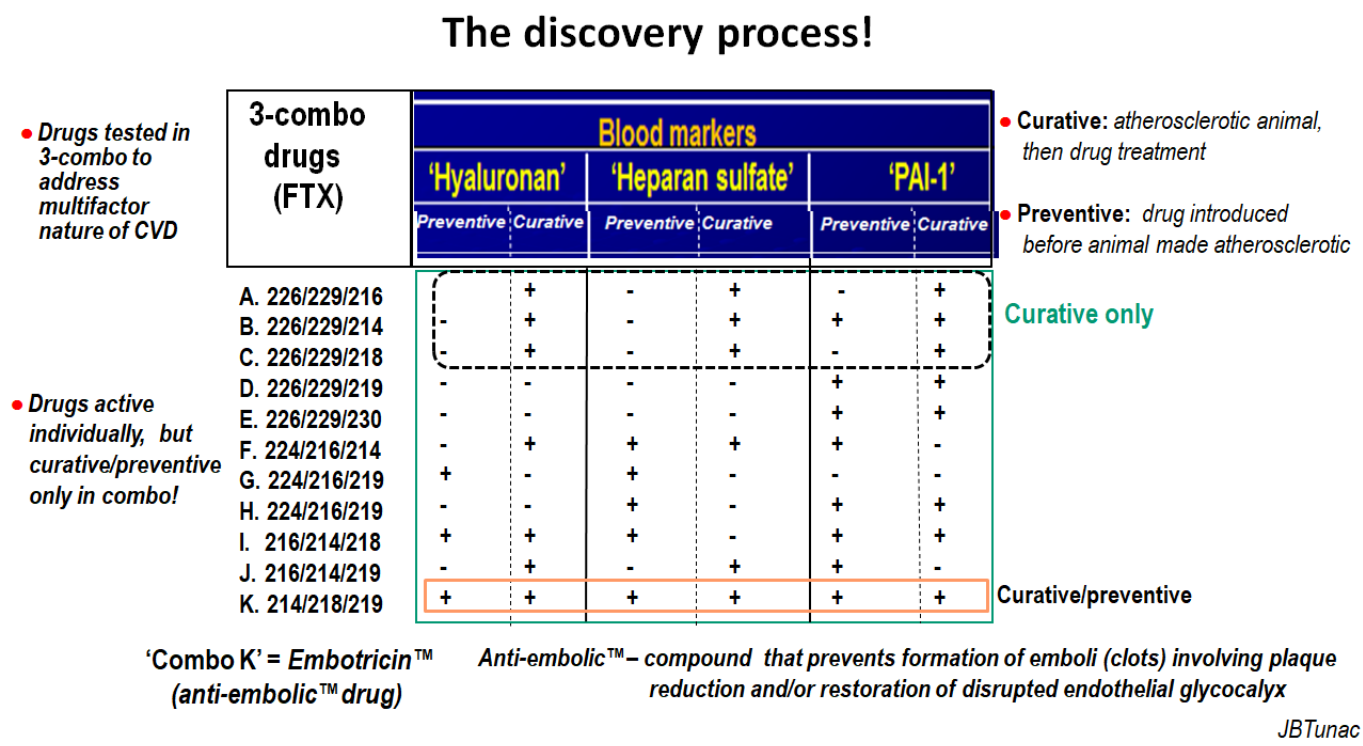

Figure 59. An abbreviated factorial 3-drug combo design. Note, individual drugs showed activity but combo FTX-214, -218, and -219 (K) was curative and preventive of plaques

Combo K is hereafter designated *Embotricin*™ (Ebn). Ebn was both preventive and curative of plaque (Fig 60)

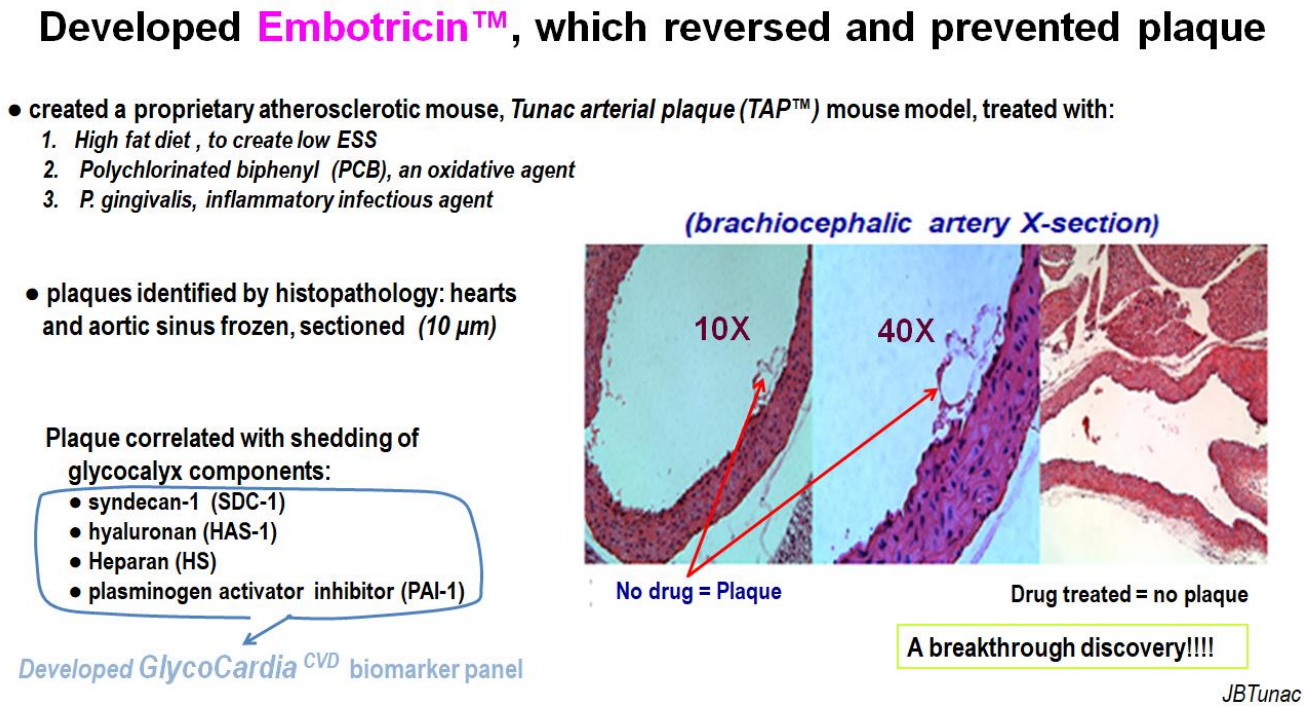

Figure 60. Micrograph of a mouse brachiocephalic artery showing plaque in the non-treated TAP™ mouse

Drug treatment with Embotricin™ removed the plaque as evidenced by the prevention or restoration of glycocalyx components including hyaluronan (HAS-1), heparan SO_4 (HS), and syndecan-1 (SDC-1) (Fig. 61).

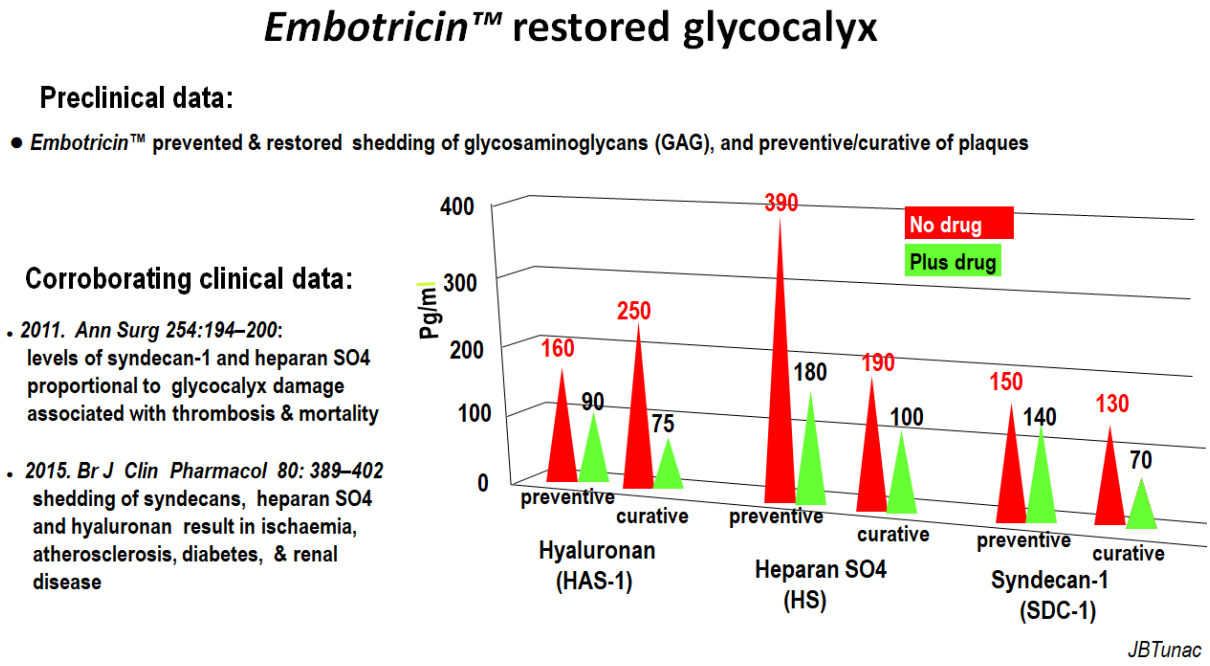

Figure 61. Embotricin™ prevented and restored shedding of glycosaminoglycans, which, corroborates published clinical data

Also, Embotricin™ was both curative and preventive of clot formation as evidenced by the marker plasminogen activator inhibitor -1 (PAI-1), which corroborates clinical data (Fig. 62).

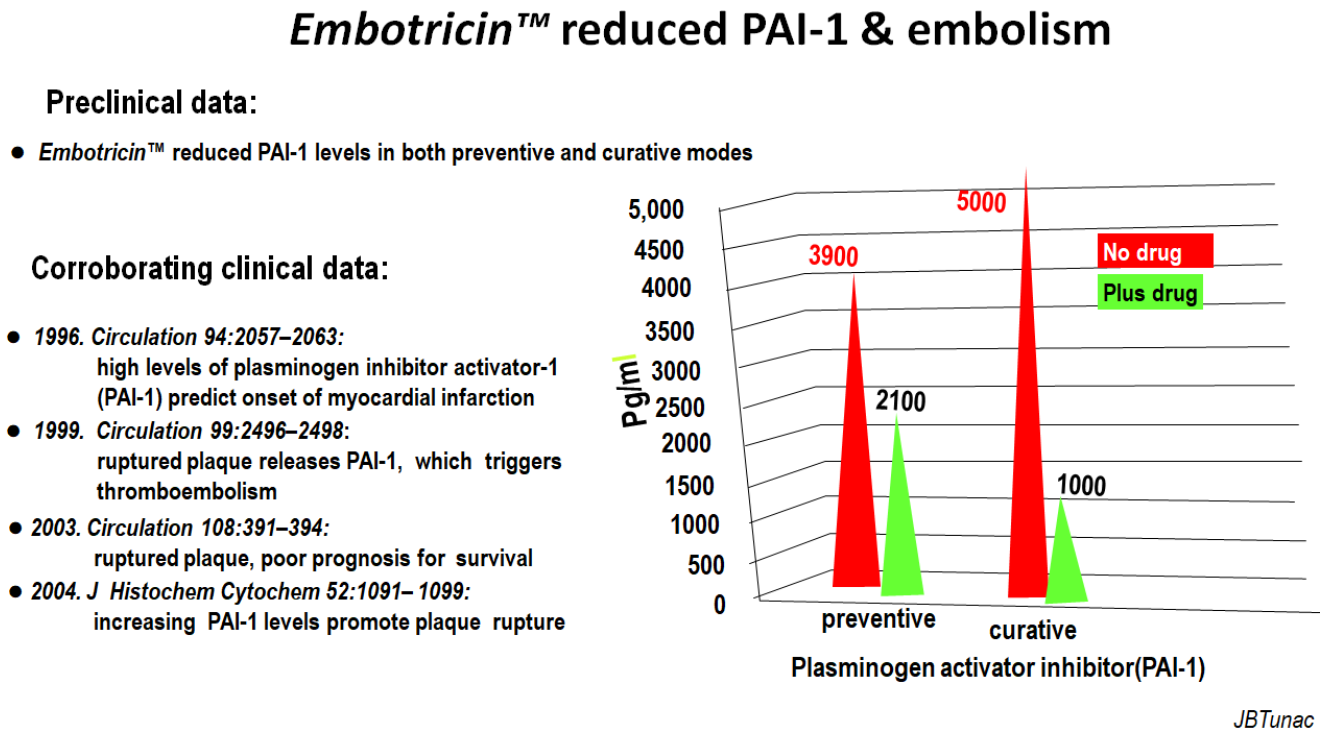

Figure 62. Embotricin™ was curative and preventive of clot (embolus) formation as evidence by the marker plasminogen activator inhibitor-1 (PAI-1),

Based on these experimental observations, the proposed mode of action of Embotricin™ includes restoration of the glycocalyx (FTX-214), anti-oxidant (FTX-218), and antiinflammatory (FTX-219) activities (Fig 63).

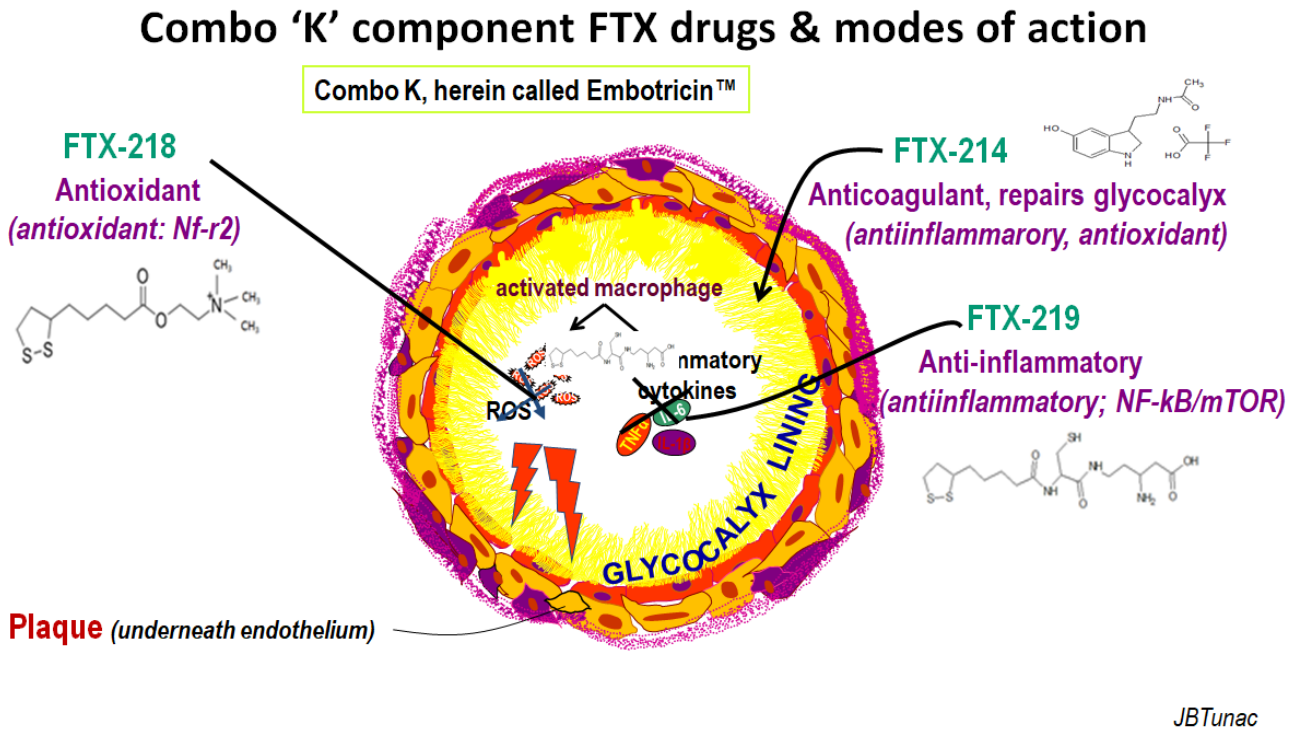

Figure 63. Chemical structure and site of action of the drug components of Embotricin™

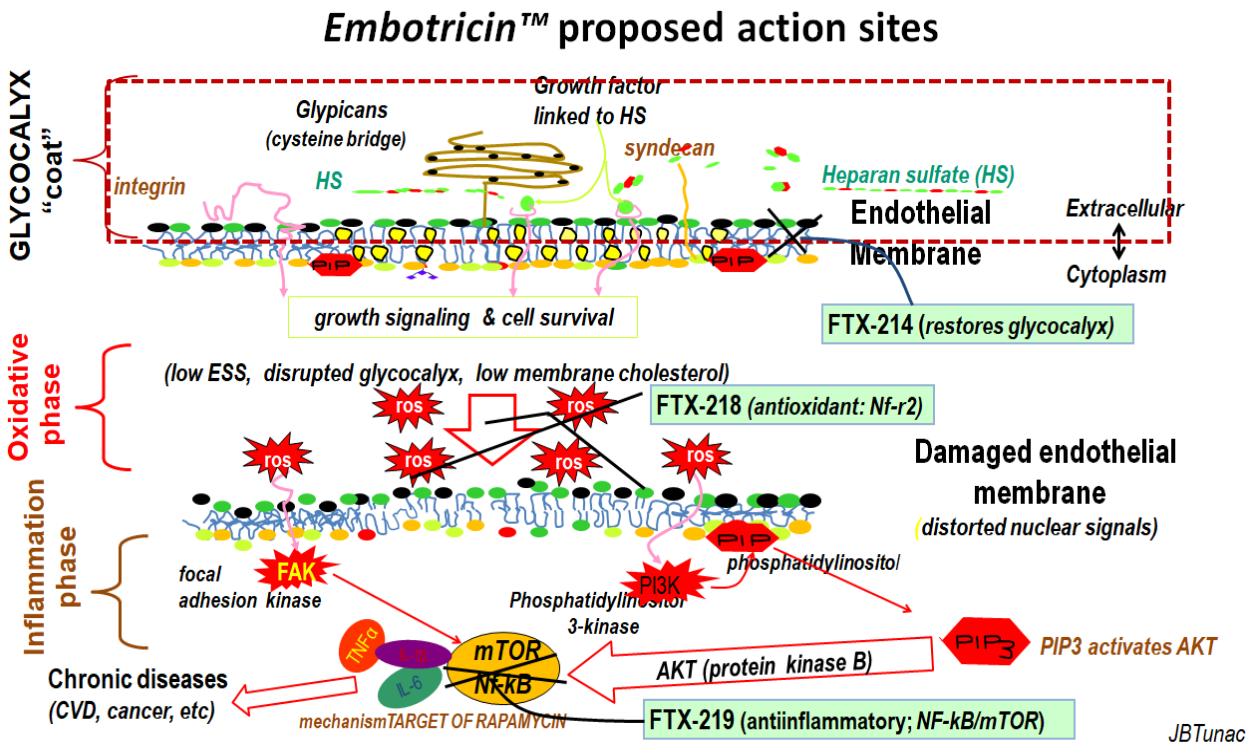

Figure 64 Proposed molecular mode of action of the three drug components of Embotricinn™

Proof of principle of validity of glycocalyx as drug target:

A number of compounds have been implicated in the reversal of plaques by restoring the health of the glycocalyx. The integrity of the glycocalyx is maintained by a balanced synthesis and shedding of its component parts. While excess shedding is associated with cardiovascular risks, it is possible to reverse shedding as shown in the following examples (Fig. 65):

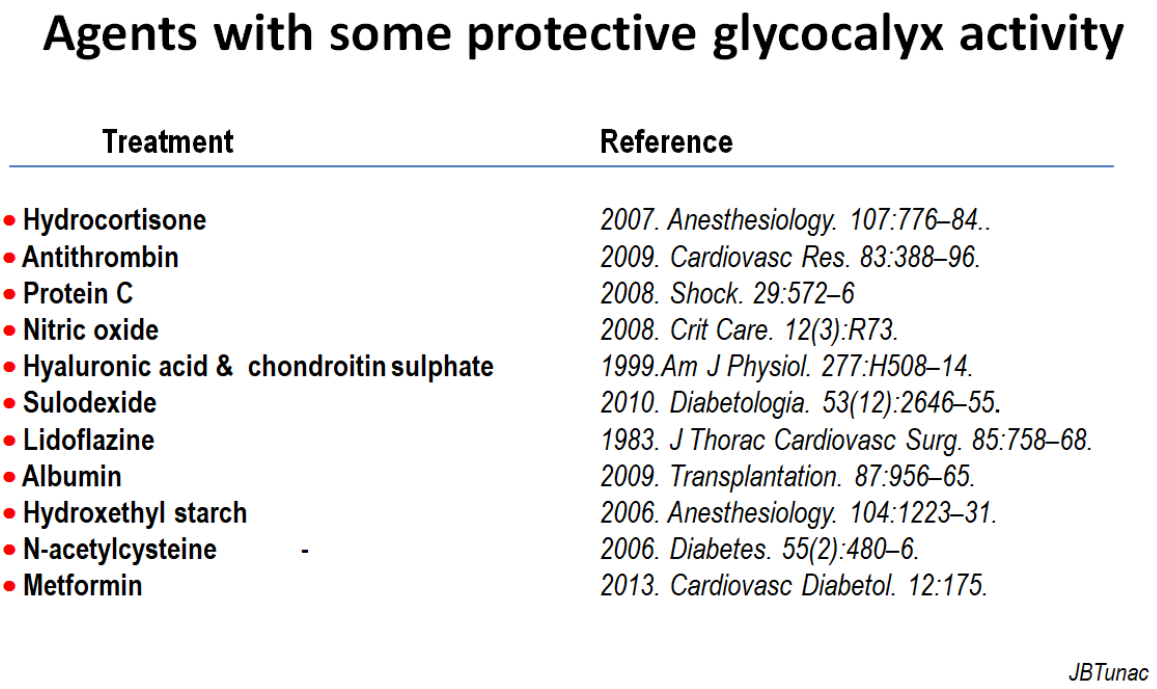

Figure 65. Published literature describing clinical potential of various compounds in ttreating glycocalyx

Embotricin™ is the first drug that systematically addresses glycocalyx restoration or repair and the other predisposing risk factors of CVD including oxidation and inflammation. The robust preclinical data may well prove Embotricin™ to be the first curative drug (anti-embolic™) against CVD with the same impact as penicillin to infectious disease (Fig. 66)

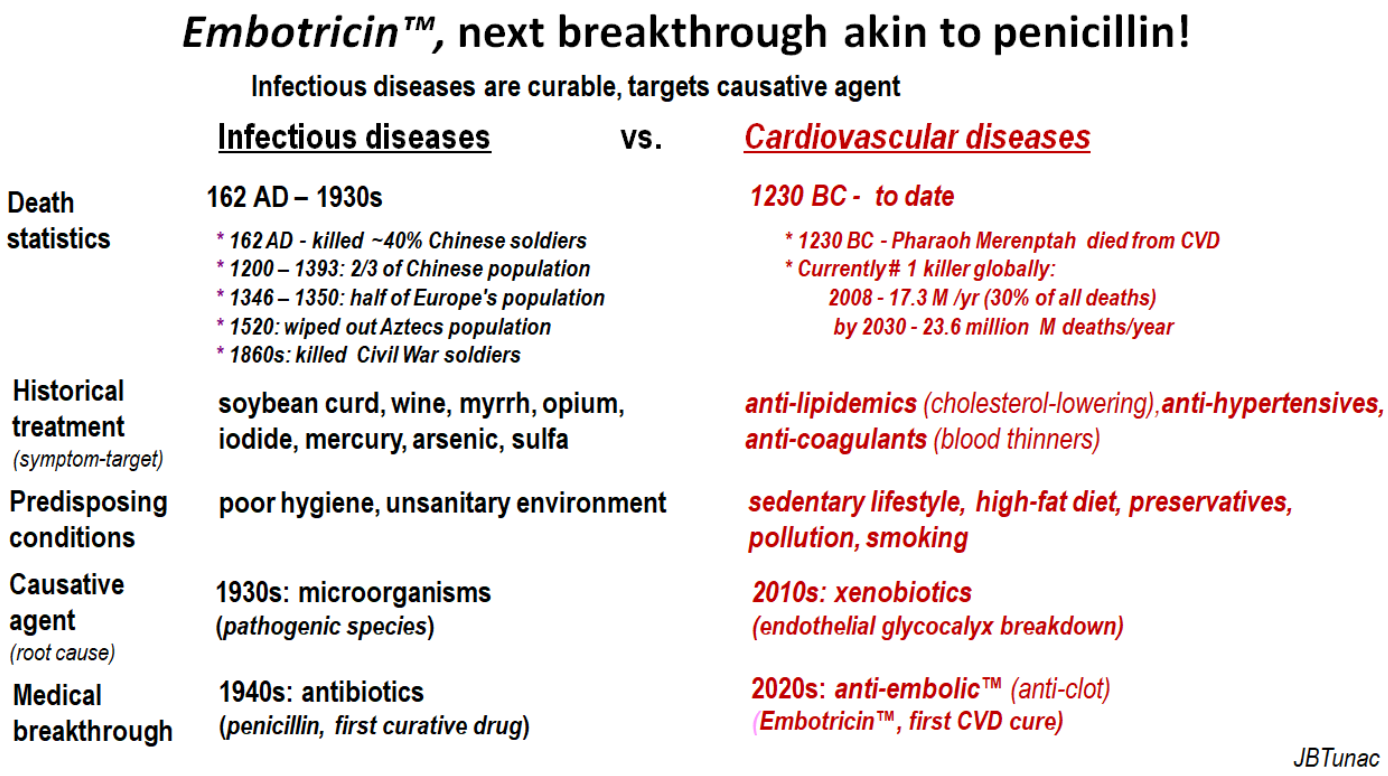

Figure 66. Historical development of antibiotic targeting microorganisms leading to cure of infectious disease. An equivalent approach is to target endothelial glycocalyx to cure CVD by an antiembolic™ mechanism